MicroRNA-494-3p Exacerbates Renal Epithelial Cell Dysfunction by Targeting SOCS6 under High Glucose Treatment

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Keywords
Diabetic nephropathy · miR-494-3p · Suppressor of cytokine signaling 6 · Renal fibrosis

Abstract
Background: Diabetic nephropathy is a common complication of the kidneys induced by diabetes and is the main cause of end-stage renal disease. MicroRNA-494-3p was reported to be upregulated in renal tissues collected from db/db mice, but its specific role in diabetic nephropathy was still unclear. This study aimed to explore the effect of miR-494-3p on renal fibrosis using an in vitro cell model of diabetic nephropathy. Methods: After human renal tubular epithelial cells (HK-2) were treated with high glucose (HG), the viability and apoptosis of cells were examined by CCK-8 assays and flow cytometry analyses. Additionally, protein levels of fibronectin, collagen I, collagen III, collagen IV, and epithelial-mesenchymal transition (EMT) markers in HG-induced HK-2 cells were quantified by Western blotting. miR-494-3p expression in HK-2 cells was detected by reverse-transcription quantitative polymerase chain reaction. The binding relation between miR-494-3p and the messenger RNA suppressor of cytokine signaling 6 (SOCS6) was detected by luciferase reporter assays. Results: HG reduced cell viability and enhanced cell apoptosis in a time- or concentration-dependent manner. Additionally, HG induced collagen accumulation and triggered the EMT process. miR-494-3p was upregulated in HG-treated HK-2 cells. miR-494-3p inhibition alleviated HG-induced cell dysfunction. Mechanistically, miR-494-3p bound with SOCS6 and negatively regulated SOCS6 expression. Moreover, silencing SOCS6 rescued the suppressive effect of miR-499-5p inhibition on HG-induced cell dysfunction. Conclusion: miR-494-3p aggravates renal fibrosis, EMT process, and cell apoptosis by targeting SOCS6, suggesting that the miR-494-3p/SOCS6 axis may become a potential strategy for the treatment of diabetic nephropathy.

Introduction
Diabetic nephropathy (DN) is a microvascular complication of diabetes mellitus and is a leading cause of chronic kidney disease, occupying 45% of end-stage renal diseases [1, 2]. The typical pathological features of DN include functional and structural changes in the kidney such as the thickening of the glomerular basement membrane, glomerular or tubular hypertrophy, and extracellular matrix (ECM) accumulation [3, 4]. Renal fibrosis is characterized by the accumulation of ECM proteins in
glomerular basement membranes and the tubulointerstitium [5]. Studies reveal that renal epithelial cells are induced to undergo epithelial-mesenchymal transition (EMT) via the activation of Smad2/3 signaling, thereby promoting ECM synthesis as well as deposition and finally resulting in extensive renal tissue fibrosis [6]. Known factors including high glucose-induced metabolic abnormalities, hypertension, and genetic influence were reported to be implicated with the development of DN [7, 8]. To improve outcomes for patients with DN, innovations such as characterization of new biomarkers and exploration of molecular mechanisms underlying DN progression are essential.

MicroRNA (miRNA) is a class of short and single-stranded noncoding transcripts [9]. miRNA has been intensively studied since 2 decades ago, leading to a drastic change in our understanding of gene regulation and epigenetic processes [10]. Although lacking the protein-coding capacity, miRNAs are capable of inhibiting messenger RNA (mRNA) translation or promoting mRNA degradation by binding with the complementary 3′ untranslated region (3′UTR) of mRNAs at the posttranscriptional level [11, 12]. In DN, a multitude of miRNAs were discovered as critical regulators of several phases [13, 14]. For example, miRNA-29c exerts an effect on the expression of inflammatory cytokines by targeting tristetraprolin in DN [15]. Moreover, miR-23c was reported to inhibit pyroptosis of renal tubular epithelial cells by targeting embryonic lethal abnormal vision-like RNA binding protein 1 in DN [16]. Recently, miR-494-3p was identified to play a specific role and mechanism of SOCS6 in DN remain uncharacterized.

Suppressor of cytokine signaling 6 (SOCS6), containing an SH2 domain and a CIS homolog domain, belongs to the cytokine-induced signal transducer and activator of transcription inhibitor [20]. Additionally, SOCS6 was also reported to be involved in the regulation of renal fibrosis in end-stage kidney failure [21]. Nevertheless, the specific role and mechanism of SOCS6 in DN require further exploration.

In the current study, we aimed to figure out the biological functions and molecular mechanism of miR-494-3p in DN. The study might provide a promising biomarker for DN treatment.

Materials and Methods

Cell Culture and Cell Treatment

The human renal tubular epithelial cell line (HK-2; Chinese Academy of Sciences, Shanghai branch, China) was incubated with Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Invitrogen) at 37°C with 5% CO2. Cell suspensions were seeded onto tissue culture flasks. After trypsinization, cells were incubated with DMEM containing 0.5% FBS for 24 h until reaching 70–80% confluence and then subjected to cell treatment.

For cell treatment, high glucose (HG) was added into cell medium to induce cell injury according to a previous study [22]. In the osmotic control group, cells were treated with 24.5 mM mannitol and 5.5 normal mM glucose. In the control (Con) group, 5.5 mM glucose was used. Cells treated with 15 mM, 25 mM, or 35 mM HG were incubated for 24 h and then subjected to cell viability detection.

Cell Transfection

miR-494-3p inhibitor/mimics were used to knockdown or overexpress miR-494-3p in HK-2 cells, and their negative controls (NC) were named as NC inhibitor/NC mimics. The short hairpin RNA targeting SOCS6 (sh-SOCS6) was used to knock down SOCS6 with sh-NC as the negative control. All these vectors were synthesized by GenePharma (Shanghai, China). Before transfection, cells (2 × 105) were seeded onto a 6-cm culture dish and starved for 24 h. Next, the above vectors were transfected into HK-2 cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s recommendations. After 48 h of transfection, reverse-transcription quantitative polymerase chain reaction (RT-qPCR) or Western blotting was performed to examine the transfection efficiency.

Reverse-Transcription Quantitative Polymerase Chain Reaction

The extraction of total RNA from HK-2 cells was performed using TRIzol reagent (Invitrogen). The reverse transcription of total RNA into cDNA was conducted using a PrimeScript® RT reagent Kit (Takara, Dalian, China). qPCR was implemented using the SYBR green assay (Invitrogen) on Applied Biosystems 7500 (ABI, Foster City, CA, USA). The relative gene expression was calculated using the 2−ΔΔct method. miR-494-3p expression was normalized to U6, while mRNA expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All RT-qPCR reactions were performed three times. Sequences of primers used for PCR were as follows: miR-494-3p forward, 5′-GGG TTA AAC ACA CAC GGG AA-3′, and reverse, 5′-GCC AGG TCC GAG GT-3′; U6 forward, 5′-ATT GTA ACG ATA CAG AGA AGA TT-3′, and reverse, 5′-GGA ACG CTT CAC GAA TTT G-3′; SOCS6 forward, 5′-CTC TCA CCA TTG CTA CCT C-3′, and reverse, 5′-TGA GTC CAC TGA AGT TCC T-3′; GAPDH forward, 5′-TGG ACA GTC AGC CGC ATC TTC TTT-3′, and reverse, 5′-ACC AAA TCC GTT GAC TAC GCC ATT-3′.

Western Blotting

Protein samples were collected from HK-2 cells using RIPA lysis buffer (Beyotime, Beijing, China) and isolated on 10% SDS-PAGE. Then, samples were moved onto PVDF membranes and blocked with 5% nonfat milk. Afterward, the membranes were in-
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cubated with primary antibodies against collagen I (ab34710, 1:1,000; Abcam, Cambridge, MA, USA), E-cadherin (ab1416, 1:50; Abcam), collagen III (ab7778, 1:5,000; Abcam), N-cadherin (ab18203, 1:1,000; Abcam), collagen IV (ab6586, 1:100; Abcam), fibronectin (ab2413, 1:1,000; Abcam), SOCS6 (ab197335, 1:200; Abcam), and GAPDH (ab8245, 1:500; Abcam) overnight at 4°C. Next, the membranes were incubated with secondary antibody Goat Anti-Rabbit IgG H&L (ab6721, 1:2,000; Abcam) at darkness for 2 h. At last, protein bands were visualized by using ECL reagent (Millipore, Shanghai, China), and the intensity of blots was quantified by using ImageJ software (National Institutes of Health, Bethesda, MA, USA).

**Cell Counting Kit-8 Assay**
After cells were treated with HG (15 mM, 25 mM, or 35 mM HG for 24 h; 25 mM HG for 0 h, 12 h, 24 h, 48 h, or 72 h) and trans-

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Fig. 1. HG induces HK-2 cell dysfunction, and miR-494-3p expression is increased in HG-treated cells. The viability of HK-2 cells treated with 15 mM, 25 mM, and 35 mM HG for 24 h (a) and HK-2 cells treated with 25 mM HG for 12, 24, 48, or 72 h (b) were measured by CCK-8 assays. c The apoptosis of HK-2 cells treated with 25 mM HG for 12, 24, 48, or 72 h was evaluated by flow cytometry analyses. d Western blotting was used to quantify protein levels of collagen I, collagen III, collagen IV, fibronectin, E-cadherin, and N-cadherin in HK-2 cells treated with HG (25 mM, 24 h), mannitol (24.5 mM, 24 h), or normal glucose (5.5 mM, 24 h). e RT-qPCR analysis was used to examine miR-494-3p expression in HK-2 cells treated with HG (25 mM, 24 h), mannitol (24.5 mM, 24 h), or normal glucose (5.5 mM, 24 h). *p < 0.05, **p < 0.01, ***p < 0.001.
cells were treated with 15 mM, 25 mM, and 35 mM HG for viability of HK-2 cells was gradually decreased after HK-2 to mimic cell injury in vitro. According to Figure 1a, the 24 h. Under the treatment of 25 mM HG, cell viability was treated with 25 mM HG for indicated time (0, 12, 24, 48, or 72 h) or indicated transfection (NC inhibitor, miR-494-3p inhibitor, or sh-SOCS6) were washed with cold phosphate-buffered saline and then resuspended in binding buffer. Subsequently, HK-2 cells were stained with annexin V-FITC and PI for 15 min in the dark at room temperature. The apoptotic rate of HK-2 cells was analyzed by using the FACScan Flow Cytometer (BD Biosciences). Cells in (Annexin V-FITC)+/PI− (Q3) area and (Annexin V-FITC)+/PI− (Q3) area were regarded as apoptotic cells.

Luciferase Reporter Assay
The binding site between miR-494-3p and SOCS6 was predicted with TargetScan (http://www.targetscan.org/vert_72/). The wild-type (WT) or mutant (Mut) sequence of SOCS6 3′UTR was subcloned into the pmirGLO luciferase reporter (Promega, Shanghai, China). miR-494-3p inhibitor/mimics (or NC inhibitor/mimics) were co-transfected with pmirGLO-SOCS6-Wt or pmirGLO-SOCS6-Mut into HK-2 cells using Lipofectamine 3000 (Invitrogen). The detection of luciferase activities was operated using a Dual-Luciferase Reporter System (Promega, Shanghai, China). The activity of firefly luciferase was normalized to that of Renilla luciferase.

Statistical Analysis
All experiments were repeated in triplicate. The data are shown as the mean ± standard deviation. The statistical analysis was performed using SPSS software (IBM, Armonk, NY, USA). The unpaired Student’s t test was employed to compare the differences between 2 groups, and one-way ANOVA followed by Tukey’s post hoc analysis was applied to the comparison among more than 2 groups. A value of p < 0.05 was considered statistically significant.

Results

HG Induces HK-2 Cell Dysfunction, and miR-494-3p Expression Is Increased in HG-Treated Cells
In the current study, HK-2 cells were treated with HG to mimic cell injury in vitro. According to Figure 1a, the viability of HK-2 cells was gradually decreased after HK-2 cells were treated with 15 mM, 25 mM, and 35 mM HG for 24 h. Under the treatment of 25 mM HG, cell viability was approximately 50% of the control group. Next, cells were treated with 25 mM HG for indicated time (0, 12, 24, 48, or 72 h), and we found that HK-2 cell viability was decreased by 25 mM HG in a time-dependent manner (Fig. 1b). Correspondingly, the apoptosis rate of HK-2 cells was gradually enhanced by 25 mM HG with the increase in incubation time (Fig. 1c). For subsequent experiments, HK-2 cells were treated with 25 mM HG for 24 h. Furthermore, Western blotting revealed that HG treatment obviously upregulated protein levels of collagen I, collagen III, collagen IV, and fibronectin in cells (Fig. 1d). Since mounting reports claimed that HG induced renal fibrosis by activating EMT process, we measured protein levels of EMT markers (E-cadherin and N-cadherin) in HK-2 cells. The results of Western blotting showed that HG treatment triggered a decrease in E-cadherin protein level and an increase in N-cadherin protein level (Fig. 1e). The data above demonstrated that an in vitro HG-induced cell model was successfully established. Then, we evaluated the expression of miR-494-3p in HK-2 cells. RT-qPCR results demonstrated that miR-494-3p expression was higher in HG groups than that in the control group or mannitol group (Fig. 1f). In conclusion, HG treatment induces HK-2 cell dysfunction, and miR-494-3p was upregulated in HG-treated HK-2 cells.

miR-494-3p Knockdown Inhibits ECM Accumulation, Cell Apoptosis, and EMT Process
We then assessed the effects of miR-494-3p on HK-2 cell phenotypes. First, miR-494-3p expression was knocked down by transfecting miR-494-3p inhibitor into HG-induced HK-2 cells (Fig. 2a). Subsequently, knockdown of miR-494-3p increased cell viability but reduced the cell apoptosis rate (Fig. 2b–c). Moreover, the reduced protein levels of fibronectin, collagen I, collagen III, and collagen IV were observed in the miR-494-3p inhibitor group (Fig. 2d). Additionally, in response to miR-494-3p inhibition, E-cadherin protein level was increased while N-cadherin protein level was decreased in cells (Fig. 2e). Altogether, miR-494-3p depletion suppresses collagen accumulation, cell apoptosis, and EMT process.

miR-494-3p Directly Targets and Negatively Regulates SOCS6
Considerable studies proposed that miRNA can bind with 3′UTR of mRNA to regulate the expression of downstream targets [11, 12]. Hence, we anticipated that miR-494-3p also functioned in the same pattern in HK-2 cells. Based on the miRDB website (http://mirdb.org/), a total of 7 potential target mRNAs were predicted to possess the binding sequences on miR-494-3p (condition: binding score ≥99). According to RT-qPCR analysis, in response to miR-494-3p inhibition, SOCS6

DOI: 10.1159/000521647

Kidney Blood Press Res 2022;47:247–255

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was significantly upregulated in HK-2 cells among candidate mRNAs (Fig. 3a). Hence, SOCS6 was selected for the following experiments. As shown in Figure 3b, the binding site between miR-494-3p and SOCS6 was predicted with Targetscan (http://www.targetscan.org/vert_72/). Then, we overexpressed miR-494-3p by transfection of miR-494-3p mimics into HK-2 cells (Fig. 3c). The luciferase reporter assay showed that the luciferase activity of wild-type pmirGLO-SOCS6 was significantly increased by the miR-494-3p inhibitor but decreased by miR-494-3p mimics. Additionally, no significant alteration was detected in mutant pmirGLO-SOCS6 groups (Fig. 3d). Similarly, miR-494-3p mimics also reduced SOCS6 protein level in HK-2 cells (Fig. 3e). At last, RT-qPCR and Western blotting indicated that SOCS6 mRNA and protein levels were downregulated in the HG group compared with those in the control group (Fig. 3f). Overall, miR-494-3p directly targets SOCS6 3’UTR and negatively regulates SOCS6 expression.

**SOCS6 Silencing Reverses the Inhibitory Impact of the miR-494-3p Inhibitor on ECM Accumulation, Cell Apoptosis, and EMT Process**

To validate whether miR-494-3p exerted its biological function by targeting SOCS6, rescue assays were carried out. Western blotting illustrated that SOCS6 protein level was obviously knocked down by transfection of sh-SOCS6 in HG-induced HK-2 cells (Fig. 4a). In addition, the suppressive effect of the miR-494-3p inhibitor on cell

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**Fig. 2.** miR-494-3p knockdown inhibits ECM accumulation, cell apoptosis, and EMT process. **a** The knockdown efficacy of the miR-494-3p inhibitor was evaluated by RT-qPCR analysis in HG-treated HK-2 cells. **b, c** CCK-8 assays and flow cytometry analyses were utilized to detect cell viability and apoptosis in the context of miR-494-3p inhibition. **d, e** Western blotting was used to examine the effects of the miR-494-3p inhibitor on protein levels of collagen I, collagen III, collagen IV, fibronectin, E-cadherin, and N-cadherin. *p < 0.05, **p < 0.001.
apoptosis was reversed by SOCS6 inhibition (Fig. 4b). Furthermore, the reduction of collagen I, collagen III, collagen IV, and fibronectin protein levels induced by the miR-494-3p inhibitor was partially offset by knockdown of SOCS6 (Fig. 4c). Eventually, the depletion of SOCS6 counteracted the increase in E-cadherin protein level and the reduction of N-cadherin protein level induced by the miR-494-3p inhibitor under HG treatment (Fig. 4d). In summary, SOCS6 silencing attenuates the suppressive effect of miR-494-3p inhibition on ECM accumulation, cell apoptosis, and EMT process.

**Discussion**

DN has become the second leading cause of end-stage renal disease, second only to glomerulonephritis [23]. Due to its inherent complexity, the treatment of DN is more difficult than that of other kidney diseases [23, 24]. Therapies like blood glucose administration, blood pressure controlling, or organ transplant were used to improve DN patients’ symptoms [25]. However, the overall 5-year survival rate remains unsatisfied [26]. Thus, identifying novel biomarkers for DN is urgently needed.
Multiple dysregulated miRNAs have been identified as potential biomarkers for DN treatment [27–29]. For example, miR-192 inhibits renal tubulointerstitial fibrosis by targeting early growth response factor 1 in DN [30]. miR-142-5p is involved in the activation of autophagy and mesangial cell injury by targeting SOCS6 in DN [31].
A previous study reported that miR-494-3p displays high expression in renal tissues of db/db mice and in HG-induced mesangial cells [19]. Similarly, miR-494-3p was also upregulated in HK-2 cells under HG treatment. Additionally, miR-494-3p inhibition significantly alleviated HG-induced ECM accumulation, EMT process, and cell apoptosis. In mechanism, considerable miRNAs including miR-185, miR-206, and miR-140 can exert their biological functions in DN by binding with mRNA 3′UTR [32–34]. According to bioinformatics analysis, SOCS6 is predicted to possess the binding site on miR-494-3p. Importantly, our experiments validated that miR-494-3p directly targeted SOCS6 3′UTR and negatively regulated SOCS6. Additionally, SOCS6 displayed relatively low expression in HG-treated HK-2 cells.

Previously, SOCS6 has been reported to inhibit EMT process in colorectal cancer cells and implicates in the pathogenesis of renal fibrosis [21, 35]. Likewise, our study validated that the suppressive effect of the miR-494-3p inhibitor on ECM accumulation, cell apoptosis, and EMT process was significantly rescued by SOCS6 silencing. The results indicated that miR-494-3p promotes HK-2 cell injury under HG treatment by targeting SOCS6.

**Conclusion**

We revealed that miR-494-3p exacerbates renal fibrosis by targeting SOCS6 in DN for the first time. This novel discovery suggests that miR-494-3p may serve as a potential biomarker for DN treatment. However, the roles of miR-494-3p and SOCS6 in DN still need to be explored in vivo. The pathogenesis of DN is complicated, and other mechanisms of miR-494-3p deserve to be investigated in the future.

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**Acknowledgments**

We appreciate all participants in this work.

**Statement of Ethics**

An ethics statement was not required for this study type, and no human or animal subjects or materials were used.

**Conflict of Interest Statement**

The authors declare that there are no competing interests in this study.

**Funding Sources**

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

**Author Contributions**

Study design: Xianjun Xue and Minjie Liu; data analysis: Xianjun Xue, Minjie Liu, Yulu Wang, Yanlei Yang, Zhiping Li, Ruifang Shi, and Yueting Miao; manuscript writing: Xianjun Xue; data collection supervision: Yulu Wang, Yanlei Yang, Zhiping Li, Ruifang Shi, and Yueting Miao. All authors saw and approved the final manuscript.

**Data Availability Statement**

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.
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