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ORIGINAL ARTICLE

Basic Study

MiR-29a-3p inhibits fibrosis of diabetic kidney disease in diabetic mice *via* downregulation of DNA methyl transferase 3A and 3B

Ying Yang, Yi Chen, Jian-Ying Tang, Jian Chen, Gui-Qing Li, Bing Feng, Jiao Mu

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Abstract

BACKGROUND

At present, the incidence of diabetic nephropathy is increasing year by year, and there are many studies on the pathogenesis of diabetic nephropathy, but it is still not completely clear. The final pathological result of diabetic nephropathy is mainly glomerular cell fibrosis, and the roles of micro-RNA (miRNA)-29 and DNA methyl transferase (DNMTs) in cell fibrosis have been confirmed in other studies, but there is a lack of relevant research in the kidney at present.

AIM

To study the potential involvement of miRNA-29a-3p in fibrosis related to diabetic kidney disease (DKD).

METHODS

The expression of miR-29a-3p, DNMT3A/3B, fibrosis-related molecules, Wnt3a, β-catenin, Janus kinase 2, and signal transducer and activator of transcription 3 was assessed in SV40MES13 cells and diabetic mice using quantitative real-time PCR and western blotting. Furthermore, the expression changes of fibrosis-related molecules were further analyzed using immunofluorescence and immunohistochemical blotting. The renal pathological changes of DKD in each group were also studied using hematoxylin-eosin and periodate-Schiff reaction staining.

RESULTS
In both the *in vivo* and *in vitro* experiments, it was observed that high glucose induction significantly decreased miR-29a-3p expression. As a result of this downregulation, DKD-related fibrosis was found to be promoted, as confirmed by elevated expression levels of α-smooth muscle actin, collagen type I, and fibronectin. MiR-29a-3p targets the 3′ non-coding regions of *DNMT3A* and *DNMT3B* and inhibits their expression. Inhibition of *DNMT3A* and *DNMT3B* can reverse the effect of miR-29a-3p downregulation on DKD-related fibrosis.

CONCLUSION
MiR-29a-3p can regulate Wnt/β-catenin and Janus kinase/signal transducer and activator of transcription signal pathways by regulating and inhibiting the expression of DNMT3A/3B and thus participate in the inhibition of DKD-related fibrosis.

Key Words: Diabetic kidney disease; Mir-29a-3p; DNA methylation; Janus kinase/signal transducer and activator of transcription; Wnt/β-catenin; Renal fibrosis

Core Tip: This study was the first to verify that MiR-29a-3p can regulate Wnt/β-catenin and Janus kinase/signal transducer and activator of transcription signal pathways by regulating and inhibiting the expression of DNMT3A/3B and thus participate in the inhibition of diabetic kidney disease-related fibrosis by *in vitro* and *in vivo* experiments. These findings indicated that targeting miR-29a-3p and DNMT3A/3B may hold promise for diabetic kidney disease prevention and treatment.

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INTRODUCTION

Diabetic kidney disease (DKD), being one of the severe microvascular complications of diabetes, poses a significant peril to human health, and it is accountable for causing end-stage renal disease[1]. Research evidence indicates that epigenetic modifications, including DNA promoter region methylation, non-coding RNA, and histone regulation, affect factors related to the pathogenesis of DKD. These modifications play a critical role in DKD pathogenesis and ultimately result in vascular complications by regulating downstream target genes and activating various gene signal transduction pathways [2-4]. In various epigenetic modifications, abnormal DNA methylation is considered to be the main regulator affecting the transcriptional activity of DKD-related target genes and presents a metabolic memory mechanism[5,6].

The DNA methyl transferase (DNMT) family catalyzes this process. There are three types of DNMTs present in mammals, including *DNMT1*, *DNMT3A*, and *DNMT3B*. Previous research suggests that *DNMT1* aids in preserving DNA methylation patterns, whereas *DNMT3A* and *DNMT3B* facilitate methylation at previously unmethylated CpG sites[7]. Elevated levels of DNMT3A/3B have been identified in patients with DKD and appear to be associated with renal fibrosis [8-10]. However, the mechanism of the rise of DNMT3A/3B under high glucose (HG) remains unclear.

MicroRNA (miRNA), a small piece of RNA produced during the synthesis of endogenous nucleotides, has the potential to silence hundreds of genes after transcription and participates in a variety of biological processes such as apoptosis, differentiation, development, proliferation, and metabolism. Members of the miR-29 family (miR-29a, miR-29b, miR-29c) share the same seed region and interfere with tissue fibrosis by targeting collagen and various extracellular matrix proteins[11-13]. Zamani *et al*[14] proposed that miR-29a can induce hypomethylation of DNA and re-expression of tumor suppressor genes in hepatocellular carcinoma by targeting *DNMT3A* and *DNMT3B*. Qin *et al*[15] confirmed that miR-29a inhibits myocardial fibrosis in Sprague-Dawley rats by downregulating the expression of *DNMT3A*.

According to previous studies, miR-29a is associated with DNMT3A/3B, so we predict that miR-29a may be involved in the etiology of DKD. In this study, we utilized SV40MES13 cells and diabetic (db/db) mice as *in vitro* and *in vivo* models, respectively. We aimed to investigate the regulatory effect of miR-29a on DNMT3A/3B and renal fibrosis as well as its potential pathways by monitoring the expression of DNMT3A/3B and fibrosis-related indexes during the dynamic changes of miR-29a. This study was the first to explore the role of miR-29a-3p in the occurrence and development of DKD. The findings may offer insight for further investigation and examination of clinical approaches for swift intervention and reversal of DKD.

MATERIALS AND METHODS

Materials

Mouse glomerular mesangial cells (SV40MES13) were purchased from Wuhan Procell Life Science and 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 negative control (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: SV40MES13 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 (HG 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 the control group (CON), CON + inhibitor NC, and CON + inhibitor-miR-29a-3p groups. In the HG group, the cells were transfected with miR-29a-3p mimics or mimics NC (50 pmol/L) and divided into HG group, 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 4 weeks, the mice were euthanized by cervical dislocation, and blood was 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 were carried out in accordance with the relevant laws and institutional guidelines and have been approved by the appropriate institutional committee, No. LL-202125.

Real-time quantitative PCR: Total RNA was extracted from the transfected cells 24 h post-transfection, using the stem-loop method for reverse transcription. U6 was used as an internal reference, with real-time quantitative PCR (qRT-PCR) 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 tissue protein extraction reagent method. Protein concentration was measured using the NanoDrop method. The proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane by electro transfer. After blocking with 5% bovine albumin, the membrane was incubated overnight at 4 °C with primary antibodies (dilution ratio of 1:1000). After washing with tris buffered saline with tween-20, the corresponding horseradish peroxidase-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 using the detection data of an 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 °C. 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-eosin 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 staining: The tissue sections were deparaffinized and rehydrated, then stained with periodic acid-Schiff (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 (potential of hydrogen: 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,

Table 1 Detailed information on antibodies

Molecular	Molecular weight	Source	Reactive species	Company
DNMT3A (ab188470)	102 kDa	Rabbit	Human/mouse/rat	Abcam
DNMT3B (L2011188)	28 kDa	Mouse	Mouse	US Biological
α-SMA (ab205719)	42 kDa	Mouse	Mouse/rat/human/African green monkey	Abcam
Collagen I (ab270993)	139 Da	Rabbit	Mouse/rat	Abcam
Fibronectin (ab268020)	262 kDa	Rabbit	Human/mouse/rat	Abcam
Wnt3a (ab219412)	39 kDa	Rabbit	Human/mouse/rat	Abcam
β-catenin (D10A8)	92 kDa	Rabbit	Human/mouse/rat/monkey	Cell signaling technology
JAK2 (D2E12)	125 kDa	Rabbit	Human/mouse/rat/monkey	Cell signaling technology
STAT3 (124H6)	79 kDa/86 kDa	Mouse	Human/mouse/rat/monkey	Cell signaling technology
Tubulin (ab7291)	55 kDa	Mouse	Mouse/rat/human	Abcam
GAPDH (ab8245S)	37 kDa	Mouse	Mouse/rat/human	Abcam

DNMT3A: DNA methyltransferase 3A; DNMT3B: DNA methyltransferase 3B; JAK 2: Janus kinase 2; STAT3: Signal transducer and activator of transcription 3; α-SMA: Alpha smooth muscle actin.

Table 2 Primer sequences

Primer	Sequence (5' to 3')
MiR-29a-3p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAACCG
MiR-29a-3p-F	CGCGTAGCACCATCTGAAAT
MiR-29a-3p-R	AGTGCAGGGTCCGAGGTATT
U6-F	AGAGAAGATTAGCATGGCCCCTG
U6-R	AGTGCAGGGTCCGAGGTATT

followed by the addition of 5% bovine albumin 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 horseradish peroxidase-conjugated secondary antibody solution was added and incubated for 30 min. After a further wash with PBS, diaminobenzidine staining was performed, and the nuclei were 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 ± SD. Intergroup comparisons were performed using the unpaired *t*-test, and correlation analysis was performed using the χ^2 test. Statistical significance was indicated as $P < 0.05$, $P < 0.01$, and $P < 0.001$.

RESULTS

The expression of miR-29a-3p was downregulated, while the expression of DNMT3A/3B and fibrosis-related molecules were upregulated after HG 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, SV40MES13 cells were cultured with HG to establish an *in vitro* DKD cell model. qRT-PCR analysis was used to detect the expression of miR-29a-3p, *DNMT3A/3B* and fibrosis-related molecules (Figure 1).

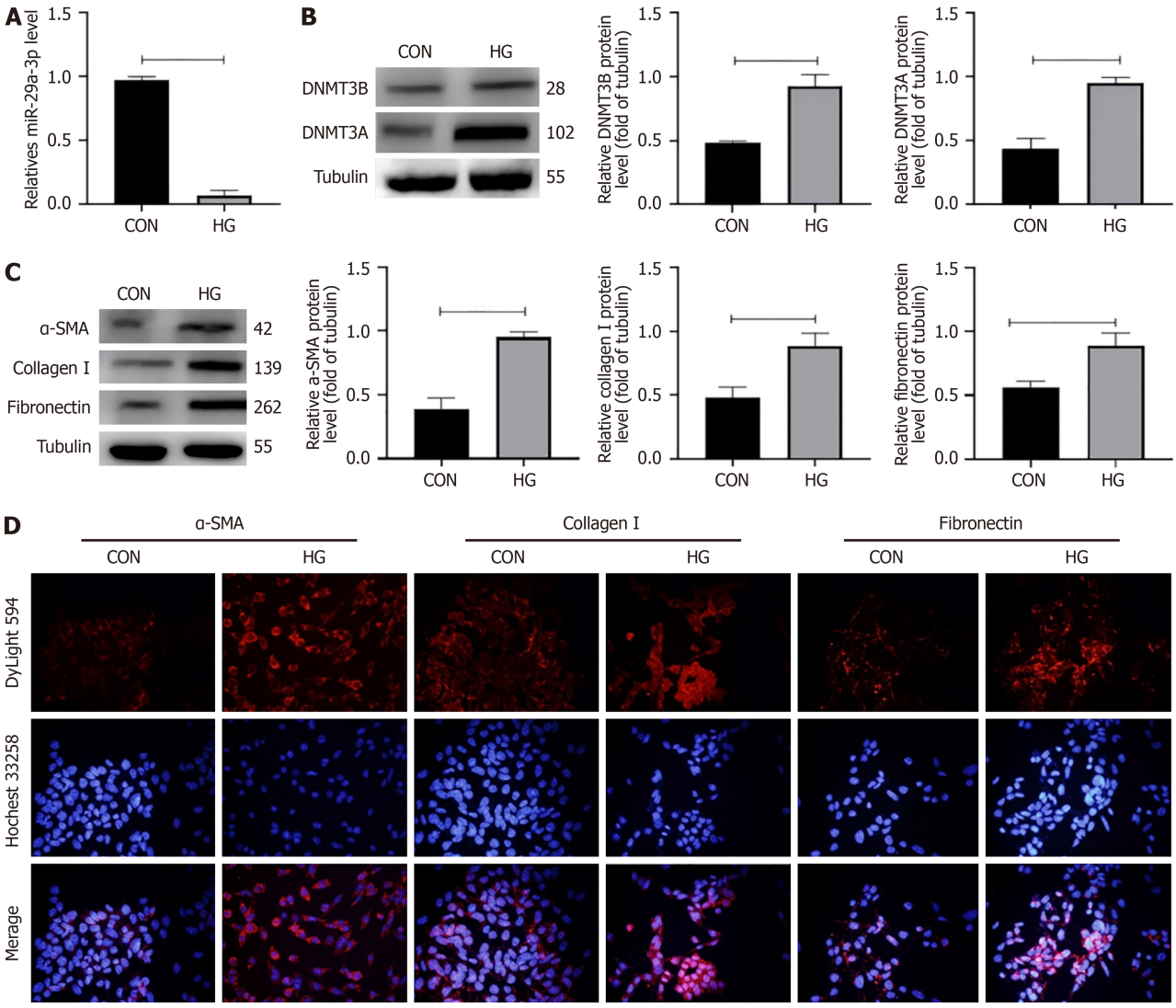


Figure 1 Expression of DNA methyl transferase 3A/3B and fibrosis-related molecules. A: Quantitative reverse transcription PCR to detect the expression of miR-29a-3p; B: Western blotting to detect the expression of DNA methyl transferase 3A/3B (DNMT3A/3B), and the right picture shows the statistical analysis map; C: Western blotting; D: Immunofluorescence (× 400) to detect alpha smooth muscle actin (α-SMA), collagen I, and fibronectin expression, and the right picture shows the statistical analysis chart (n = 3). CON: Control group; HG: High glucose group.

MiR-29a-3p negatively regulated the expression of alpha smooth muscle actin, collagen I, and fibronectin under HG

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 SV40MES13 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 the normal glucose group, the expression levels of alpha smooth muscle actin (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 the HG group, the expression of α-SMA, type I collagen, and fibronectin decreased after adding miR-29a-3p mimic ($^cP < 0.001$, Figure 3). MiR-29a-3p mimics improved the progression of fibrosis. The overexpression of miR-29a in the HG group can improve the expression of fibrosis-related markers.

Negative regulation of DNMT3A/3B expression by miR-29a-3p under HG

After transfection with inhibitor-miR-29a-3p, qRT-PCR was utilized to measure the expression of several factors. The expression of miR-29a-3p in the normal glucose group was significantly decreased ($^cP < 0.001$), while the expression of *DNMT3A* and *DNMT3B* ($^cP < 0.001$) was significantly upregulated (Figure 4A and B). Similarly, we found that after the HG group 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 downregulated ($^bP < 0.01$) (Figure 4D). It suggested that miR-29a-3p may negatively regulate the expression of DNMT3A/3B protein in SV40MES13 cells.

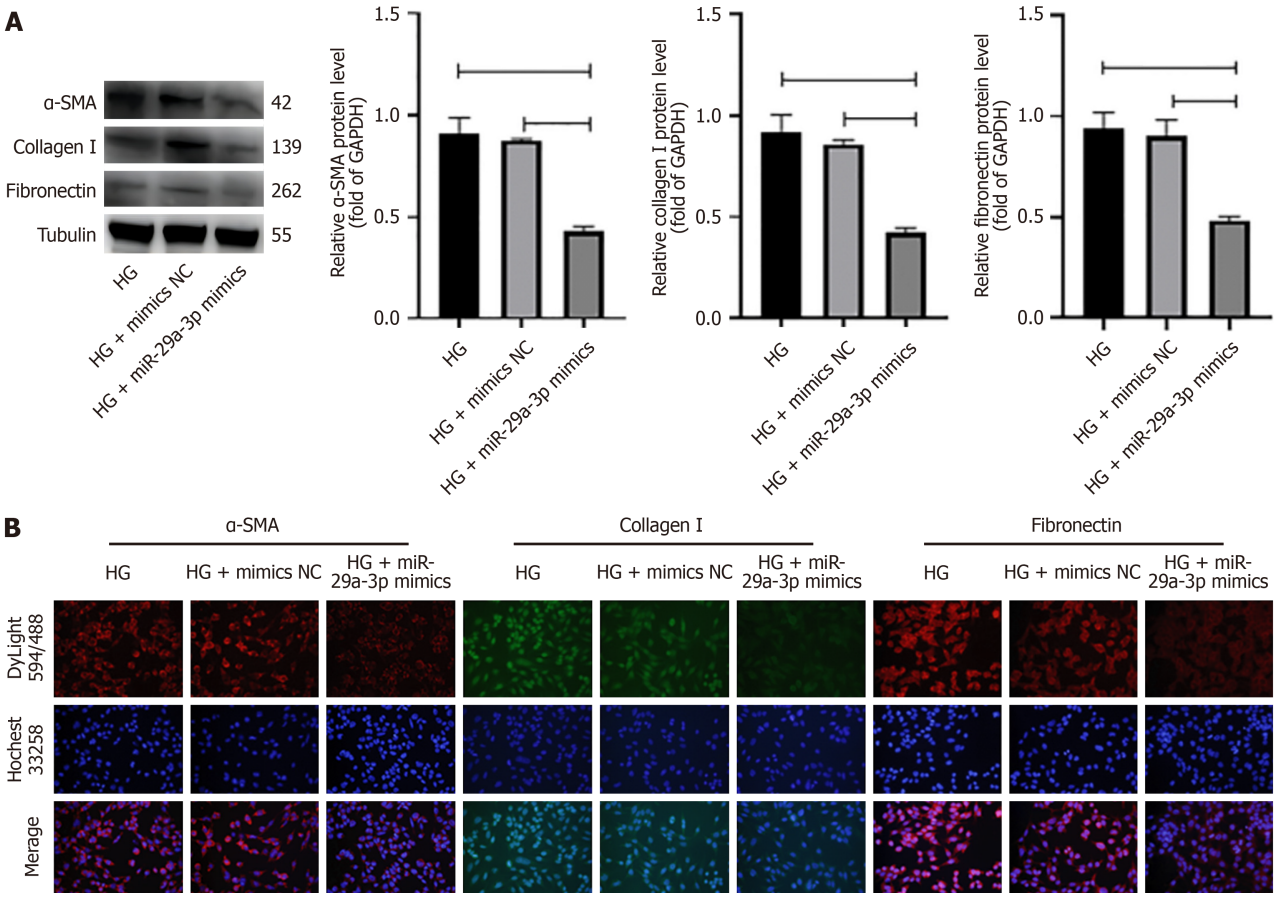


Figure 2 Expression of alpha smooth muscle actin, collagen I, and fibronectin. Alpha smooth muscle actin (α-SMA), collagen I, and fibronectin were significantly decreased after transfection of cells in the high glucose (HG) group with miR-29a-3p mimics. A: Western blot including the statistical analysis chart; B: Immunofluorescence (× 400) (n = 3). HG: High glucose group; NC: Negative control.

Inhibition of DNMT3A and DNMT3B reversed the downregulation of miR-29a-3p on diabetic nephropathy-related fibrosis in SV40MES13 cells cultured with HG

In order to further study whether miR-29a-3p regulates DKD by targeting DNMT3A and DNMT3B, SV40MES13 cells were treated with SGI-1027 after transfection with miR-29a-3p inhibitor to inhibit the expression of DNMT3A and DNMT3B. The results of qRT-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 downregulate DNMT3A and DNMT3B to inhibit DKD.

The effect of antagomiR-29a-3p on the kidney index, serum creatinine and blood urea nitrogen 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 SV40MES13 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 serum creatinine (normal range: 10.81-34.74 mg/dL) and blood urea nitrogen (normal range: 10.91-85.09 μ/L), which were shown to be outside the normal range (Figure 6B and 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 increased significantly, indicating that kidney atrophy had reduced after treatment (Figure 6D). Serum biochemical tests showed that the levels of serum creatinine and blood urea nitrogen 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 and 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).

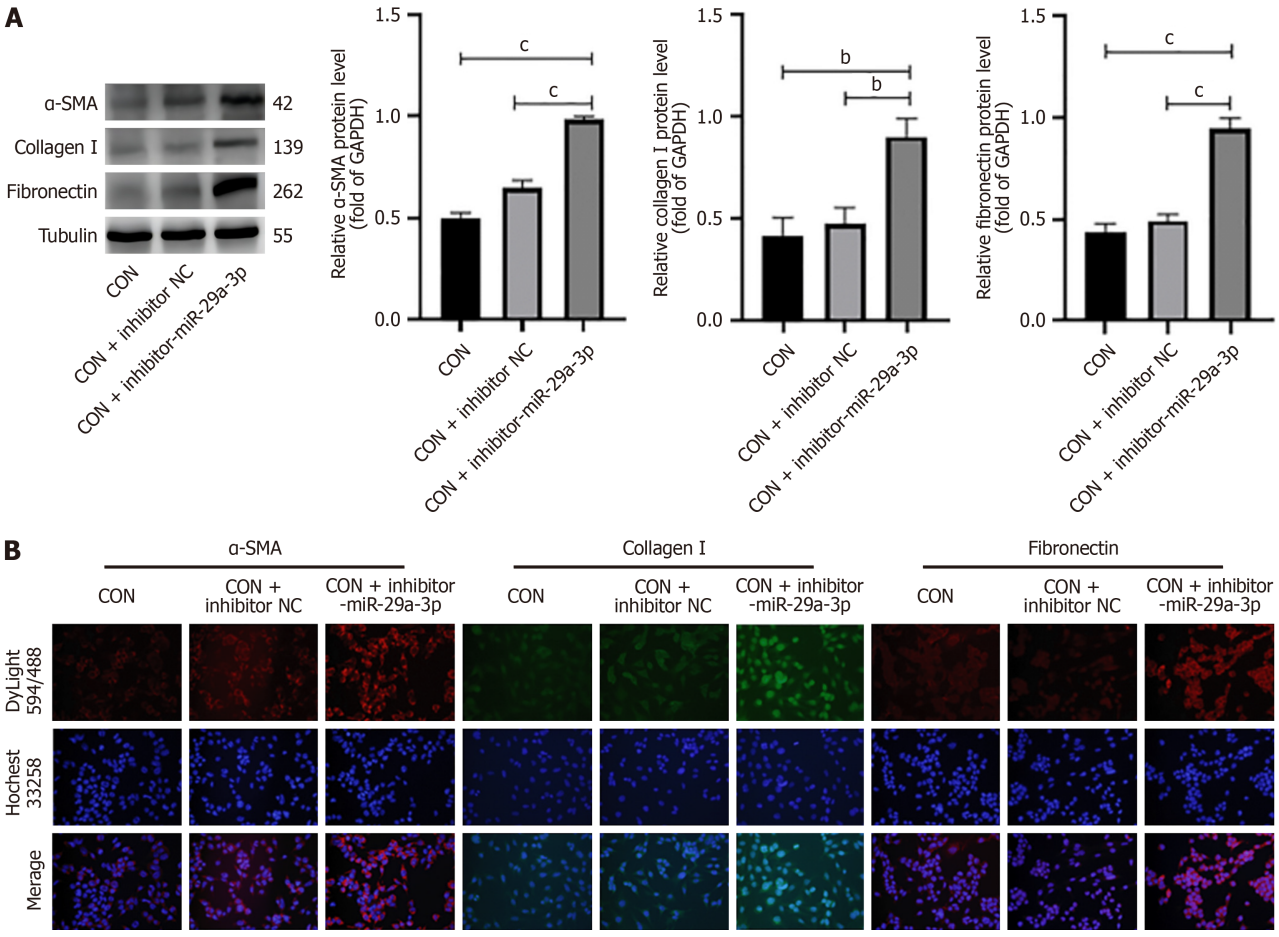


Figure 3 Expression of alpha smooth muscle actin, collagen I, and fibronectin. Alpha smooth muscle actin (α-SMA), collagen I, and fibronectin were significantly increased in cells transfected with inhibitor-miR-29a-3p in the normal glucose group. A: Western blot including the statistical analysis chart; B: Immunofluorescence (× 400) (n = 3). $^{b}P < 0.01$. $^{c}P < 0.001$. CON: Control group; NC: Negative control.

Hematoxylin and eosin staining 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-J). 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 negatively regulated 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 negatively regulated DNMT3A/3B in db/db mice and is involved in the pathogenesis of renal fibrosis through the Wnt/β-catenin and Janus kinase/signal transducer and activator of transcription signal pathways

In order to determine potential signaling pathways that may be regulated, we examined the expression of Wnt3a, β-catenin, Janus kinase (JAK) 2, and signal transducer and activator of transcription (STAT) 3 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. 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.

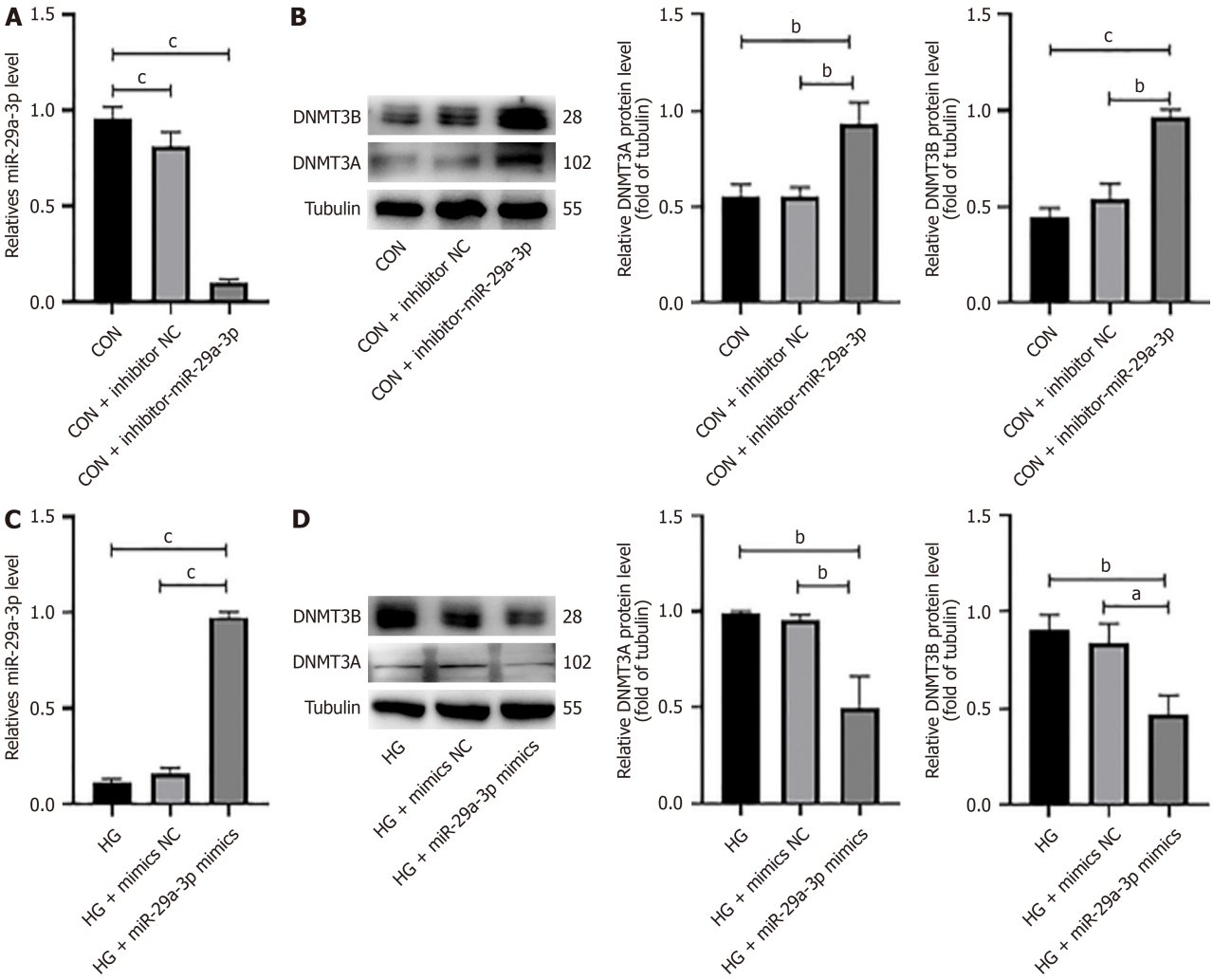


Figure 4 Effect of adding inhibitor-mir-29a-3p to cells in the normal glucose group or adding miR-29a-3p mimics to cells in the high glucose group on the expression of DNA methyl transferase 3A/3B. A and C: Quantitative reverse transcription PCR for detecting the transfection efficiency; B and D: Western blotting to detect the expression of DNA methyl transferase 3A/3B (DNMT3A/3B) (n = 3). $^{a}P < 0.05$. $^{b}P < 0.01$. $^{c}P < 0.001$. CON: Control group; HG: High glucose group; NC: Negative control.

DISCUSSION

MiRNA is considered to be the key regulatory layer of basic biological processes that regulate differentiation, proliferation, embryogenesis, development, and apoptosis by binding to the 3′ untranslated region (UTR) site of its target gene. Several miRNAs are reportedly involved in different tissue fibrosis processes. For instance, miR-21 can induce podocyte differentiation and mesangial cell activation, thereby enhancing renal fibrosis[16]. Meanwhile, miR-325-3p restricts excessive cell proliferation, inflammatory infiltration, and fibrosis *in vivo*, thus hindering the progression of DKD[17]. MiR-542-3p may have a role in renal fibrosis by inhibiting the expression of *AGO1*[18].

Additional recent evidence supports the regulatory role of the miR29 gene cluster in tissue fibrosis[9,19-22]. MiR-29a, a highly conserved member of the miR-29 family, is known for its anti-fibrosis properties and has been studied for its mechanism in myocardial, lung, liver, and skin fibrosis[23-26]. However, its role and mechanism in DKD remain unclear. Shi *et al*[27] observed a reduction in miR-29a-3p expression in early and late diabetic mouse models, whereas no alteration was observed in miR-29b or miR-29c levels. Additionally, Ebadi *et al*[28] discovered that captopril and spironolactone reduced transforming growth factor β and microalbuminuria levels while enhancing the downregulated expression of miR-29a-3p in db/db mice.

Similarly, we observed a significant decrease in the expression of miR-29a-3p in SV40MES13 cells following stimulation by HG and a significant increase in the expression of fibrosis factors (α-SMA, type I collagen, and fibronectin) (Figure 1). However, the addition of miR-29a-3p mimics resulted in a notable decrease in the expression of fibrosis factors compared with the CON group (Figure 2). To confirm that abnormal expression of fibrosis factors was not caused by pathways mediated by HG, we also conducted verification in the normal glucose group. After inhibiting miR-29a-3p expression, we observed synchronous negative changes in fibrosis factors (Figure 3). This demonstrated that miR-29a-3p is an effective anti-fibrosis molecule as it negatively regulates the expression of fibrosis-related factors, preventing the occurrence and development of fibrosis.

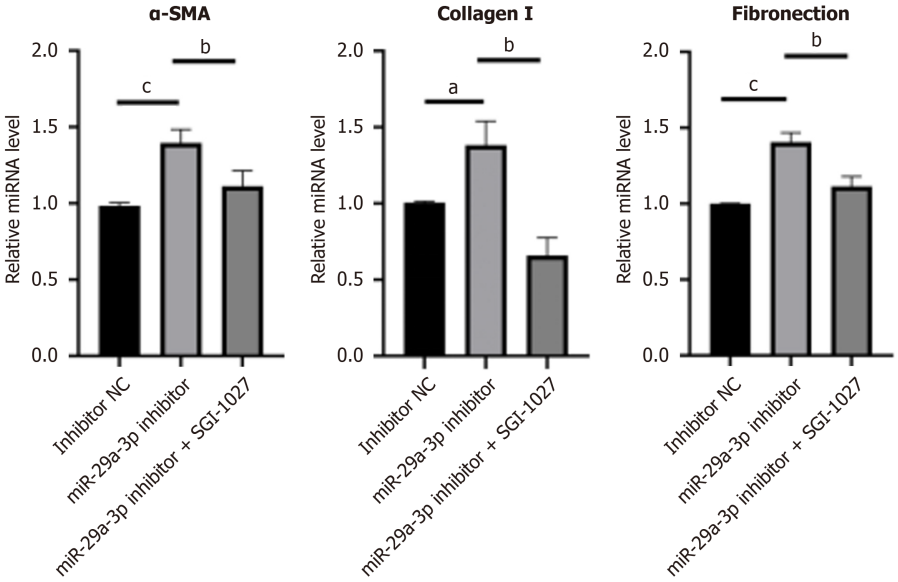


Figure 5 Inhibition of DNA methyltransferase 3A/3B can reverse the effect of downregulation of miR-29a-3p on diabetic kidney disease-related fibrosis in SV40MES13 cells cultured with high glucose. Quantitative reverse transcription PCR detected the expression levels of alpha smooth muscle actin (α-SMA), collagen I, and fibronectin in the diabetic kidney disease model. Results are represented as mean ± SD (n = 3). $^{a}P < 0.05$. $^{b}P < 0.01$. $^{c}P < 0.001$. NC: Negative control.

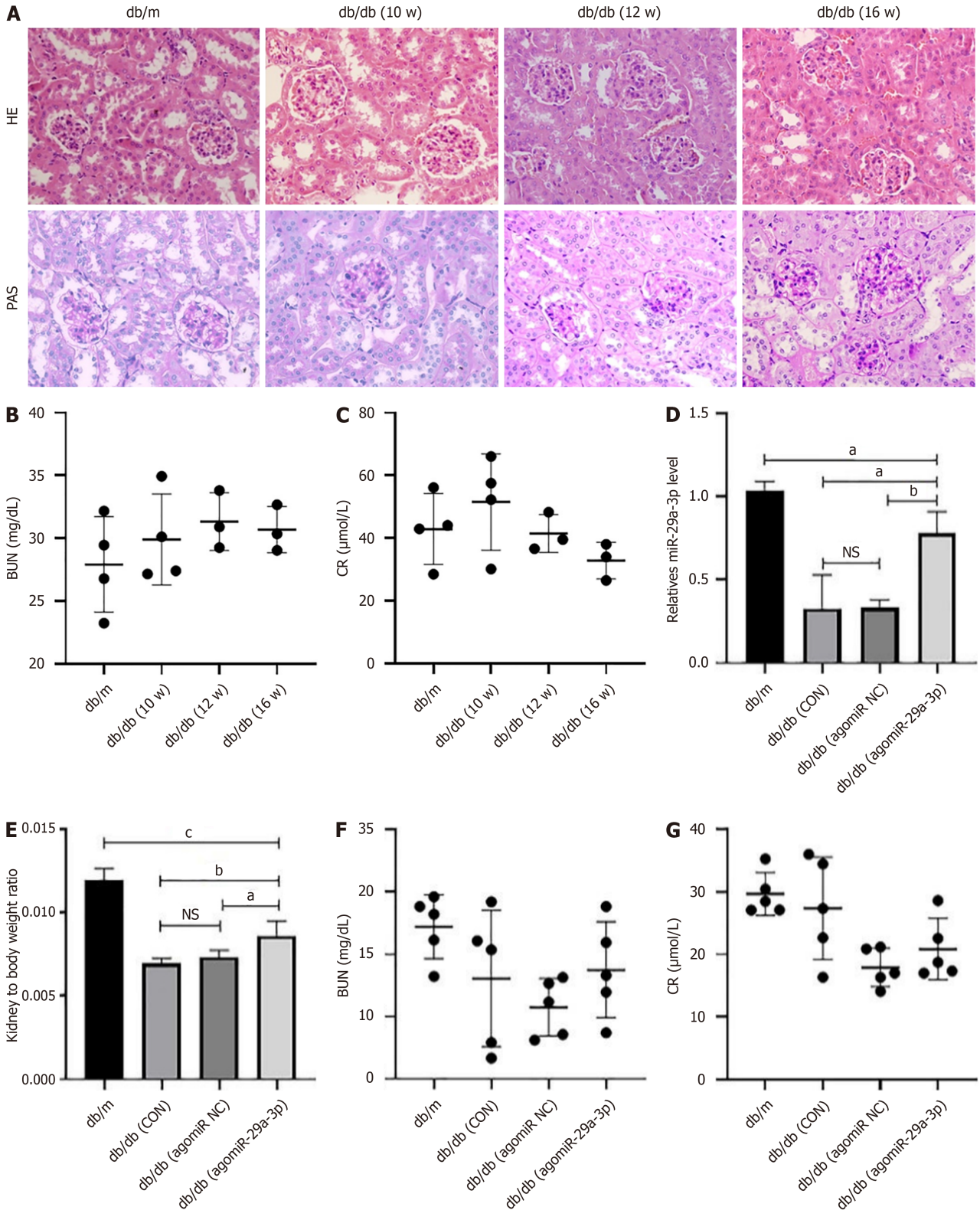
The present study provided additional support to the aforementioned perspective through *in vivo* experiments. Our findings revealed a notable decrease in the level of fibrosis factor expression within the renal tissue of db/db mice following antagomiR-29a-3p treatment compared with the CON group. DNA methylation is a well-researched epigenetic marker. Several pieces of evidence strongly suggest a close link between miRNA and DNA methylation, particularly in fibrotic diseases[29].

For instance, Fabbri *et al*[30] uncovered that the regulation of *DNMT3A* and *DNMT3B* by miR-29 mediated the reversal of abnormal methylation in lung cancer. The expression of *DNMT3A* and *DNMT3B*, two key ab initio DNMTs, is often upregulated in patients with idiopathic pulmonary fibrosis[30]. Wang *et al*[31] discovered that inhibiting miR-29c enhanced biliary atresia-related fibrosis, induced by transforming growth factor β, by targeting *DNMT3A* and *DNMT3B*. In addition, Wu *et al*[32] found that miR-29b expression in cardiac tissues of patients with coronary heart disease was negatively correlated with *DNMT3A* and *DNMT3B* expression. They further demonstrated that miR-29b could regulate the methylation status of cardiomyocytes by directly targeting DNMT, which in turn regulated cardiomyocyte proliferation and fibrosis progression.

We conducted additional verification, finding that HG stimulation notably increased the expression of DNMT3A/3B in mesangial cells. Additionally, the expression of fibrosis factors changed synchronously with it. Subsequently, after DNMT3A/3B inhibition, fibrosis factors decreased synchronously, suggesting that it also facilitates cell fibrosis development. Then we used online tools (such as TargetScan, miRDB, *etc*.) to predict the target genes of miR-29a-3p and found that the 3′-UTR of *DNMT3A* (862-868 bp, 1305-1311 bp, 5559-5565 bp) and the 3′-UTR of *DNMT3B* (1202-1909 bp) contained miR-29a-3p (UGGUGCU)-capable sites. It is said that DNMT3A/3B is a possible target of miR-29a-3p, which has also been confirmed by other scholars[15,33-35], but the interaction between them in diabetic nephropathy is not clear.

We further investigated the regulatory relationship between miR-29a-3p and DNMT3A/3B in SV40MES13 cells by inhibiting or overexpressing the former. Our findings revealed that suppressing miR-29a-3p expression resulted in upregulated *DNMT3A/3B* expression and aggravated cell fibrosis in the normal glucose group. Conversely, overexpressing miR-29a-3p in the HG group led to downregulated *DNMT3A/3B* expression and decreased cell fibrosis (Figure 4). MiR-29a-3p is proposed to contribute to cellular fibrosis by negatively regulating DNMTs. Furthermore, the expression of fibrosis molecules was observed to decrease upon addition of a DNMT3A/DNMT3B inhibitor to the HG + inhibition miR-29a-3p group, corroborating DNMT3A/DNMT3B as a direct target of miR-29a-3p (Figure 5).

We performed additional *in vivo* verification by inducing expression of antagomiR-29a-3p in mice through injections. The results demonstrated a significant decrease in the levels of DNMT3A/3B in the kidneys of the antagomiR-29a-3p group compared with the CON group. Additionally, the pathological changes were mild, and no instances of glomerulosclerosis were observed. In contrast, the CON group of db/db mice exhibited more severe renal pathological damage and glomerulosclerosis (Figures 6 and 7). It is postulated that elevated levels of miR-29a-3p in DKD may impede the progression of kidney pathology by suppressing the expression of DNMT3A/3B. Here, we have identified *DNMT3A* and *DNMT3B* as the direct targets of miR-29a-3p. The effect of miR-29a-3p on fibrosis factors can be significantly reversed through the use of DNMT inhibitors. Genomic stability is predominantly regulated by genetic and epigenetic mechanisms. Inactivation of genes is mainly caused by abnormalities in miRNA and DNMT-mediated promoter methylation.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D
Relatives miR-29a-3p level
a
a
b
NS
db/m
db/db (CON)
db/db (agomiR NC)
db/db (agomiR-29a-3p)
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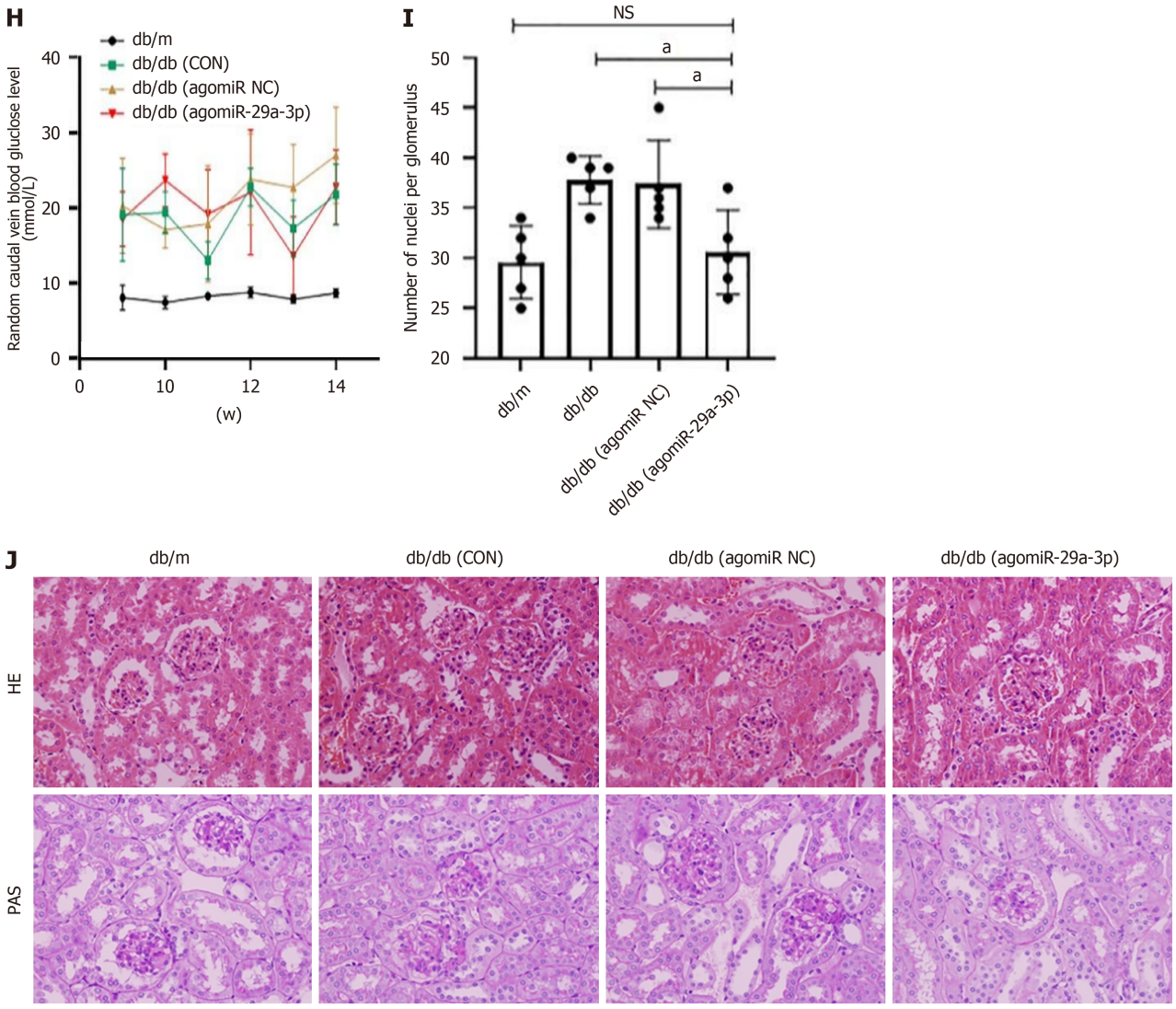

Figure 6 After antagomiR-29a-3p therapy, the renal index of diabetic mice increased, serum creatinine and blood urea nitrogen levels were within the normal range, and the renal pathological changes were relieved. A: Renal pathological changes in mice at 10, 12, and 16 weeks hematoxylin-eosin staining (HE), periodic acid-Schiff staining (PAS), × 400]; B and C: Results of serum creatinine (CR) and blood urea nitrogen (BUN) levels in mice at different weeks of age; D: Quantitative reverse transcription PCR to detect the expression of miR-29a-3p; E: Renal index of diabetic (db/db) mice in each group (bilateral renal body weight/mouse body weight), (n = 5); F and G: Serum biochemical test results for mice in each group; H: Random caudal vein blood glucose levels; I and J: Renal pathological changes in the mice: The pathological changes in db/db (agomiR-29a-3p) mice were more severe than that in the db/m mice, but were less severe than that in the db/db mice without agomiR-29a-3p treatment. $^{a}P < 0.05$. $^{b}P < 0.01$. $^{c}P < 0.001$. NS: Not significant; NC: Negative control; CON: Control group.

Recent findings indicated that miR-29a-3p induces colon cancer cell apoptosis *via* the Wnt/β-catenin signaling pathway, inhibits the proliferation of parathyroid cells, and facilitates migration and invasion of ameloblastoma by targeting *CTNNBIP1*[36-38]. The Wnt/β-catenin signaling pathway is significant in the development of diabetic nephropathy. For instance, this pathway is involved in the anti-apoptotic effect of zinc in type 2 diabetic nephropathy[39]. The reduced expression of miR-29a is related to decreased signal transduction of Wnt/β-catenin in the glomeruli of diabetic mice[40]. Both *in vitro* and *in vivo* experiments have demonstrated that miR-29a serves as the upstream regulator of Wnt/β-catenin signal transduction and plays a critical role in protecting mesangial cells against apoptosis and fibrosis [41].

Additionally, several studies suggest that the JAK/STAT pathway may contribute to the development of DKD through its regulation of cell proliferation, inflammation, and fibrosis[42,43]. For instance, an increase in *SOCS1* expression can effectively inhibit the JAK/STAT pathway, resulting in reduced levels of serum creatinine, proteinuria, and renal damage [44,45]. Accordingly, it is speculated that post-inhibition of DNMT3A/3B expression, miR-29a-3p may impede DKD-related chronic inflammation and renal fibrosis by suppressing the Wnt/β-catenin and JKA/STAT signal pathways (Figure 8).

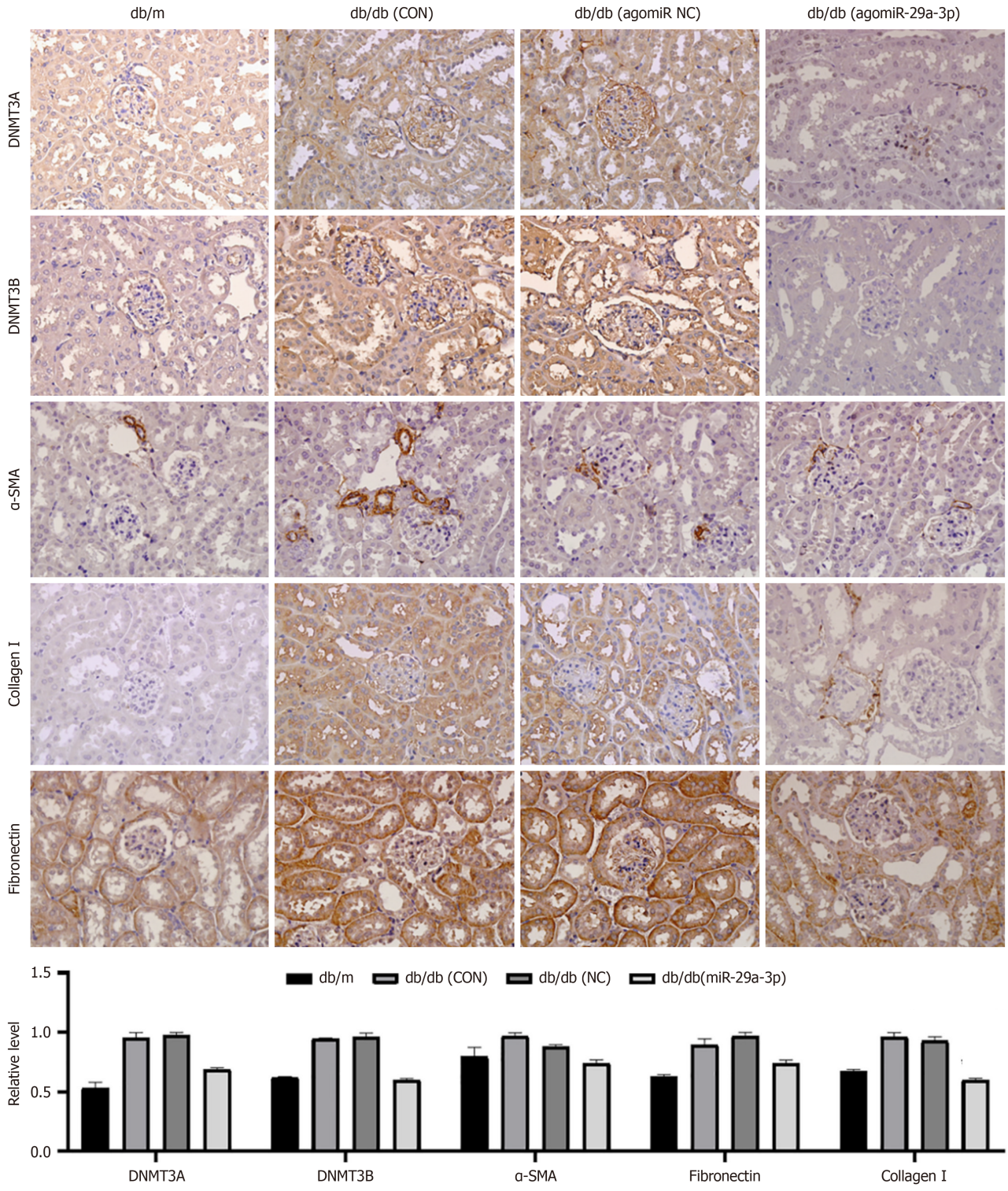

Figure 7 Change in the expression of DNA methyl transferase 3A/3B and fibrosis-related molecules in the mice. The expression levels of DNA methyl transferase 3A/3B (DNMT3A/3B), alpha smooth muscle actin (α-SMA), collagen I, and fibronectin in diabetic (db/db) mice treated with agomiR-29a-3p were significantly lower than those in the db/db mice without agomiR-29a-3p treatment (n = 3). CON: Control group; NC: Negative control.

CONCLUSION

We identified miR-29a-3p as an inhibitor of renal fibrosis in DKD, achieved by its downregulation of DNMT3A/3B. We speculated that its downstream regulatory mechanism might involve feedback from Wnt/β-catenin and JKA/STAT signaling pathways. Notably, *DNMT3A* and *DNMT3B* inhibition can counteract the effect of miR-29a-3p downregulation in DKD-related fibrosis. These findings indicate that targeting miR-29a-3p and DNMT3A/3B may hold promise for DKD prevention and treatment.

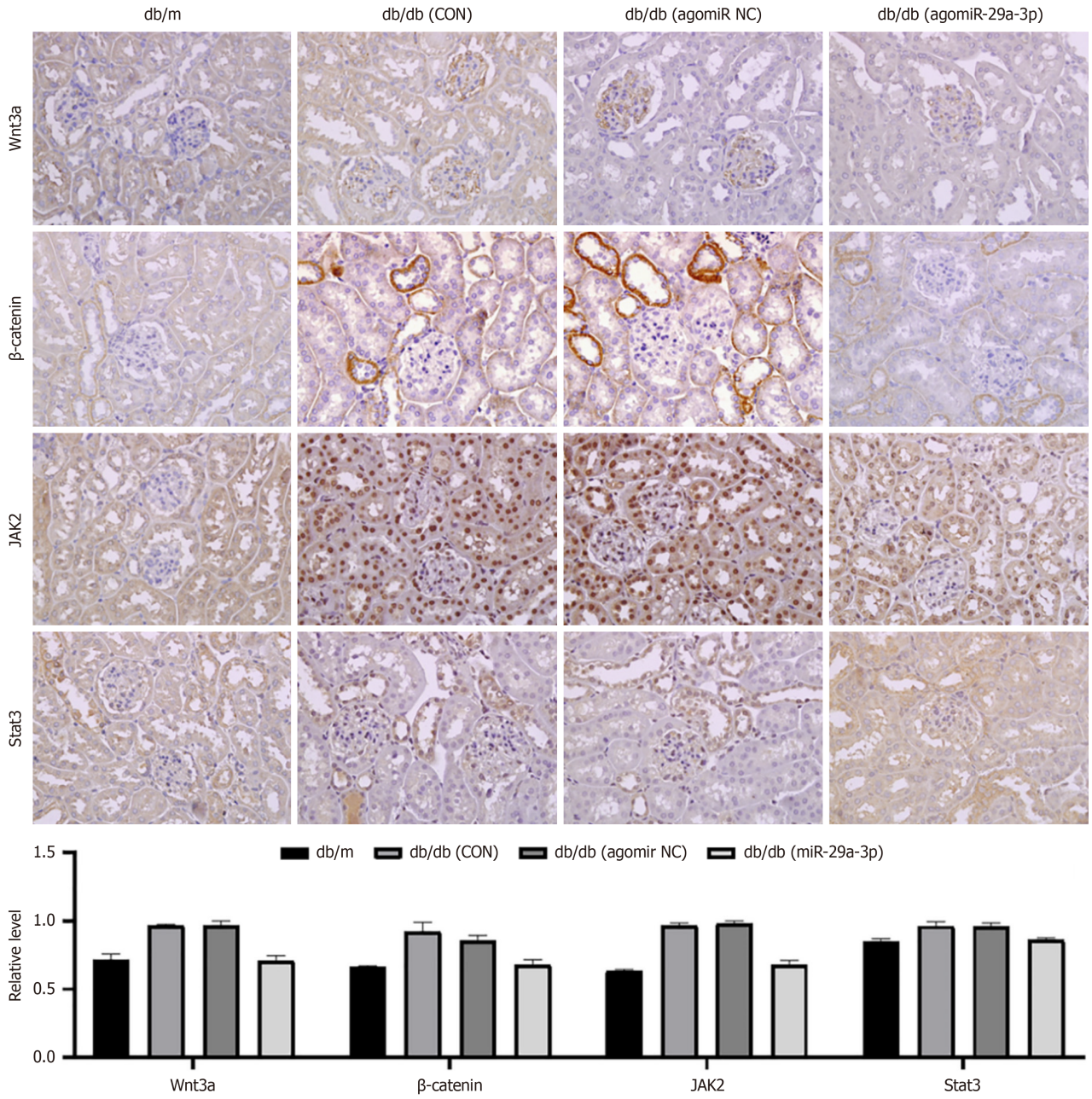

Figure 8 Change in the expression of Wnt3a/β-catenin and Janus kinase/signal transducer and activator of transcription pathways in the diabetic mice with agomiR-29a-3p treatment. The expression of Wnt3a, β-catenin, Janus kinase (JAK) 2, and signal transducer and activator of transcription (Stat) 3 in the diabetic (agomiR-29a-3p) mice were significantly lower than that in the diabetic mice without agomiR-29a-3p treatment (n = 3). CON: Control group; NC: Negative control.

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FOOTNOTES

Author contributions: Yang Y and Chen Y contributed to the design of the study, acquiring and analyzing data from experiments, and writing of the manuscript and jointly wrote this manuscript; Chen J, Li GQ, Tang JY, and Feng B provided suggestions on the design of the experiment and the interpretation of the results; Mu J, Feng B, Yang Y, and Chen Y conceived the project; Mu J and Feng B received funding for the project; Mu J is the guarantor of this work; All authors commented and edited the manuscript and approved the final version.

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