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Original Article

Study of the regulation and mechanism of SOX9 on podocytes in diabetic nephropathy by regulation of Nrf2-Keap1-ARE signaling pathway

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Abstract: Diabetic nephropathy (DN) is a common complication of diabetes in the clinic. Podocyte injury is key to the occurrence and development of DN. SRY-associated high mobility group box 9 (SOX9) is involved in the development and progression of various diseases. However, the role and mechanism of SOX9 in DN podocytes has not been elucidated. Mouse podocyte MCP-1 was cultured *in vitro* and randomly divided into 4 groups; including control group that was cultured in normal glucose concentration (5.5 mmol/L), high glucose group that was cultured in high glucose (30 mmol/L), SOX9 overexpression group, and SOX9 siRNA group that were transfected with pcDNA-SOX9 plasmid and SOX9 siRNA in the high glucose environment, respectively. Real-time PCR and Western blot were used to detect SOX9 expression. MTT assay was selected to assess cell proliferation. Caspase 3 activity was tested by Caspase-3 Activity Assay kit. Myeloperoxidase (MPO), superoxide dismutase (SOD) activities, and reactive oxygen species (ROS) levels were measured. The expression of Nrf2, Keap1 and ARE mRNA were determined by real-time PCR. The expression of inflammatory factors tumor necrosis factor-α (TNF-α) and interleukin-2 (IL-2) were detected by enzyme-linked immunosorbent assay (ELISA). In high glucose environment, SOX9 expression was significantly increased, MCP-1 cell proliferation was inhibited, Caspase 3 activity was increased, SOD activity was decreased, ROS and MPO were elevated, TNF-α and IL-2 expression was upregulated, Keap1 expression was enhanced, and Nrf2 and ARE levels were decreased compared with the control group ($P < 0.05$). Their changes were more significant in SOX9 overexpressed MCP-1 cells in the high glucose environment ($P < 0.01$). On the contrary, downregulation of SOX9 expression by SOX9 siRNA promoted the proliferation of MCP-1 cells, decreased the activity of Caspase 3, enhanced the activity of SOD, reduced ROS and MPO, downregulated the expressions of TNF-α and IL-2, declined the expression of Keap1, and elevated the expressions of Nrf2 and ARE, compared with the high glucose group ($P < 0.05$). SOX9 expression increased in the high glucose environment. Down-regulation of SOX9 expression can inhibit apoptosis, restrain oxidative stress and inflammation, and promote podocyte proliferation by regulating the Nrf2-Keap1-ARE signaling pathway.

Keywords: Diabetic nephropathy, SOX9, apoptosis, oxidative stress, Nrf2-Keap1-ARE, inflammation

Introduction

As one of the major metabolic diseases, the incidence of diabetes is increasing. There are nearly 300 million people with diabetes in the world, and more than 100 million people with diabetes in China [1, 2]. Diabetic nephropathy (DN) is one of the most common and important diabetic complications in clinical practice. It has a high incidence and slow progress. It has become the second most important cause of end-stage renal disease, which can lead to end-stage renal failure [3]. The incidence of DN is increasing year by year in China, only second to various glomerulonephritis [4]. Due to the existence of metabolic disorders and complicated conditions, it is very difficult during the end stage DN, which has become one of the medical treatment conundrums [5]. Diabetes and other factors cause renal vascular damage, leading to extracellular matrix hyperplasia, thickening of glomerular basement membrane, and glomerular sclerosis [6, 7]. Glucose and lipid metabolism disorders, inflammation, oxidative stress, and apoptosis are all important factors in the occurrence and development of

DN [8]. It is confirmed that podocyte injury plays a key role in the occurrence of DN [9]. Podocytes are highly differentiated cells in kidney tissue with poor proliferative capacity and compensatory capacity, which can lead to disruption of the integrity of the filtration membrane, causing proteinuria and glomerular sclerosis [9, 10]. DN patients suffer from a short survival period and a poor prognosis, which brings mental and economic pressure [11, 12]. Therefore, elucidation of the relevant regulatory mechanisms of DN is conducive to the delay and treatment of the disease.

SRY-related high mobility group box 9 (SOX9) plays an important role in mammalian development [13]. SOX9 is a member of the sex-determining region Y family. Its encoded transcription factor participates in the development and differentiation of multiple cell lines, and plays a key role in embryonic and neurogenesis, neural crest development, tumorigenesis and invasion, and stem cell self-renewal [14, 15]. SOX9 is involved in the occurrence and development of various diseases [16]. However, the role and mechanism of SOX9 in DN podocytes has not been elucidated.

Materials and methods

Main reagents and instruments

Mouse podocyte MCP-1 cells were preserved by the laboratory and stored in liquid nitrogen. DMEM-F12 medium and penicillin-streptomycin were purchased from Hyclone, USA. Dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), and MTT powder were purchased from Gibco. Trypsin-EDTA was purchased from Sigma. PVDF membrane was purchased from Pall Life Sciences. EDTA was purchased from Hyclone. RNA extraction kit, reverse transcription kit, and lipo2000 transfection reagent were purchased from Invitrogen. Western blot related chemical reagent was purchased from Beyotime. ECL reagent was purchased from Amersham Biosciences. Rabbit anti-human SOX9 monoclonal and goat anti-rabbit horseradish peroxidase (HRP) labeled IgG secondary antibodies were purchased from Cell Signaling. The Caspase 3 activity detection kit was purchased from Pall Life Sciences. TNF-α and IL-2 ELISA kits were purchased from R&D. The pcDNA-SOX9 plasmid and the SOX9 siRNA sequence were synthesized by Genepharma. The Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad. The ABI 7700 Fast Quantitative PCR reactor was purchased from ABI. Other commonly used reagents were purchased from Sangon.

Methods

MCP-1 cell culture and grouping: Mouse podocyte MCP-1 cells were seeded in a culture dish at 1×10^6 cells/cm^2 with DMEM-F12 medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 5.5 mmol/L glucose. The cells were passaged every 2-3 days and randomly divided into 4 groups; including control group that was cultured in normal glucose concentration (5.5 mmol/L), high glucose group that was cultured in high glucose (30 mmol/L), SOX9 overexpression group, and SOX9 siRNA group that were transfected with pcDNA-SOX9 plasmid and SOX9 siRNA in the high glucose environment, respectively.

pcDNA-SOX9 plasmid and SOX9 siRNA transfection: The pcDNA-SOX9 plasmid and the SOX9 siRNA sequence were transfected into MCP-1 cells under high glucose conditions. The pcDNA-SOX9 plasmid sequence was 5'-TAGGCCAACCAACACAAAACTTCATA-3'. The SOX9 siRNA sequence was 5'-TGCATCCGCGAGGCGGTCAGCCAGGTGCT-3'. The pcDNA-SOX9 plasmid and SOX9 siRNA were transfected by lipo2000 transfection reagent. The MCP-1 cells in logarithmic phase were collected and inoculated in a 6-well plate at 3×10^6/ml for 12 h. Next, 5 μl lipo2000 was mixed with 200 μl serum-free DMEM-F12 medium at room temperature for 15 min. The mixed lipo2000 was mixed with pcDNA-SOX9 plasmid or SOX9 siRNA at room temperature for 30 min. The mixture together with 1.6 ml serum-free DMEM-F12 medium was added to the cells and incubated at 5% CO_2 and 37°C for 6 hours. Then the cells were changed to DMEM-F12 medium containing FBS for further cultivation.

Real-time PCR: Total RNA was extracted from the cells by Trizol and reverse transcribed to cDNA. The primers were designed and synthetized (**Table 1**). Real-time PCR was performed at 52°C for 1 min, followed by 35 cycles of 92°C for 30 s, 58°C for 45 s, and 72°C for 35 s. GAPDH was selected as the internal reference. The relative expression of mRNA was calculated by $2^{-\Delta Ct}$ method.

Table 1. Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
GADPH	AGTGCCAGCCTCGTCTCATAG	CGTTGAACTTGCCGTGGGTAG
SOX9	AGCGGCTCATCTAAACAATGG	GGCGCACATTCTCTCCGTA
Nrf2	CTCATCGTAACAATCATCGGG	GCACCGTTCTTAGCGTG
Keap1	TCGGAAAGAGGAACGGAATG	ACATCATCTATTCTCATCATCT
ARE	TCATAGTCATCTAGCCTC	ACTTAGCTTGCACGGTAGG

MTT assay: The cells in logarithmic phase were seeded in a 96-well plate at 3000 cells/well and combined with 20 μl MTT for 4 h. Then, 150 μl DMSO was added to the plate for 10 min and tested at 570 nm to obtain the absorbance value (A). The proliferation rate = A in test group/A in control × 100%.

Western blot: The cells were lysed by RIPA (150 mM NaCL, 1% NP-40, 0.1% SDS, 2 ug/ml Aprotinin, 2 μg/ml Leupeptin, 1 mM PMSF, 1.5 mM EDTA, 1 mM NaVanadate) and quantified by BCA method. The isolated proteins were electrophoresed using 10% SDS-PAGE. The gel was transferred to PVDF membrane by semi-dry transfer method at 100 mA for 1.5 h. After blocking for 1 h, the membrane was incubated in SOX9 (1:1000) primary antibody at 4°C overnight. After incubation in secondary antibody (1:2000) without light for 30 min, the membrane was imaged using chemiluminescence reagent for 1 min and analyzed by image processing system software and Quantity one software. The experiment was repeated for four times (n = 4).

Caspase 3 activity detection: Caspase 3 activity in each group of cells was examined by the kit according to the instructions. The cells were trypsinized and centrifuged at 4°C and 600 g for 5 min. Next, the cells were cmbined with lysate on ice for 15 min and centrifuged at 4°C and 20000 g for 5 min. Then the cells were treated with 2 mM Ac-DEVD-pNA and the optical density (OD) value was detected at 405 nm wavelength.

ELISA: ELISA was used to test TNF-α and IL-2 contents in the serum. A total of 50 μl diluted standard substance was added to each well to establish the standard curve. Next, the plate was filled with 50 μl sample and washed for five times. Then the plate was incubated in 50 μl conjugate reagent at 37°C for 30 min. At last, the plate was tested at 450 nm to obtain the OD value. The OD value of standard substance was used to prepare the linear regression equation, which was adopted to calculate the concentration of samples.

ROS content detection: The treated cells were bathed in 95°C water for 40 min and rinsed with cold water. After being centrifuged at 4000 rpm for 10 min, the cells were incubated in 2', 7'-dichlorofluorescein diacetate (DCF-DA) at 37°C for 15 min and centrifuged at 10,000 rpm for 15 min. The sediment was resuspended in sterile PBS buffer and incubated at 37°C for 60 min. The level of ROS was measured by a microplate reader.

MPO and SOD activities detection: SOD activity was examined according to the kit instructions. The protein was extracted and washed in a 95°C water bath. After 40 min, it was taken out and rinsed with cold water. After being centrifuged at 4000 rpm for 10 min, the sample was extracted using an ethanol-chloroform mixture (5:3, v/v volume ratio 5:3) to detect total SOD activity. The sample was mixed and moved into an Ep tube. After being bathed at 95°C for 40 minutes, it was taken out and rinsed with cold water. After being centrifuged at 4000 rpm for 10 min, the sample was mixed in a buffer containing 30 mM H_2O_2 at pH 7.0. The complex was bathed in water for 10 min. The enzyme activity was determined by detecting the reduced absorption optical density of H_2O_2 at 240 nm.

Statistical analysis

All data analyses were performed on SPSS 16.0 software. The measurement data were presented as mean ± standard deviation and compared by one-way ANOVA. $P < 0.05$ was considered as a significant statistical difference.

Results

SOX9 expression in podocytes in high glucose environment

The expression of SOX9 mRNA and protein in normal and high glucose environments in podocyte MCP-1 cells were detected by real-time PCR and Western blot. It was showed that SOX9 mRNA and protein expression were significantly increased compared with the normal control group ($P < 0.05$) (**Figure 1**).

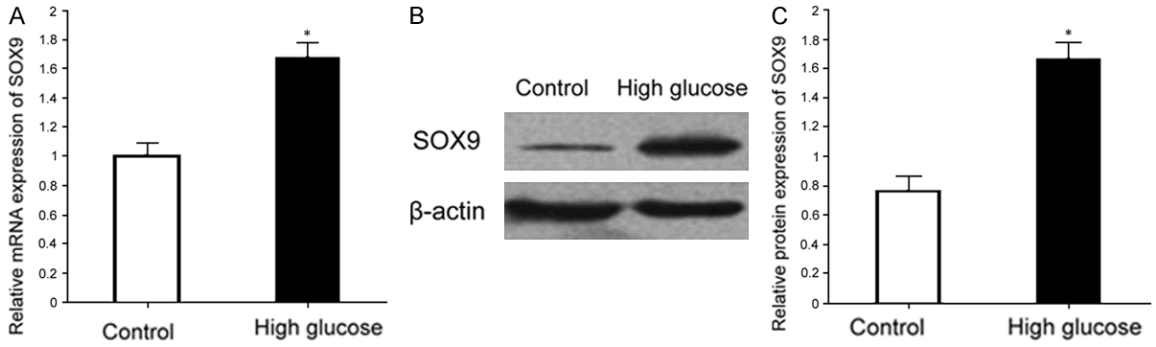

Figure 1. SOX9 expression in podocytes in a high glucose environment. (A) Real-time PCR detection of SOX9 mRNA expression; (B) Western blot detection of SOX9 protein expression; (C) SOX9 protein expression analysis. *P < 0.05, compared with control.

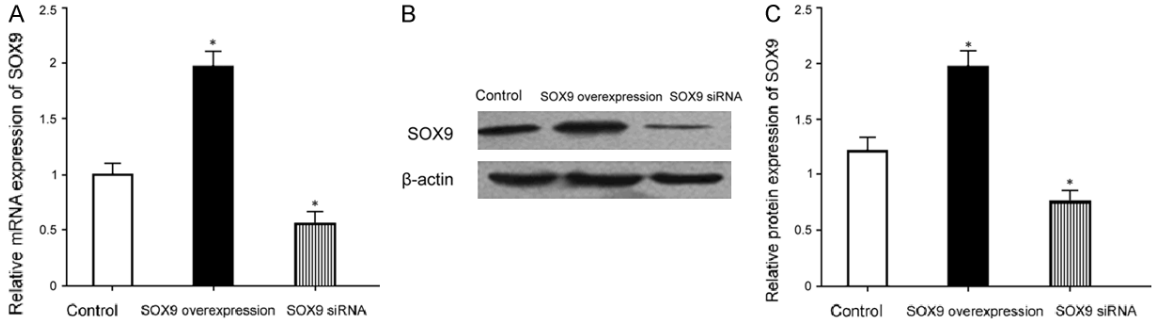

Figure 2. The impact of regulating SOX9 expression in podocytes in a high glucose environment. (A) Real-time PCR detection of SOX9 mRNA expression; (B) Western blot detection of SOX9 protein expression; (C) SOX9 protein expression analysis. *P < 0.05, compared with high glucose group.

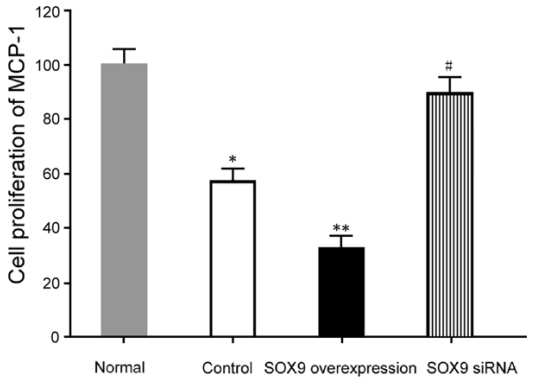

Figure 3. The influence of regulating SOX9 expression on podocytes proliferation in a high glucose environment. *P < 0.05, **P < 0.01, compared with normal. #P < 0.05, compared with control group.

The impact of regulating SOX9 expression in podocytes in high glucose environment

The pcDNA-SOX9 plasmid and SOX9 siRNA were transfected into mouse podocyte MCP-1 in a high glucose environment. It was found that the transfection of pcDNA-SOX9 plasmid upregulated SOX9 expression in podocyte MCP-1 in a high glucose environment. SOX9 siRNA transfection reduced the expression of SOX9 in podocyte MCP-1 in a high glucose environment ($P < 0.05$) (**Figure 2**).

The influence of regulating SOX9 expression on podocyte proliferation in high glucose environment

In a high glucose environment, SOX9 expression was clearly elevated and cell proliferation was inhibited compared with the normal group ($P < 0.05$); overexpression of SOX9 in high glucose environment MCP-1 cells can further inhibit cell proliferation ($P < 0.01$). Downregulation of SOX9 expression by SOX9 siRNA markedly promoted MCP-1 proliferation ($P < 0.05$) (**Figure 3**).

The effect of regulating SOX9 expression on Caspase 3 activity in podocytes in high glucose environment

In a high glucose environment, SOX9 expression was increased in MCP-1 cells, which clear-

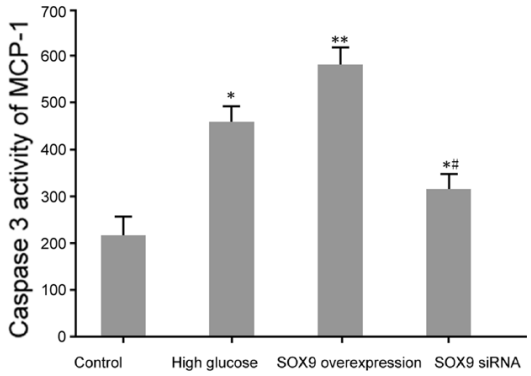

Figure 4. The effect of regulating SOX9 expression on Caspase 3 activity in podocytes in high glucose environment. *P < 0.05, **P < 0.01, compared with control. #P < 0.05, compared with high glucose group.

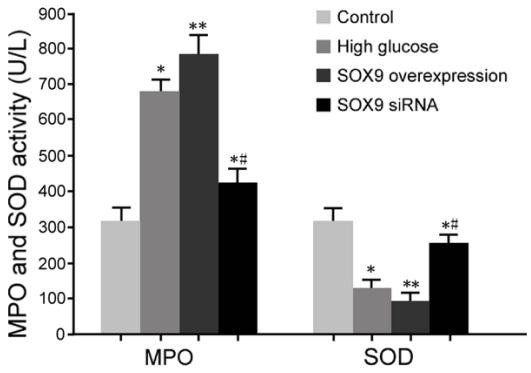

Figure 5. The impact of regulating SOX9 expression on MPO and SOD activities in podocytes in high glucose environment. *P < 0.05, **P < 0.01, compared with control. #P < 0.05, compared with high glucose group.

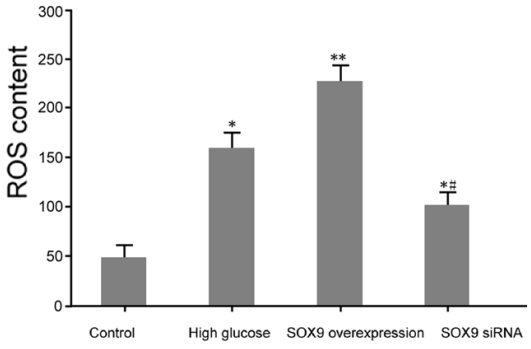

Figure 6. The influence of regulating SOX9 expression on ROS content in podocytes high glucose environment. *P < 0.05, **P < 0.01, compared with control. #P < 0.05, compared with high glucose group.

ly enhanced Caspase 3 activity (P < 0.05). SOX9 overexpression promoted Caspase 3 activity in MCP-1 cells in high glucose environment (P < 0.01). SOX9 siRNA transfection downregulated SOX9 expression and inhibited Caspase 3 activity in MCP-1 cells compared with control (P < 0.05) (**Figure 4**).

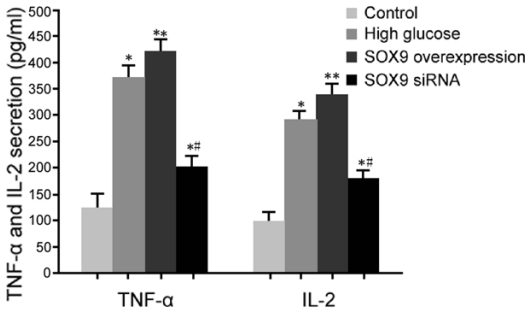

Figure 7. The effect of regulating SOX9 expression on TNF-α and IL-2 levels in the supernatant of podocytes in high glucose environment. *P < 0.05, **P < 0.01, compared with control. #P < 0.05, compared with high glucose group.

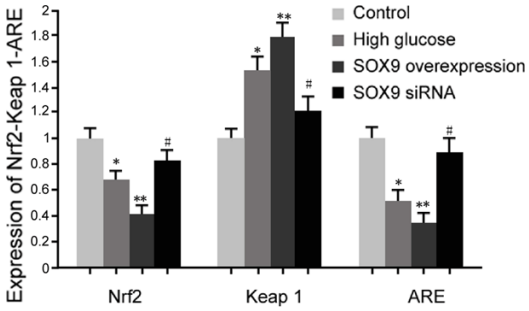

Figure 8. The impact of regulating SOX9 expression on Nrf2-Keap1-ARE pathway in the supernatant of podocytes in high glucose environment. *P < 0.05, **P < 0.01, compared with control. #P < 0.05, compared with high glucose group.

The impact of regulating SOX9 expression on MPO and SOD activities in podocytes in high glucose environment

In a high glucose environment, SOX9 expression was apparently increased in MCP-1 cells, which markedly declined SOD activity, and elevated ROS and MPO activities (P < 0.05). SOX9 overexpression further induced SOD reduction and upregulated MPO activities in MCP-1 cells in high glucose environment (P < 0.01). SOX9 siRNA transfection facilitated SOD activity and decreased MPO activity in MCP-1 cells compared with control (P < 0.05) (**Figure 5**).

The influence of regulating SOX9 expression on ROS content in podocytes in high glucose environment

In a high glucose environment, SOX9 expression was apparently increased in MCP-1 cells, which significantly increased ROS content ($P < 0.05$). SOX9 overexpression further upregulated ROS content in MCP-1 cells in high glucose environment ($P < 0.01$). SOX9 siRNA transfection declined ROS content in MCP-1 cells compared with control ($P < 0.05$) (**Figure 6**).

The effect of regulating SOX9 expression on TNF-α and IL-2 levels in the supernatant of podocytes in high glucose environment

In a high glucose environment, SOX9 expression was apparently increased in MCP-1 cells, which markedly enhanced TNF-α and IL-2 secretions ($P < 0.05$). SOX9 overexpression further elevated TNF-α and IL-2 secretions in MCP-1 cells in a high glucose environment ($P < 0.01$). SOX9 siRNA transfection downregulated TNF-α and IL-2 secretions in MCP-1 cells compared with control ($P < 0.05$) (**Figure 7**).

The impact of regulating SOX9 expression on the Nrf2-Keap1-ARE pathway in the supernatant of podocytes in high glucose environment

In a high glucose environment, SOX9 expression was apparently increased in MCP-1 cells, which significantly upregulated Keap1 expression, whereas it declined Nrf2 and ARE expressions ($P < 0.05$). SOX9 overexpression further enhanced these changes in MCP-1 cells in a high glucose environment ($P < 0.01$). SOX9 siRNA transfection downregulated Keap1 expression, while increased Nrf2 and ARE expressions in MCP-1 cells compared with control ($P < 0.05$) (**Figure 8**).

Discussion

Microvascular disease occurs in diabetes, causing a large amount of albuminuria, accompanied by progressive renal impairment and DN [17]. The podocytes appear to have irreversible damage under the stimulation of various pathogenic factors, such as high sugar and inflammation. The podocytes are detached from the basement membrane, leading to the glomerular filtration barrier destruction and induced proteinuria. Increased secretion of collagen from podocytes results in accumulation of extracellular matrix, thickening of glomerular basement membrane (GBM), and glomerular sclerosis [18]. Therefore, podocytes play an important role in the development of DN. SOX9 plays a crucial role in fetal development and in the development of stem and progenitor cells in the liver, pancreas, and hair follicles [19, 20]. Abnormal mutations in SOX9 expression can lead to skeletal malformations, central nervous system dysfunction, multiple defects in other organs, and even tumors [21, 22]. In this study, it was found that the expression of SOX9 was increased, the proliferation of MCP-1 cells was inhibited, and the activity of Caspase 3 was enhanced in the high glucose environment. The above changes were more significant in MCP-1 cells overexpressing SOX9 in high glucose environment, suggesting that SOX9 participates in the regulation of MCP-1 cell activity and proliferation.

Furthermore, this study explored the role of SOX9 in podocytes in high glucose environments and related mechanisms. During the process of DN, the body is in an oxidative stressed state, which leads to excessive free radicals such as ROS, dynamic imbalance of oxidation and antioxidant systems. It further induces tissue inflammation and damage, and a large increase in ROS, leading to podocyte apoptosis and damage [23]. SOD is one of the important antioxidant enzymes for scavenging oxygen free radicals in the body. It plays a vital role in the oxidation and anti-oxidation balance. When cells are damaged or dying, MPO can be released through the cell membrane and aggravate inflammation [24, 25]. The results showed that SOX9 expression was significantly increased, SOD activity was reduced, ROS and MPO elevated, TNF-α and IL-2 expressions were upregulated in a high glucose environment. SOX9 was overexpressed in a high glucose environment further enhancing the change. Downregulation of SOX9 expression by SOX9 siRNA enhanced SOD activity, decreased ROS and MPO, and declined TNF-α and IL-2 expression, indicating that SOX9 can participate in the role of podocytes in DN by regulating oxidative stress and inflammation. The Nrf2-Keap1-ARE signaling pathway is involved in the regulation of redox balance. After activation, it can inhibit external oxidation and chemical substances, thereby restraining oxi-

dative stress and exerting a defense effect. It is currently considered to be one of the most important endogenous antioxidant signaling pathways [26, 27].

This study demonstrated the increased expression of Keap1 and decreased expression of Nrf2 and ARE in high glucose environment, while downregulation of SOX9 significantly inhibited Keap1 expression and promoted expressions of Nrf2 and ARE, revealing that the Nrf2-Keap1-ARE signaling pathway plays a role in DN oxidative stress response. SOX9 can affect the activity of podocytes in DN by regulating the Nrf2-Keap1-ARE signaling pathway. In further research, it is proposed to use clinical samples to analyze the expression and related mechanisms of SOX9, thus to provide a theoretical basis for elucidating the pathogenesis of DN and the choice of treatment strategies.

Conclusion

SOX9 expression increased in high glucose environment. Down-regulation of SOX9 expression can inhibit apoptosis, restrain oxidative stress and inflammation, and promote podocyte proliferation by regulating the Nrf2-Keap1-ARE signaling pathway.

Disclosure of conflict of interest

None.

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