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MiR-203 improved renal cell injury in diabetic nephropathy by targeting SOCS6/SOCS7 and inhibiting JAK/STAT pathway activation

You Wang, Yuan Wang, Pei Pi, Dan Luo, Min Ning✉ & Gang Ye✉

This study investigates the role of miR-203 in regulating renal cell injury in diabetic nephropathy by targeting the suppressor of cytokine signaling (SOCS) proteins SOCS6 and SOCS7. Using NRK cells, we assessed apoptosis through flow cytometry and TUNEL assays, while real-time quantitative PCR (RT-PCR) quantified miRNA and mRNA expressions. Cell viability was measured using the CCK-8 assay, and cytokine levels were determined through ELISA. We also evaluated reactive oxygen species (ROS) and malondialdehyde (MDA) levels with specific assay kits. The dual luciferase assay confirmed the interaction of miR-203 with SOCS6 and SOCS7. Western blotting analyzed the protein levels of key signaling molecules including JAK1, p-JAK1, JAK2, p-JAK2, STAT3, and p-STAT3.Our findings revealed that high glucose (HG) treatment reduced miR-203 levels, leading to decreased NRK cell proliferation, increased cytokine concentrations (TNF-α, IL-1β, IL-4, IL-6), heightened ROS and MDA levels, and increased cell apoptosis. Notably, miR-203 mimics counteracted HG's detrimental effects, while miR-203 inhibitors exacerbated them. Mechanistically, miR-203 directly decreased SOCS6 and SOCS7 expression, thereby inhibiting JAK/STAT3 signaling. Thus, miR-203 provides protective effects against renal cell injury by modulating SOCS and their associated pathways.

Keywords Diabetic nephropathy, miR-203, SOCS6/SOCS7, JAK/STAT pathway

Diabetes is a common type of metabolic diseases and can lead to a series of complications[1]. Diabetic nephropathy (DN) is a serious microvascular complication primarily caused by proteinuria and is also a common secondary cause of late-stage renal disease[2]. Hyperglycemia, lipid metabolism disorders, glomerular hemodynamics, inflammatory responses, and fibrosis are related to the potential pathogenesis and complexity of DN[3]. Therefore, there is an urgent need for the prevention and treatment of DN.

Inflammatory responses, oxidative stress, and cell apoptosis are involved in the occurrence of DN. Previous studies showed a large amount of ROS accumulated in the kidneys of mice with DN, which would result in the formation of lipid peroxidation, such as malondialdehyde (MDA), and lead to cytotoxicity[4]. Eliminating ROS could improve the symptoms of DN and attenuate the apoptosis of NRK cells[5]. In addition, the concentrations of inflammatory factors, such as IL-6 and TNFα, were obviously increased in the blood of patients with DN[6]. Alleviating the productions of inflammatory factors could possess protective effects against DN[7]. Moreover, both the oxidative stress and inflammatory responses could increase the apoptosis rate of renal cells, which would finally accelerate DN progression[4]. However, the regulation of the inflammatory responses, oxidative stress, and cell apoptosis in DN is not fully understood.

Multiple evidences have shown that miRNAs may be involved in the pathogenesis of DN and can serve as biomarkers for DN treatment[8]. Besides, miRNAs have the ability to regulate inflammatory responses and cellular apoptosis in DN. Among them, miR-203, which was widely distributed in various tissues and organs of the human body, was firstly identified to be associated with diabetic foot[9]. Recently, miR-203 was identified as a new risk factor of DN[10]. And miR-203-3p, a subtype of miR-203, played anti-inflammation, anti-oxidation, and anti-apoptosis roles in mice podocytes treated with high glucose (HG)[11]. Besides, studies have found that miR-203-3p may negatively regulate the occurrence and development of DN[8], indicating miR-203 may have a critical role in

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the development of DN. Moreover, miR-203 played a significant role in controlling cell proliferation, apoptosis, and inflammation[12,13]. However, the underlying mechanism by which miR-203 affects DN development and the targeted genes of miR-203 in renal cell are still unknown.

The suppressor of the cytokine signaling (SOCS) family is a recently discovered protein family induced by cytokines. It acts as a negative regulator of the Janus kinase (JAK)/signal transducers and activators of the transcription (STAT) pathway. Additionally, SOCS proteins can inhibit cytokine signaling and reduce the JAK/STAT-mediated response in diabetic kidney cells[14], and SOCS6 deficiency improves the injury of renal cell induced by HG[15], suggesting the potential benefits of SOCS in preventing the progression of diabetic nephropathy. The JAK/STAT pathway is critical in regulating gene expression and cellular activation, proliferation, and differentiation in response to cytokines and other stimuli[16]. Recent study has reported that exposing kidney cells to high glucose activates the JAK/STAT signaling cascade[17]. Once activated, the JAK/STAT pathway contributes to the occurrence of atherosclerosis[18], hypertension[19], and diabetic kidney and vascular complications[20]. Overall, the JAK/STAT signaling system is important to connect cell surface receptors to nuclear transcription and transmit extracellular signals into the nucleus, leading to cell growth, inflammatory responses, and fibrotic reactions.

In this study, a diabetic nephropathy rat kidney injury cell model was established in vitro to explore the role and mechanism of miR-203 in DN development. We aimed to reveal whether miR-203 could affect the proliferation, apoptosis, and inflammatory state of renal cell treated with HG via regulating the expression of SOCS6/ SOCS7 and activating its downstream JAK/STAT signaling. And whether SOCS6 overexpression could eliminate the protective effects of miR-203 on renal cell. Our study provides a basis for clinical targets in the treatment of diabetic nephropathy and kidney injury.

Materials and methods

Reagents and instruments

The Annexin V-FITC/PI apoptosis detection kit (FS-79505) was purchased from Shanghai Fuson Industrial Co., Ltd. (Shanghai, China). Opti-MEM (M5650) was purchased from Sigma (St Louis, MO). Lipofectamine® RNAiMAX (13778-100) was purchased from Life Technologies (San Diego, CA). RIPA (strong) tissue and cell rapid lysis buffer (R0010) and the BCA protein concentration assay kit (PC0020) were purchased from Solarbio (Beijing, China). The antibodies of JAK1 (MAB43810), p-JAK1 (PAB43509-P), JAK2 (PAB30711), p-JAK2 (PAB43510-P), STAT3 (PAB30641), p-STAT3 (PAB36302-P), and GAPDH (PAB36269) were purchased from Bioswamp (Wuhan, China). Lipofectamine 2000 (11668-027) was purchased from Invitrogen (Waltham, MA). The dual-luciferase reporter assay kit (RG027) was purchased from Beyotime Biotechnology (Shanghai, China). And the flow cytometer was purchased from Agilent (CA, USA).

Cell culture

Rat kidney cells (NRK cells) were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences. And the cells were cultured in low glucose-containing medium with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin and placed in an incubator for static culture.

Cell apoptosis detection by flow cytometry

NRK cells in logarithmic phase were divided into three groups: low glucose (LG, 5.8 mM), high glucose (HG, 30 mM), and low glucose + mannitol (M, 5.8 mM glucose + 24.2 mM mannitol). Prior to experiment, cells were serum-starved for 12 h for synchronization. Then the cells were collected at 12, 24, 48, and 72 h for apoptosis detection using the Annexin V-FITC/PI apoptosis detection kit according to the manufacturer's instruction. Then the cells were analyzed by flow cytometry to determine the time point for inducing apoptosis under high glucose condition.

MiRNA transfection

NRK cells in logarithmic phase were divided into 6 experimental groups: low glucose (LG, 5.8 mM), high glucose (HG, 30 mM), mimic group (30 mM + miR-203 mimics), mimic NC group (30 mM + miR-NC mimics), inhibitor group (30 mM + miR-203 inhibitor), and inhibitor NC group (30 mM + inhibitor-NC). MiR-203 mimics and inhibitors were chemically synthesized for transfection. Cells were cultured to logarithmic phase for 72 h in different medium, followed by a 4 h-transfection. After transfection, the medium was replaced with fresh culture medium, and the cells were cultured for an additional 72 h. Samples were collected for subsequent analysis.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted by TRIzol, followed by accessing the purity and concentration. Then the RNA was reverse transcribed into cDNA using the Takara reverse transcription kit according to the instructions. The Real-Time PCR amplification system with total volume of 20 μL was as follows: SYBR FAST qPCR Master Mix 10 μL, forward primer (10 μM) 0.5 μL, reverse primer (10 μM) 0.5 μL, cDNA template 1 μL, ddH_2O 8 μL. And the reaction conditions were: initial denaturation at 95 °C for 3 min, followed by 40 cycles of amplification at 95 °C for 5 s, 56 °C for 10 s, and 72 °C for 25 s. Finally, the melting curve was conducted from 65 °C to 95 °C. U6 was used as internal reference gene for miRNA, and GAPDH was used as internal reference gene for SOCS6 and SOCS7 to calculate the relative gene expression level. The primer sequences for the target genes were listed in Table 1.

Primer Name	Sequence
miR-203-F	GGGGGAGTGGTTCTTAACAG
miR-203-R	CTGGTGTCGTGGAGTCGG
U6-F	CTCGCTTCGGCAGCACATATACT
U6-R	ACGCTTCACGAATTTGCGTGTC
SOCS6-F	CCTTGCTGGTGACTTCGTA
SOCS6-R	GTCTTCTGCTTGGCGGA
SOCS7-F	GACGTGGACATATCTCAGCG
SOCS7-R	GCATCTGGGGCGTGG
GAPDH-F	CAAGTTCAACGGCACAG
GAPDH-R	CCAGTAGACTCCACGACAT

Table 1. Primer sequences.

Cell counting kit-8 (CCK-8) assay

A total of 1×10^5 cells in 100 μL culture medium were seeded into each well of a 96-well plate. The cells were added with 10 μL of CCK-8 solution (DCM7122) and cultured for another 1 h. Then the absorbance at 450 nm was measured using a microplate reader (BIO-RAD).

Enzyme-linked immunosorbent assay (ELISA)

ELISA was preformed to detect the concentrations of TNF-α (MD120490), IL-1β (MD132598), IL-4 (MD120128), and IL-6 (MD132597) by using the corresponding ELISA kits according to the manufacturer's instruction. Briefly, 100 μL of serially diluted standard solution, sample dilution, and the samples was added to different wells and incubated for 90 min at 37 °C. After removing the liquid, 100 μL of horseradish peroxidase (HRP)-conjugated detection antibody was added to each well and incubated for 60 min at 37 °C. Then the wells were washed by the wash buffer for 3 times. Each well was added with 100 μL of enzyme-substrate working solution and incubated for 30 min at 37 °C. After being washed by the wash buffer for 3 times, 90 μL of substrate solution (TMB) was added to each well and incubated at 37 °C in the dark for 15 min. Finally, 50 μL of stop solution was added to each well to terminate the reaction. The optical density (OD) was measured at a wavelength of 450 nm.

Reactive oxygen species (ROS) and malondialdehyde (MDA) detections

MDA detection was performed using the MDA Test Kit (A003-1, Nanjing Jiancheng, China), and ROS detection was conducted using the Reactive Oxygen Species Assay Kit (S0033S, Beyotime, Shanghai, China) strictly following the instructions provided in the manuals.

Terminal Deoxynucleotidyl transferase-mediated dUTP Nick end labeling (TUNEL) staining

TUNEL staining was applied to detect cell apoptosis using a TUNEL assay kit (40306ES60, Yisheng Biotechnology Co., Ltd., Shanghai, China). Briefly, the cells were covered by 100 μL of 1× Equilibration Buffer and incubated at room temperature for 10 min, followed by reaction with 50 μL of TdT incubation buffer at 37 °C for 60 min. After being washed by PBS for 3 times, the cells were stained with PI solution (1 μg/mL) in the dark for 5 min. Then the cells were washed by deionized water for 3 times and further stained with DAPI. Then the signals were detected under a fluorescence microscope (Leica).

Plasmid construction and luciferase assay

The 3'-UTR sequences of SOCS6 and SOCS7 were cloned into luciferase reporter vectors to construct wild-type (SOCS6 3'-UTR-WT, SOCS7 3'-UTR-WT) or mutant-type (SOCS6 3'-UTR-MUT, SOCS7 3'-UTR-MUT) plasmids to investigate whether SOCS6 and SOCS7 were targets of miR-203. The cells were co-transfected with miR-203 and the constructed plasmids or their respective controls by Lipofectamine 2000. After 48 h of transfection, a dual-luciferase reporter gene assay kit was applied to measure the luciferase activity, and the relative luciferase activity was calculated.

Western blot

The proteins were extracted from cells by 200 μL of lysis buffer containing protease and phosphatase inhibitors. Then the protein concentration was determined by the BCA protein assay kit. Protein samples were separated by SDS-PAGE and transferred to membranes. Then the membranes were blocked with 5% skim milk in PBST at room temperature overnight, followed by incubation with primary antibodies with dilutions suggested by the manufacturer's instructions at room temperature for 1 h. After blotting, membranes were washed for 3 times with PBST and then incubated with HRP-conjugated secondary antibody (1:10,000) at room temperature for 1 h. Then the luminescent signal was detected by ECL chemiluminescent substrate using an automated chemiluminescence analyzer to obtain grayscale values of relevant bands.

Statistical analysis

All experimental results were presented as mean ± standard deviation (Mean ± SD). Statistical analysis for comparisons between multiple groups was conducted using one-way analysis of variance (ANOVA) followed by Dunnett's test. Differences between means were considered statistically significant when $P < 0.05$.

Results

MiR-203a-5p could increase the proliferation of NRK cells and protect them from an inflammatory state

To explore the optimal intervention time point for high glucose-induced cell apoptosis, NRK cells in the LG, HG, and M groups were cultured for 12, 24, 48, and 72 h. Then the cell apoptosis was detected by flow cytometry. The results revealed no significant changes in apoptosis rate at 12 h among the LG, HG, and M groups ($P > 0.05$). Interestingly, the cell apoptosis rate in the high glucose groups were significantly higher than those in the LG groups at 24, 48, and 72 h, especially at 72 h. And mannitol treatment would reduce the apoptosis rate in cells treated with HG (Fig. S1A, B). These results suggested that the optimal intervention time point for high glucose-induced cell apoptosis was 72 h.

Then we investigated whether miR-203a-5p would influence the survival and inflammatory state of NRK cells. Firstly, the NRK cells were transfected with mimics, mimics-NC, inhibitor, or inhibitor-NC, respectively. As shown in Fig. 1A, the expression of miR-203a-5p was significantly lower in the HG, mimics, mimics-NC, inhibitor, and inhibitor-NC groups compared with that in the LG group ($P < 0.05$). While the mRNA level of miR-203a-5p in the mimics group was significantly higher than that in the HG group ($P < 0.05$). In the meantime, the cells transfected with inhibitor showed lower miR-203a-5p expression than that in the HG group ($P < 0.05$). These data suggested miR-203a-5p mimics could up-regulate and the inhibitor could down-regulate the mRNA expression of miR-203a-5p. Interestingly, HG treatment would decrease the proliferation of NRK cells. However, miR-203a-5p mimics obviously rescued this effect caused by HG. And the inhibitor would further inhibit the proliferation of NRK cells compared with that in the HG group (Fig. 1B). Furthermore, the concentrations of TNF-α, IL-1β, IL-4, and IL-6 were significantly increased in the HG group compared with those in the LG group. Surprisingly, miR-203a-5p mimics could reduce and the inhibitor could promote the concentrations of these 4 cytokines compared with those in the HG group (Fig. 1C). A similar results were found in the ROS and MDA productions, which showed the productions of ROS and MDA were higher in the HG group than those in the LG group. And they would be decreased in the mimic group and increased in the inhibitor group when

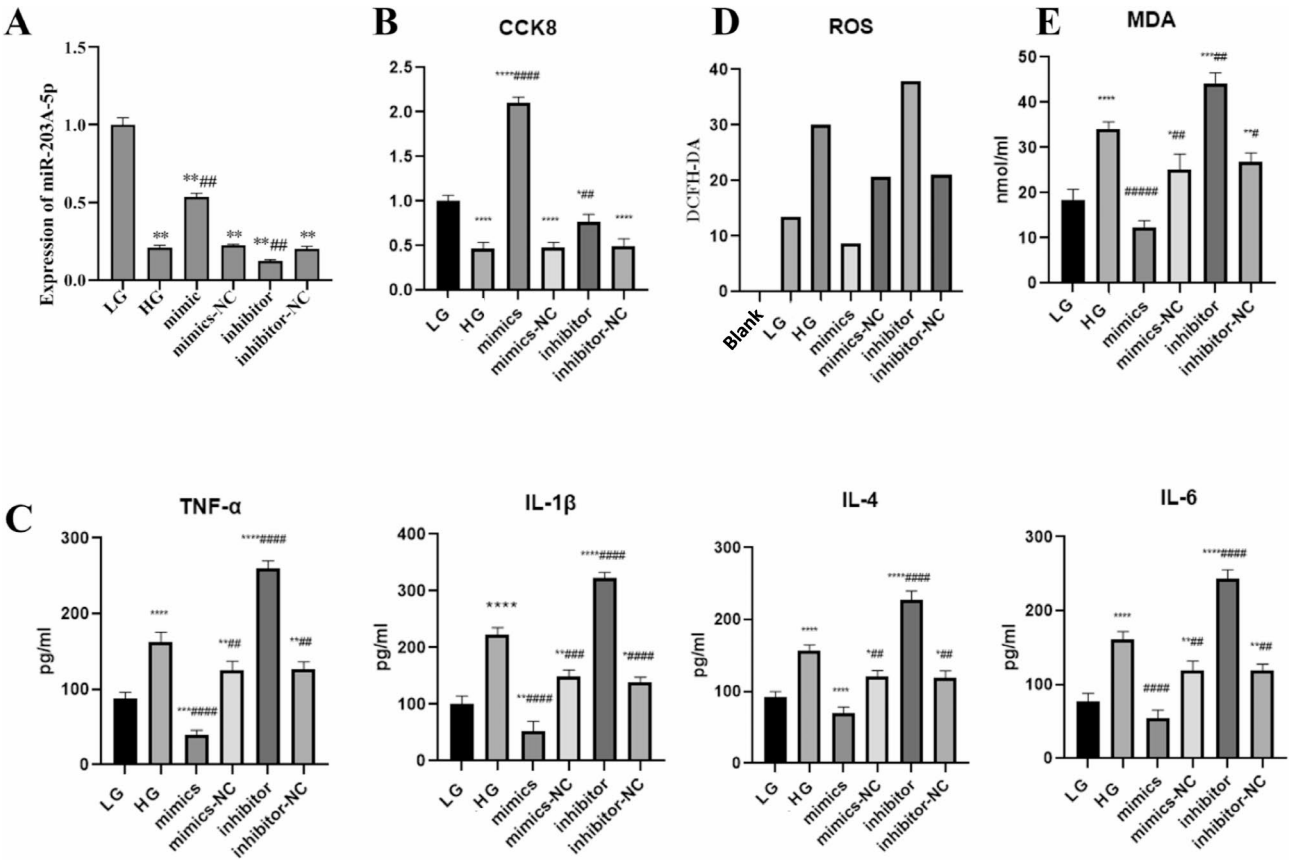

Fig. 1. Effects of miR-203 on the inflammatory and oxidative state of NRK cells. (**A**) The relative expression of miR-203a-5p in NRK cells transfected with miR-203 mimic or inhibitor was detected by RT-PCR. (**B**) The proliferation of NRK cells transfected with miR-203 mimic or inhibitor was measured by CCK8 assay. (**C**) The concentrations of TNF-α, IL-1β, IL-4, and IL-6 in NRK cells transfected with miR-203 mimic or inhibitor were detected by ELISA. (**D**, **E**) The levels of ROS (**D**) and MDA (**E**) in NRK cells transfected with miR-203 mimic or inhibitor were detected by assay kits. $N = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, compared with LG group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$, compared with HG group.

compared with those in the HG group (Figs. 1D, E, S1C). These results indicated miR-203a-5p could increase the proliferation of NRK cells and protect them from an inflammatory state.

MiR-203a-5p could decrease the apoptosis rate of NRK cells treated with HG

Flow cytometry assay was performed to further investigate the effect of miR-203a-5p on the apoptosis of NRK cells. As shown in Fig. 2A–C, HG treatment would promote the apoptosis of NRK cells. However, miR-203a-5p mimics would reverse the effect of HG on NRK cells apoptosis. In addition, the inhibitor of miR-203a-5p would further promote the pro-apoptotic effect of HG on NRK cells. These results were also confirmed by the TUNEL assay, which revealed the apoptosis rate of NRK cells was higher than that in the LG group, and that would be decreased by miR-203a-5p mimics treatment and increase by miR-203a-5p inhibitor transfection when compared with the HG group (Fig. 2D). These results suggested miR-203a-5p could decrease the apoptosis rate of NRK cells treated with HG.

SOCS6 inhibited the proliferation of NRK cells and promoted them to an inflammatory state

It was reported that SOCS6 and SOCS7 were the targeted genes of miR-203[21]. So, we further explored whether miR-203a-5p would influence the proliferation and inflammatory state of NRK cells though SOCS6 and SOCS7. As shown in Fig. 3A, the expression of SOCS6 and SOCS7 were obviously higher in the HG groups compared with those in the LG group. After being transfected with miR-203a-5p mimics, the cells showed a lower expressions of SOCS6 and SOCS7 than those in the HG group. Meanwhile, the levels of SOCS6 and SOCS7 would increase in the cells transfected with miR-203a-5p inhibitor compared with those in the HG group, indicating miR-203a-5p would decrease the expressions of SOCS6 and SOCS7 in NRK cells. Subsequently, we directly investigated the role of SOCS6 in the apoptosis and inflammatory state of NRK cells treated with HG. The results showed SOCS6 overexpression could rescued and SOCS6 deficiency would promote the pro-apoptotic effect caused by HG (Fig. 3B). In addition, the concentrations of TNF-α, IL-1β, IL-4, and IL-6 were higher in the NRK cells overexpressed with SOCS6 than those in the HG groups. And SOCS6 deficiency would lower the concentrations of these 4 cytokines compared with those in the HG group (Fig. 3C). Furthermore, the productions of ROS and MDA were increased in the NRK cells overexpressed with SOCS6 and decreased in the NRK cells with low expression of SOCS6 when compared with those in the HG groups (Fig. 3D, E, Figure S2). These data showed SOCS6 could inhibit the proliferation of NRK cells and promote them to an inflammatory state.

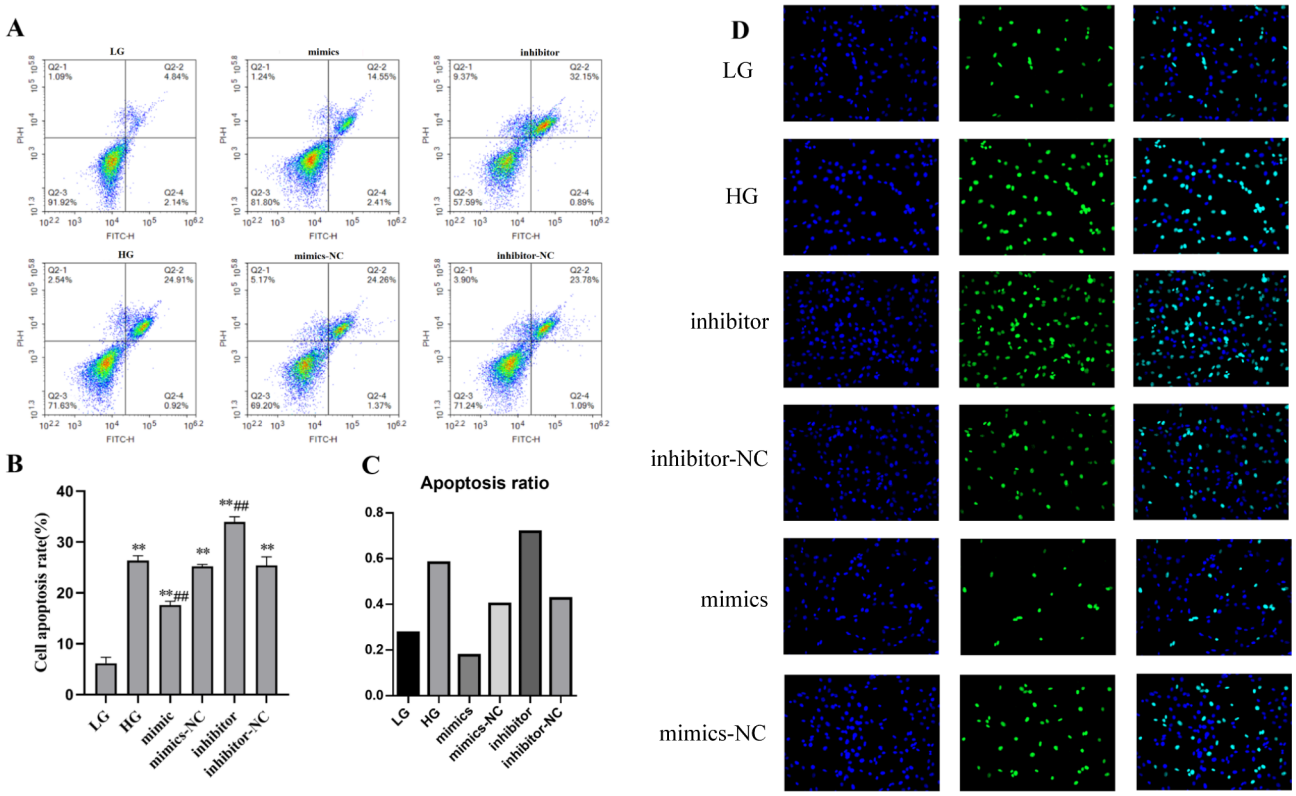

Fig. 2. Effect of miR-203 on the apoptosis of NRK cells. (**A**–**C**) The representative flow cytometry images (**A**) and quantifications (**B**, **C**) of apoptosis in NRK cells transfected with miR-203 mimic or inhibitor. (**D**) The representative TUNEL staining images of NRK cells transfected with miR-203 mimic or inhibitor. $N = 3$. $^{**}P < 0.01$, compared with LG group; $^{\#\#}P < 0.01$, compared with HG group.

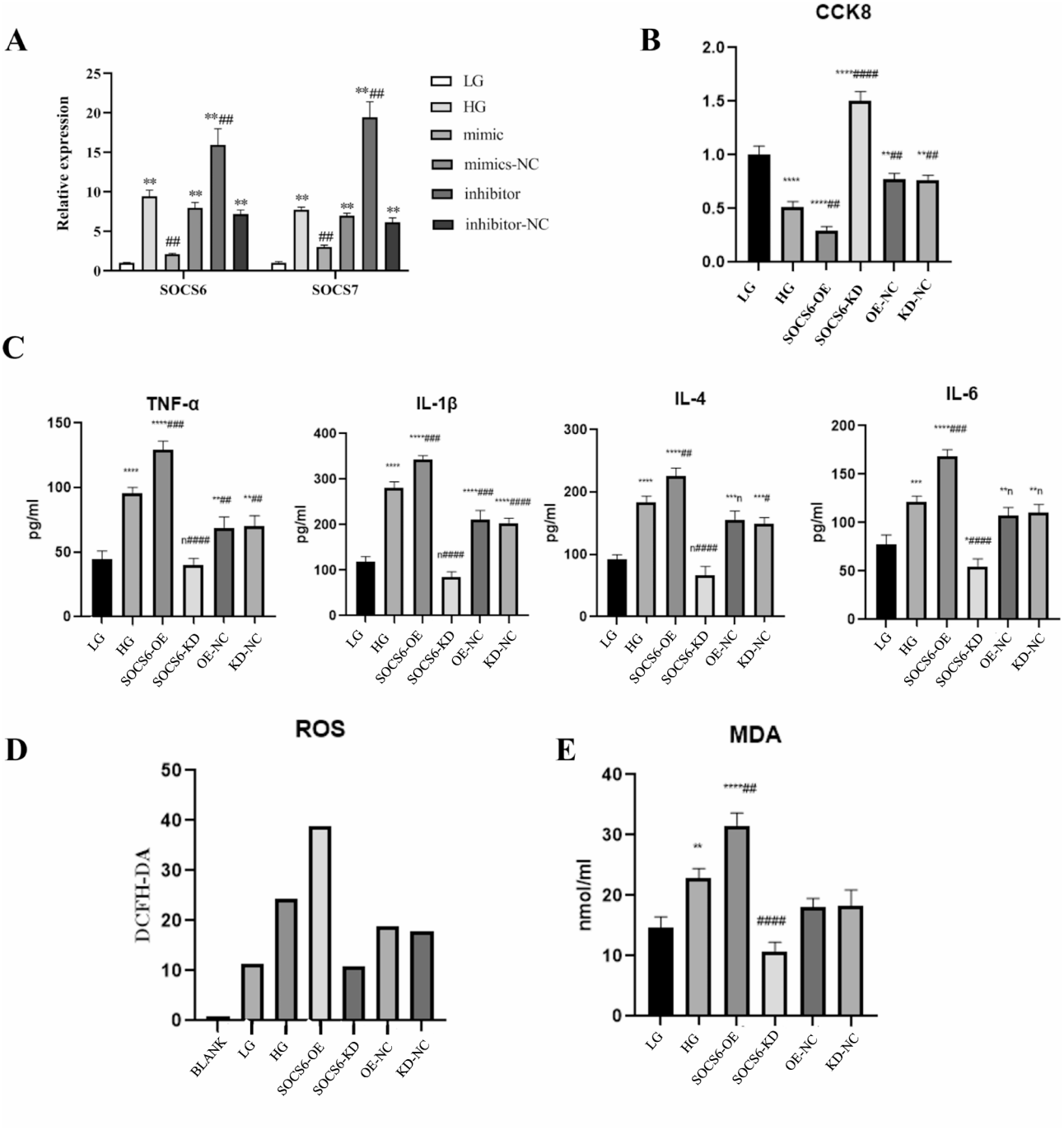

Fig. 3. Effects of SOCS6 on the inflammatory and oxidative state of NRK cells. (**A**) The relative expression of SOCS6 in NRK cells transfected with miR-203 mimic or inhibitor was detected by RT-PCR. (**B**) The proliferation of NRK cells overexpressed or deficient with SOCS6 was measured by CCK8 assay. (**C**) The concentrations of TNF-α, IL-1β, IL-4, and IL-6 in NRK cells overexpressed or deficient with SOCS6 were detected by ELISA. (**D**, **E**) The levels of ROS (**D**) and MDA (**E**) in NRK cells overexpressed or deficient with SOCS6 were detected by assay kits. $N = 3$. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, compared with LG group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$, compared with HG group.

SOCS6 promoted the apoptosis of NRK cells treated with HG

Next, we wondered whether SOCS6 would influence the apoptosis of NRK cells treated with HG. The results revealed SOCS6 overexpression could further promote the pro-apoptotic effect of HG on NRK cells. Surprisingly, the apoptosis rate of NRK cells with low expression of SOCS6 was significantly lower than that in the HG group (Fig. 4A, B), indicating SOCS6 would promote the apoptosis of NRK cells treated with HG. Then, we detected whether SOCS6 and SOCS7 were targets of miR-203 by using the luciferase assay. As shown by Fig. 4C, the

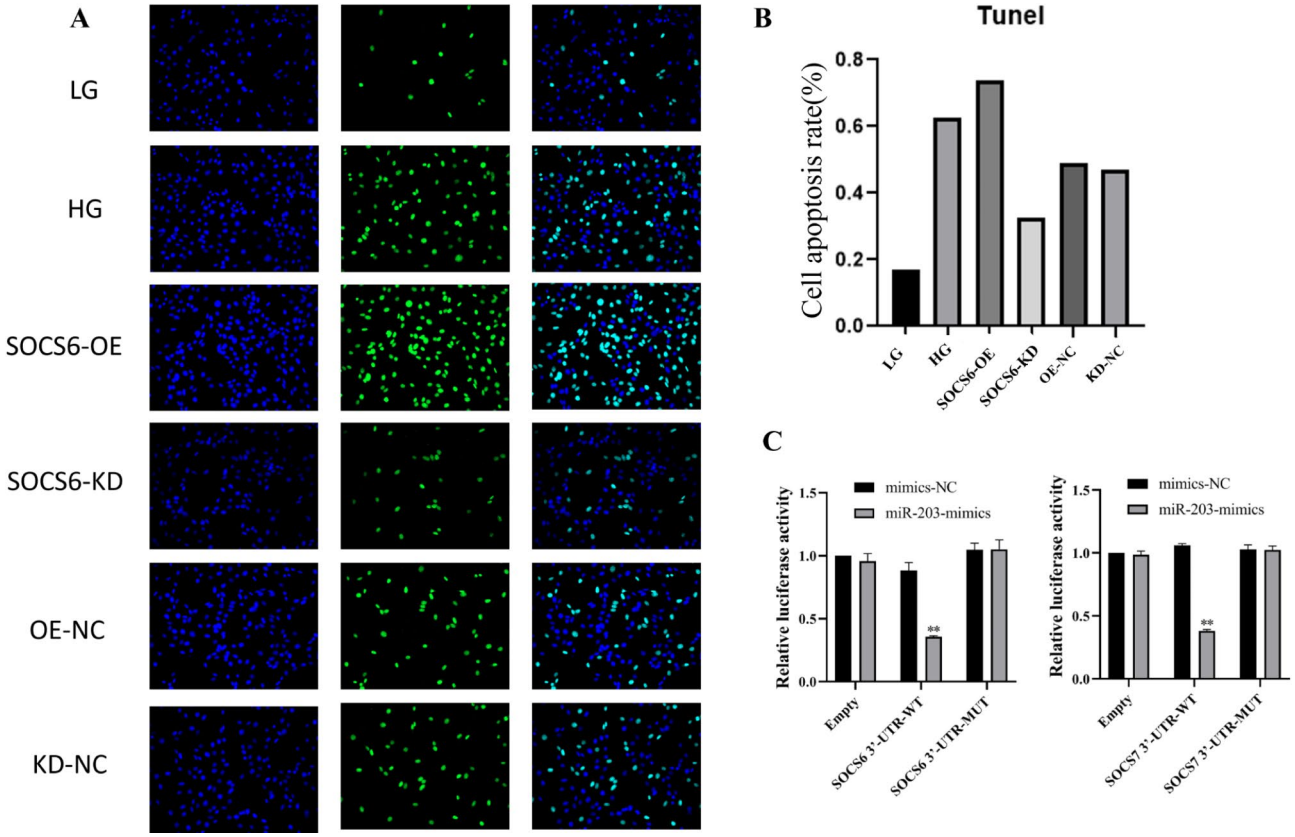

Fig. 4. Effect of SOCS6 on the apoptosis of NRK cells. (**A**, **B**) The representative TUNEL staining images (**A**) and quantifications (**B**) of NRK cells overexpressed or deficient with SOCS6. (**C**) The relative luciferase activities of NRK cells transfected with mimic-NC or miR-203-mimic and SOCS6 3'-UTR-WT or SOCS6 3'-UTR-MUT plasmids. And the relative luciferase activities of NRK cells transfected with mimic-NC or miR-203-mimic and SOCS7 3'-UTR-WT or SOCS7 3'-UTR-MUT plasmids. $N = 3$. $^{\#\#}P < 0.01$, compared with mimic-NC + SOCS6 3'-UTR-WT group for SOCS6 and mimic-NC + SOCS7 3'-UTR-WT group for SOCS7.

relative luciferase activities were significantly lower in the NRK cells transfected with miR-203 and SOCS6 3'-UTR-WT or SOCS7 3'-UTR-WT than those in the cells transfected with mimics-NC and SOCS6 3'-UTR-WT or SOCS7 3'-UTR-WT, indicating miR-203 could directly target to the 3'-UTR of SOCS6 and SOCS7, then regulating their expressions.

miR-203a-5p inhibited the JAK/STAT3 pathway in NRK cells

Previous study reported SOCS6 would work though JAK/STAT signaling[22]. However, whether miR-203a-5p could regulate JAK/STAT signaling was unknown. As shown in Fig. 5, HG treatment obviously increased the phosphorylation of JAK1 and STAT3. And these effects were inhibited by miR-203a-5p mimic transfection. Whereas the inhibitor group showed enhanced phosphorylation of JAK and STAT3 compared with those in the HG group. These results revealed 203a-5p could inhibit JAK/STAT signaling in NRK cells treated with HG.

Discussion

DN is one of the primary microvascular complications of diabetes and a major cause of late-stage renal disease (ESRD)[23]. Even though previous studies showed miR-203 may related to the development of DN, the underlying mechanism and targeted gene of miR-203 in DN are still unclear. Our current study revealed HG treatment would damage the NRK cells and down-regulate the expression of miR-203. And miR-203 mimic could rescue the injury effects on NRK cells caused by HG via targeting SOCS6 and SOCS7 and inhibiting the activation of JAK/STAT signaling.

Hyperglycemia was one of the potential pathogenic factors of DN[24]. And HG was commonly used as a classical treatment for the establishment of a kidney injury cell model[20]. It was reported that HG treatment would increase the apoptosis rate of NRK cells[25]. And the NRK cells produced abundant ROS and showed an oxidative damage state when stimulated with HG[26]. In addition, HG treatment could promote the NRK cells to secrete inflammatory factors, such as TNF-α, IL-1β, and IL-6[27]. Antioxidants such as NRF2, whose nuclear translocation played a critical role, and anti-inflammatory drugs, have shown effectiveness in the clinical treatment of DN[28]. In addition, GLP-1 could reduce the production of ROS and inhibit inflammatory response, thus showing beneficial effects on DN[29]. In line with these findings, we identified that HG treatment would lead to a series of damaging effects on NRK cells, including an increase in cell apoptosis, an up-regulation in

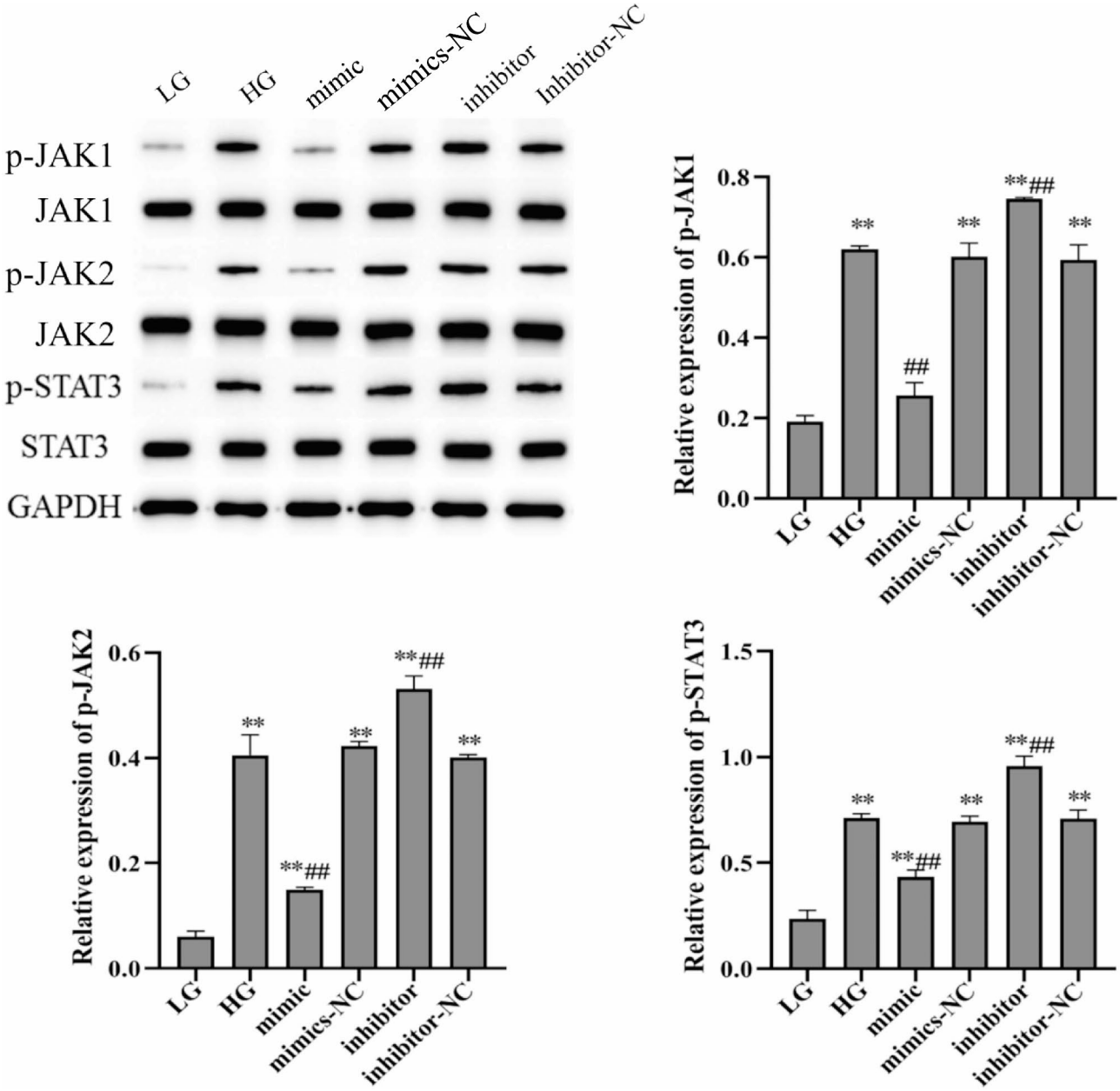

Fig. 5. Effect of miR-203 on the JAK/STAT pathway in NRK cells. The representative western blotting images and quantifications of JAK1, p-JAK1, JAK2, p-JAK2, STAT3, and p-STAT3 in NRK cells transfected with miR-203 mimic or inhibitor. $N = 3$. $^{**}P < 0.01$, compared with LG group; $^{\#\#}P < 0.01$, compared with HG group.

inflammatory factors secretion, and a promotion in ROS production, indicating the HG-induced kidney injury cell model was successfully established in vitro.

MiRNAs play roles in various cellular processes in DN, including controlling insulin resistance, inflammatory responses, and apoptosis[30]. Previous studies reported that both Mm_miR-29b and Mm_ let-7a were decreased in diabetic mice[31]. The upregulation of Mm_miR-29b contributed to the protective effects of linagliptin in the context of diabetes[32]. Further investigating the effect of other miRNAs on the development of DN may unveil more therapeutic targets of DN. Besides, miR-203 was considered as a new risk factor of DN[10]. And miR-203-3p possessed the anti-inflammatory, anti-oxidative, and anti-apoptotic abilities in mice podocytes treated with HG[11]. Moreover, miR-203-3p might improve the development of DN[8]. Similar to these reports, we here revealed that miR-203 reduced high glucose-induced apoptosis in vitro, promoting the cell proliferation, attenuating the concentrations of inflammatory factors, and blocking the production of ROS, indicating miR-203 was of benefit to alleviate DN development. It is well known that the upregulation of SOCS genes in DN can significantly improve renal function and reduce interstitial fibrosis damage[33]. Here, we found that miR-203 could directly targeted SOCS6 and SOCS7 and negatively regulated their expressions. SOCS6 deficiency effectively rescued the

damaged effects caused by HG, indicating miR-203 improved the function of NRK cells by down-regulating the expression of SOCS6.

The JAK/STAT pathway has dual functions in signal transduction and gene transcription, which can mediate inflammation transmission, cell proliferation, and fibrosis. In diabetic nephropathy, the JAK/STAT signaling pathway can be activated by various substances, including high glucose, reactive oxygen species, and advanced glycation end products. These substances continuously promote the phosphorylation of JAKs and STATs, which cause nuclear translocation and promote the transcription and translation of STAT-dependent target genes, such as inflammatory factors and growth factors. And these factors will further lead to cell growth, inflammation, and fibrosis, ultimately accelerating disease onset and progression. During the initial phase of DN, Ang II promoted the synthesis of type IV collagen, contributing to disease advancement[34]. In contrast, glucagon-like peptide-1 (GLP-1) exerted therapeutic effects by inhibiting Ang II-mediated upregulation of type IV collagen and fibronectin[35]. Interestingly, activation of the JAK/STAT pathway can stimulate excessive proliferation and growth of glomerular mesangial cells, promoting the production of cytokines and extracellular matrix proteins such as type IV collagen and fibronectin, thereby leading to DN[36]. In addition, activation of the JAK/STAT pathway induces the expression of SOCS, which acts as an internal inhibitor of the JAK/STAT pathway. Therefore, the JAK/STAT pathway and SOCS can be considered as negative feedback systems. This study reports that the expression levels of p-JAK1, p-JAK2, and p-STAT3 were declined in the mimics group. These data indicate that miR-203 down-regulates the JAK/STAT pathway by targeting SOCS6 and SOCS7 genes, thus protecting against renal damage in diabetic nephropathy.

Even though we found that miR-203 attenuated inflammation and apoptosis of NRK cells treated with HG. Actually, an in vivo DN model will be more conducive to elucidate the function of miR-203 in DN. We will confirm our results in vivo in future work. Moreover, in order to increase the clinical application, the concentration of miR-203 should be measured in the blood of patients with or without DN. Furthermore, the impact of miR-203 on other renal cells, particularly podocytes, in the progression of DN merits further exploration.

In conclusion, miR-203 blocks the JAK/STAT pathway by targeting SOCS6 and SOCS7 genes, thereby alleviating inflammation and apoptosis in renal cells of diabetic nephropathy. These findings provide new insights for exploring the clinical application of miR-203 in DN.

Data availability

All data generated or analyzed during this study are included in this published article. And the original images of western blot were available in the file named Supplementary Material.

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Author contributions

Y.W. and M.N. carried out the studies, participated in collecting data, and drafted the manuscript. P.P. and D.L., Y.W. performed the statistical analysis and participated in its design. Y.W. and M.N. participated in acquisition, analysis, or interpretation of data and draft the manuscript. All authors read and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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