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Influence of overexpression of SOCS2 on cells of DN rat

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ABSTRACT

Objective: To explore the influence and mechanism of overexpression of SOCS2 on diabetic nephropathy (DN) rats and cells.

Methods: STZ was used to induce male SD rats and SOCS2 was injected into left renal vein. Rats were divided into DN group, DN-Ad-null group and DN-Ad-SOCS2 group. Glucose with high and normal concentration was used to culture HBZY-1 cells and then transfect Ad-SOCS2. HG group, HG-Ad-null group, HG-Ad-SOCS2 group, CG group, CG-Ad-null group, and CG-Ad-SOCS2 group were created. The expression of inflammatory cytokines (MCP-1, TNF- α and IL-6) in kidney tissue of rats, fibrosis related protein (FN, Collagen IV and TGF- β) in kidney tissue and cells of rats, and JAK/STAT signaling pathway related proteins (p-JAK2 and p-STAT3) were tested by western blot. ELISA was used to test the expression of inflammatory cytokines (TNF- α and IL-6) in cells.

Results: The expression of inflammatory cytokines in DN rats (MCP-1, TNF- α and IL-6) and cell (TNF- α and IL-6) were increased ($P < 0.01$) significantly. However, SOCS2 could decrease the overexpression of mediated inflammatory cytokines in DN animal models and cell models ($P < 0.01$). The expression of fibrosis related protein in DN rats and cells increased while SOCS2 decreased the overexpression of mediated fibrosis related protein in DN model rats and cells ($P < 0.01$). The expression of JAK/STAT pathway related protein in both DN rats and cells increased and the JAK/STAT signaling pathway was activated. Yet, SOCS2 obviously suppressed the expression of the JAK/STAT signaling pathway as well as the related proteins (p-JAK2 and p-STAT3) in both DN rats and cells.

Conclusions: The overexpression of SOCS2 can decrease the expression of inflammatory cytokines and fibrosis related proteins in DN rats and cells, and meanwhile suppress the activation of JAK/STAT signaling pathway mediated by DN.

1. Introduction

Diabetic nephropathy (DN) can lead to end-stage renal disease and even mortality, with a great threat to life [1–3]. Therefore, it has been a hot issue to explore the pathogenesis of DN and the way to prevent and treat DN. The DN pathogenesises are still unclear and researches show that DN patients usually have the following characteristics: thicker

glomerular basement membrane, smaller glomerular filtration area, expanded mesangial area and specific hyaline sedimentation [4]. In addition, recent studies indicate that it is glycometabolic disorder, abnormal expression of cytokines, genetic factors, inflammatory factors, change of renal hemodynamics, oxidative stress and other factors that play essential roles in DN development [5,6]. Suppressor of cytokine signaling (SOCS), with the function to suppress the signal transduction of cytokines, can be part of negative feedback mechanism of JAK/STAT signaling pathway, and more studies are performed on SOCS1 and SOCS3 [7]. It is shown that in the development of DN, SOCS1 and SOCS3 possess the protective function with great importance [2,5]. Apart from that, overexpression of SOCS2 can suppress the activation of JAK/STAT signaling pathway so that both the fibronectin (FN) produced from mesangial cells and

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pathological status resulted from accumulation of extracellular matrix can be suppressed [8]. There are also scientific results showing that SOCS1 and SOCS3 can suppress the overexpression of IL-6, TGF- β , Collagen IV *etc.* to alleviate the kidney damage. In other studies, SOCS2 has shown the function to suppress ERK1/2 activation and sequentially the composition of Collagen IV can be suppressed [9]. However, the function of SOCS2 in DN development remains unclear, so this study aims to explore the molecular mechanism of SOCS2 in DN so that the function of SOCS2 in DN can be figured out.

2. Materials and methods

2.1. Experimental materials

Healthy SD rats aged 7 weeks with weight of 230–280 g were prepared. The DMEM low glucose culture was purchased from Gibco. Fetal calf serum was from Hyclone. Glomerulus mesangial cells of rats HBZY-1 was from Shanghai Jimian Bioscience Incorporation. Streptozotocin was from sigma Company. Ad-SOCS2 and Ad-Null were from Shanghai Hanheng Bioscience Incorporation. TNF- α , IL-6, MCP-1, TNF- β , Collagen IV, FN, p-JAK2, and p-STAT3 antibody were also from sigma. IL-6 and TNF- α ELISA kit was from Shanghai haling Bioscience Incorporation. RIPA and NP-40 lysis solution were from Beyotime Biotechnology Incorporation.

2.2. Experimental methods

2.2.1. Construction and grouping of DN animal models mediated by DN and adenovirus

A total of 24 healthy male SD rats were given intraperitoneal injection with streptozotocin 60 mg/kg (dissolved in precooling sodium citrate solution, 0.01M, pH4.4) for DN animal model construction, which was successfully achieved after 48 h following the injection with over 250 mg/dL blood glucose concentration in rats. Meanwhile, 10 healthy male SD rats were injected with the same amount of sodium citrate solution and treated as control group. The rats in DN model group were further divided into 3 groups with 8 in each and treated with 10% of chloral hydrate for anesthesia, after which left renal vein was separated through the open procedure. Among these 3 groups, one was injected with 0.2 mL of negative control adenovirus as DN-Ad-null group, one was injected with SOCS2 adenovirus as DN-Ad-SOCS2 group and the last group with open procedure was only DN group. After suture, the rats were raised in clean and proper environment for 4 weeks, after which the kidney tissue was taken out and preserved at -80°C for further experiment.

2.2.2. Grouping of HBZY-1 cells after high glucose processing and adenovirus infection

HBZY-1 (37°C 5%CO₂) was cultured in normal glucose concentration in 3 groups. When it reached 70%, the group without transfection was named CG group, the group later transfected with Ad-null was named CG-Ad-null group and the group transfected with Ad-SOCS2 was named CG-Ad-SOCS2 group. All the three groups were then cultured for 48 h. Similarly, HBZY-1 (37°C 5%CO₂) was cultured in normal glucose concentration in 3 groups. When it reached 70%, HBZY-1 was shifted to high glucose concentration for 24 h, and then the

group without transfection was named HG group, the group later transfected with Ad-null was named HG-Ad-null group and the group transfected with Ad-SOCS2 was named HG-Ad-SOCS2 group. All the three groups were then cultured for 48 h as well.

2.2.3. Test on protein expression of kidney tissue and cells in rats through western blot

Kidney tissue with RIPA was cut into pieces with scissors and made even. In the case of cells, the supernatant was abandoned and NP-40 was added. Pipettor was used to make cell suspension. Then, the tissue and cells were placed on ice to lyse for 20 min, and centrifuged at 12000 rpm for 10 min. The obtained supernatant was total protein. Bradford method was used to determine the concentration of total protein.

Separation gel with 15% concentration and spacer gel with 5% concentration were prepared. With 40 μg of loading quantity on each well, the protein sample was diluted with PBS for further use, making the loading volume of 20 μL . After the processing of 5 \times loading buffer and boiling at 100°C for 5 min, the diluted protein sample was loaded onto the wells as per the designed volume. The electricity was charged at 90 v for 30 min and at 110 v for 90 min.

After the electrophoresis, separation gel was taken down and put in Trans-blot at 60 v for 2.5 h. After the transfer, PVDF membrane was placed in 5% skimmed milk powder produced from TBST and shaken by a shaker for 1 h. Sealed PVDF membrane was put in primary antibody configured from 5% of skimmed milk powder, and shaken to incubation overnight at 4°C .

PVDF membrane was taken out and washed with TBST solution 3 times/5 min, after which PVDF membrane was put in secondary antibody configured from 5% of skimmed milk powder for 2 h. PVDF membrane was taken out and washed with TBST solution 3 times/5 min, after which luminous fluid was added.

2.2.4. Test of IL-6 and TNF- α expression in cellular supernatant by ELISA

According to the instruction, all the reagents were prepared. Cells in each group were centrifuged at 1000 rpm for 5 min and the supernatant was kept for further use. The plates were washed twice with 300 μL of washing liquor and soaking for 10 min. After the washing liquor was abandoned, 50 μL of cell culture fluid, 50 μL of standard substance/sample and 50 μL of antibodies were added to each plate. The plates were then sealed and vibrated at the room temperature at 100 rpm for 2 h. The liquid was abandoned and the plates were washed with washing liquor 5 times before the plates were suck dry and 100 μL of streptavidin labeled by horseradish peroxidase was loaded on each well. Next, the plates were sealed with microplate sealer and vibrated at the room temperature at 100 rpm for 1 h. The liquid was abandoned and the plates were sucked dry before 100 μL of chromogenic substrate TMB was loaded in each well. The incubation was undergone at the room temperature in the dark for 30 min and 100 μL of stop buffer was loaded in each well so that the OD value can be measured at the wavelength of 450 nm. The OD value in each well minus the value in the blank well is the standard concentration which served as the abscissa while the OD values were the ordinate for standard curve. The standard curve was used to calculate the concentration accordingly.

2.3. Statistical analysis

SPSS17.0 was used for statistical analysis. All the experimental statistics were expressed with “mean \pm standard deviation” and the differences between groups were analyzed by *t*-test. If $P < 0.05$, the differences were considered to be significant.

3. Results

3.1. Effect of overexpression of SOCS2 in kidney tissue and cells on inflammatory cytokines expression

The expression of inflammatory cytokines MCP-1, TNF- α and IL-6 in kidney tissue in rats was tested by western blot while the expression of TNF- α and IL-6 in cells which was induced by sorts of conditions was tested by ELISA. As shown in Figure 1, the expression of MCP-1, TNF- α and IL-6 in kidney tissue in DN group, DN + Ad-null group and DN + Ad-SOCS2 group was remarkably higher than that in control group ($P < 0.01$). The expression of MCP-1, TNF- α and IL-6 in kidney tissue in DN group and DN + Ad-null group was apparently higher than that in DN + Ad-SOCS2 group, which illustrated that DN can bring about the increasing expression of the inflammatory related cytokines MCP-1, TNF- α and IL-6 in kidney tissue of rats while SOCS2 can decrease the inflammatory cytokines in kidney tissue of DN rats and play a protective function on DN. The experimental results of cells were shown in Figure 2. The expression of TNF- α and IL-6 in cells in HG + Ad-SOCS2 group, HG + Ad-null group and HG group, all of which were induced by high glucose, was obviously higher than that in CG group with the normal glucose concentration ($P < 0.01$). The

expression of TNF- α and IL-6 in cells in HG + Ad-SOCS2 group was lower than that in HG group while the expression of TNF- α and IL-6 in cells in CG group, CG + Ad-null group and CG + Ad-SOCS2 group, all of which were cultured in normal glucose concentration appeared to have no significant difference ($P > 0.05$). It was clear to say that DN cells induced by high glucose could cause the overexpression of inflammatory cytokines which would be reduced by SOCS2, with a protective function on DN.

3.2. Effect of overexpression of SOCS2 in kidney tissue and cells on fibrosis related protein expression

The change of fibrosis related protein expression in kidney tissue and cells of all groups was tested by western blot. As shown in Figure 3, the protein expression of FN, Collagen IV and TGF- β in kidney tissue of rats in DN group, DN + Ad-null group and DN + Ad-SOCS2 group was higher than that in control group. It was noticeable that the protein expression of FN, Collagen IV and TGF- β in kidney tissue of rats in DN group and DN + Ad-null group was higher than that in DN + Ad-SOCS2 group, which illustrated that DN could cause the increasing expression of fibrosis related proteins FN, Collagen IV and TGF- β in kidney tissue of rats and that SOCS2 could suppress the expression of fibrosis related proteins in kidney tissue of DN rats with a protective function on DN. The experimental results of cells were shown in Figure 4. The expression of FN, Collagen IV and TGF- β in cells in HG + Ad-null group, HG + Ad-DOCS2 and HG group was higher than that in CG group ($P < 0.01$). Also, the expression of FN, Collagen IV and TGF- β in cells in HG + Ad-SOCS2 was lower than that in HG group ($P < 0.01$). However, the

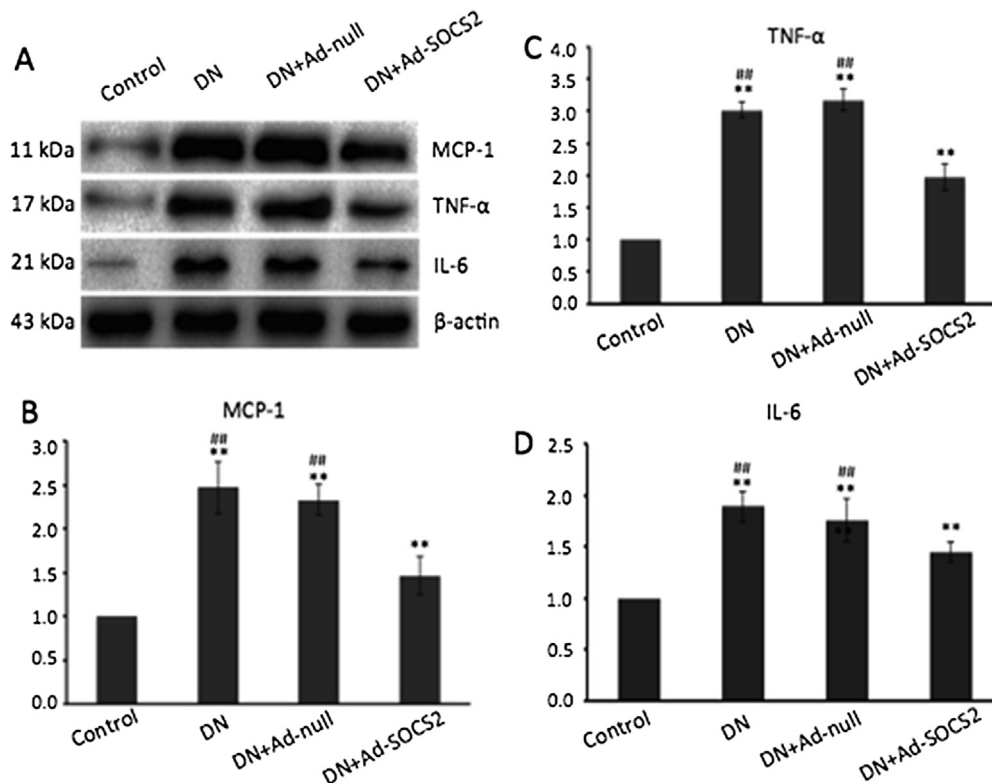


Figure 1. Expression of inflammatory cytokines in kidney tissue of rats by Western blot. Compared with control group, ** $P < 0.01$; compared with DN + Ad-SOCS2 group, ## $P < 0.01$ ($n = 8$).

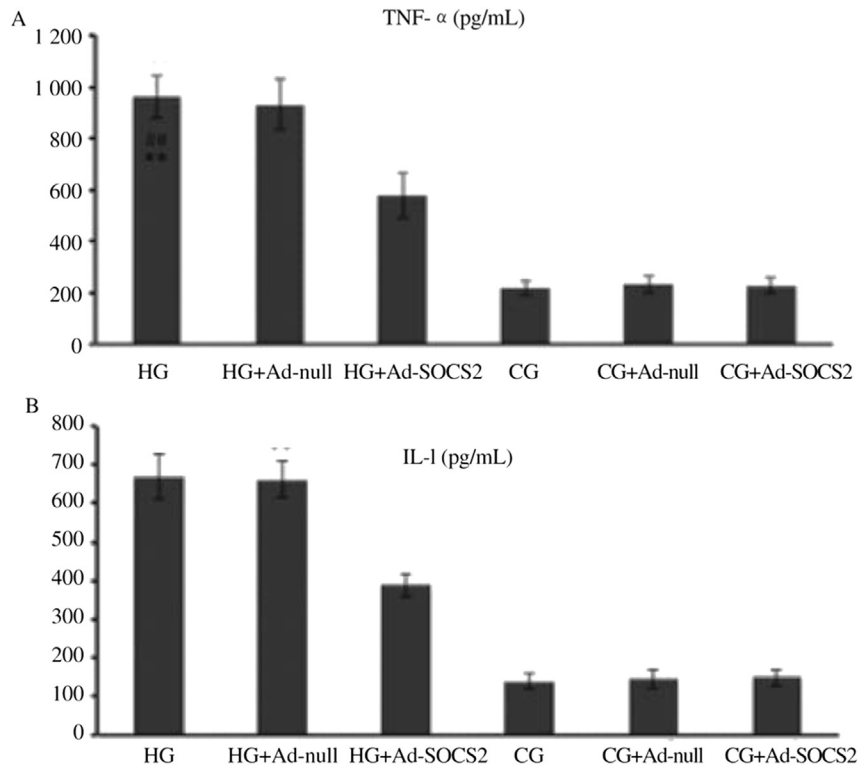


Figure 2. Expression of inflammatory cytokines in cells of rats by ELISA. Compared with CG group, ** $P < 0.01$; compared with HG group, ## $P < 0.01$ ($n = 3$).

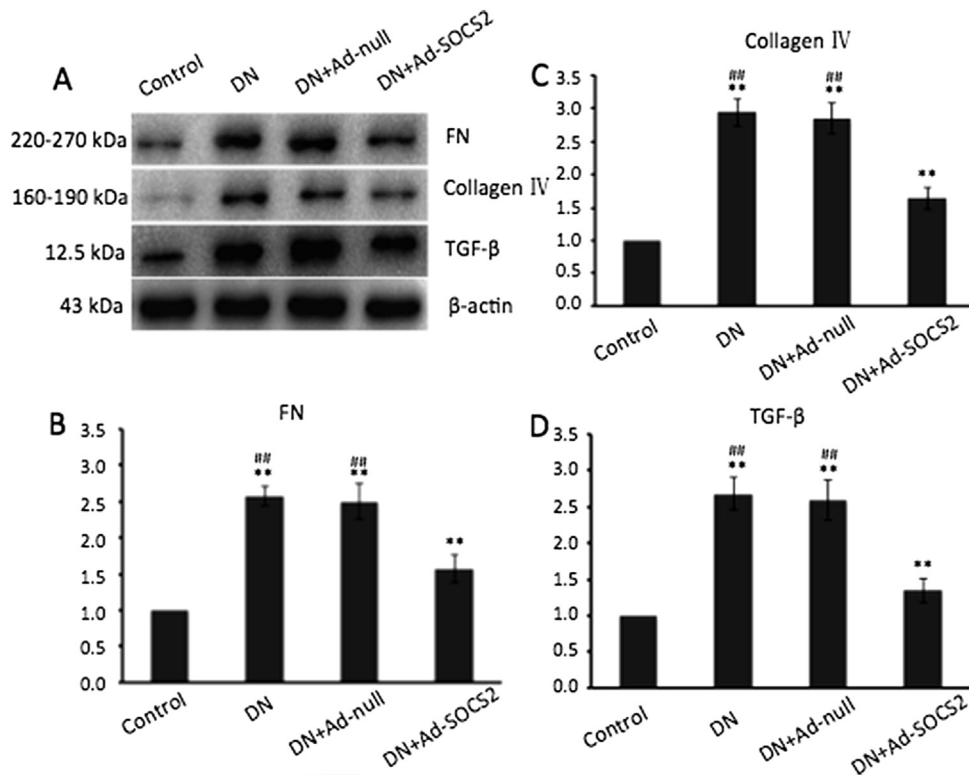


Figure 3. Expression of fibrosis related proteins in kidney tissue of rats by Western blot. Compared with control group, ** $P < 0.01$; compared with DN + Ad-SOCS2 group, ## $P < 0.01$ ($n = 8$).

expression of TNF- α and IL-6 in cells in CG group, CG + Ad-null group and CG + Ad-SOCS2 group appeared to have no significant difference ($P > 0.05$). That illustrated that DN cells induced by high glucose could bring about the overexpression

of fibrosis related proteins FN, Collagen IV and TGF- β while the overexpression of SOCS2 could suppress the expression of FN, Collagen IV and TGF- β and play a protective function on DN.

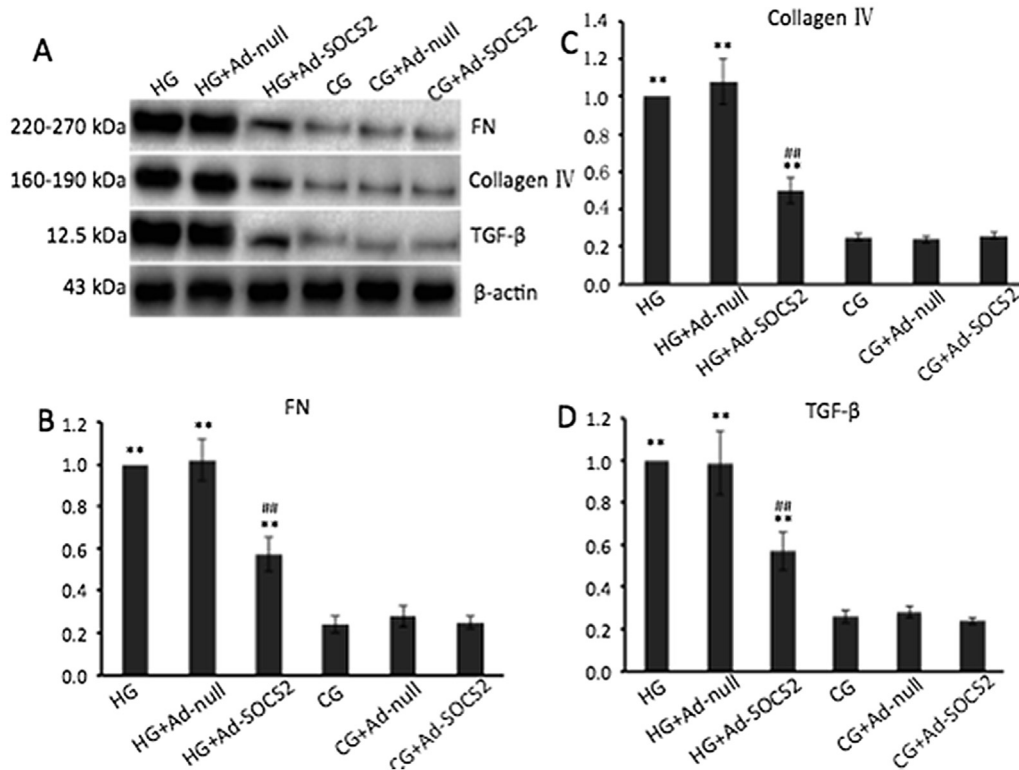


Figure 4. Expression of fibrosis related proteins in cells of rats by Western blot. Compared with CG group, ** $P < 0.01$; compared with HG group, ### $P < 0.01$ ($n = 3$).

3.3. Suppression of expression of SOCS2 in kidney tissue and cells on JAK/STAT signaling pathway

The expression of phosphorylated JAK and STAT in kidney tissue of rats was tested by western blot. As presented in Figure 5, the protein expression of p-JAK2 and p-STAT3 in kidney tissue

in DN group, DN + Ad-null group, DN + Ad-SOCS2 group was higher than that in control group ($P < 0.01$). Also, the protein expression of p-JAK2 and p-STAT3 in kidney tissue in DN group and DN + Ad-null group was higher than that in DN + Ad-SOCS2 group ($P < 0.01$). That illustrated that DN could activate the JAK and STAT signaling pathway in kidney tissue while the

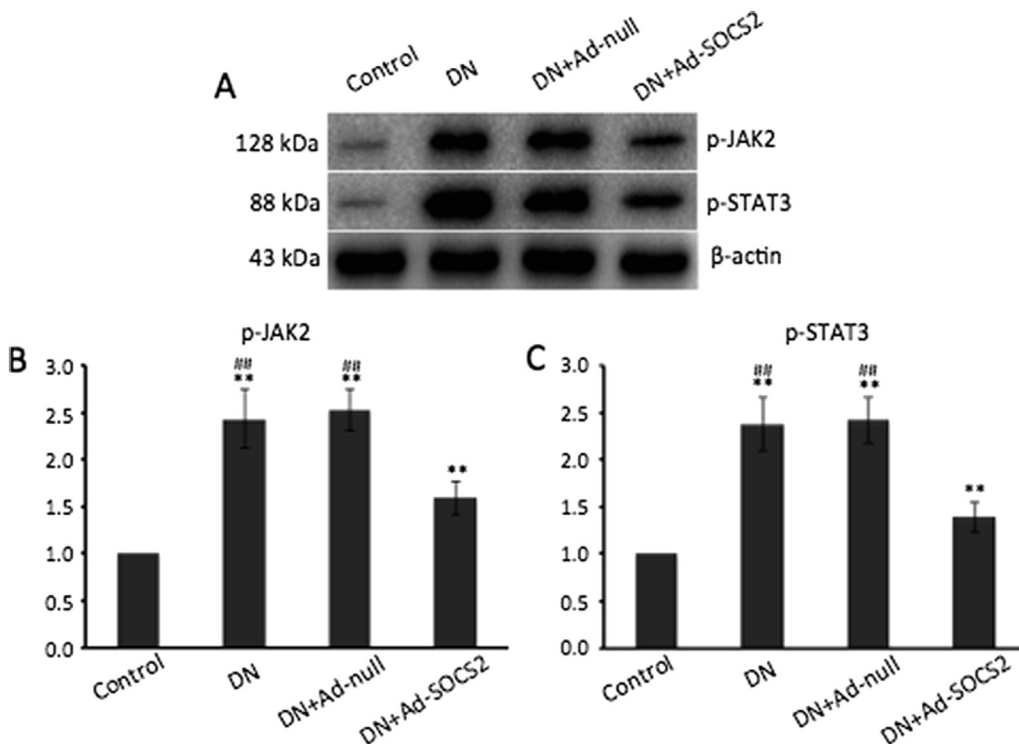


Figure 5. Expression of p-JAK2 and p-STAT3 in kidney tissue of rats by Western blot. Compared with control group, ** $P < 0.01$; compared with DN + Ad-SOCS2 group, ### $P < 0.01$ ($n = 8$).

overexpression of SOCS2 could suppress the JAK/STAT signaling pathway and meanwhile play a protective function on DN. On the other hand, the protein expression of p-JAK2 and p-STAT3 in cells of rats by western blot was presented in Figure 6. The protein expression of p-JAK2 and p-STAT3 in cells in HG group, HG + Ad-null group and HG + Ad-SOCS2 group was higher than that in CG group ($P < 0.01$). Yet, the protein expression of p-JAK2 and p-STAT3 in cells in HG group and HG + Ad-null group was higher than that in HG + Ad-SOCS2 group ($P < 0.01$). That illustrated that the DN cells induced by high glucose could activate the JAK/STAT signaling pathway while the overexpression of SOCS2 could suppress the pathway and play a protective function on DN.

4. Discussion

It is of significance to study the pathogenesis of DN in order to prevent DN. Many studies show that DN may be related to glucose metabolic disorder, oxidative stress, inflammatory reaction, abnormal hemodynamics, genetic factors and other factors [5,6,10]. SOCS1 and SOCS3 have been proved to play a protective function on DN by suppressing the JAK/STAT signaling pathway [11,12]. In recent years, some studies show that SOCS2 could be part of the development of DN, as a member of SOCS [13], with unclear mechanism of action. Therefore, this study aims to explore the molecular mechanism that SOCS2 involves in DN models *in vitro* and *in vivo*.

4.1. Effect of overexpression of SOCS2 on inflammatory cytokines and fibrosis related proteins in DN rats and cell models

The inflammatory reaction plays an important role in the development of DN. In pathological conditions, many

inflammatory cytokines would be secreted when the cells get damaged and inflammatory cascade would be initiated through autocrine and paracrine [14,15]. This study shows that DN can promote the expression of the inflammatory cytokines MCP-1, TNF- α and IL-6; and SOCS2 can suppress the overexpression of them caused by DN so that the direct damage on glomerulus and renal tubules by inflammatory cytokines MCP-1 and TNF- α can be reduced and meanwhile, SOCS2 can also suppress the generation of urine protein as well as the fibrosis of renal tubules so that it can delay and protect the development of DN [16,17]. The fibrosis of renal tubules is one of the important features in end-stage renal disease [18]. This study also explores the effect of SOCS2 on fibrosis related proteins in DN. In both animal and cell DN models, the expression of fibrosis related proteins FN, Collagen IV and TGF- β obviously increased while SOCS2 apparently decreased the fibrosis related protein expression in both models so that the fibrosis of renal tubules and transformation into the end-stage renal disease were delayed, and thereby SOCS2 play a protective function on DN.

4.2. Effect of overexpression of SOCS2 on JAK/STAT signaling pathway in DN rats and cell models

JAK/STAT signaling pathway has been proved to be important in the development of DN [7]. Many studies have shown that cytokines, like inflammatory cytokines, can activate the JAK/STAT signaling pathway to promote the fibrosis of renal tubules so that it would transfer to the end-stage renal disease [19,20]. Therefore, the study explores whether SOCS2 can participate in JAK/STAT signaling pathway in DN so that it can protect DN. The results showed that in both animal and cell DN models, the protein expression of p-JAK2 and p-STAT3 increased while SOCS2 decreased the protein expression of p-JAK2 and p-STAT3

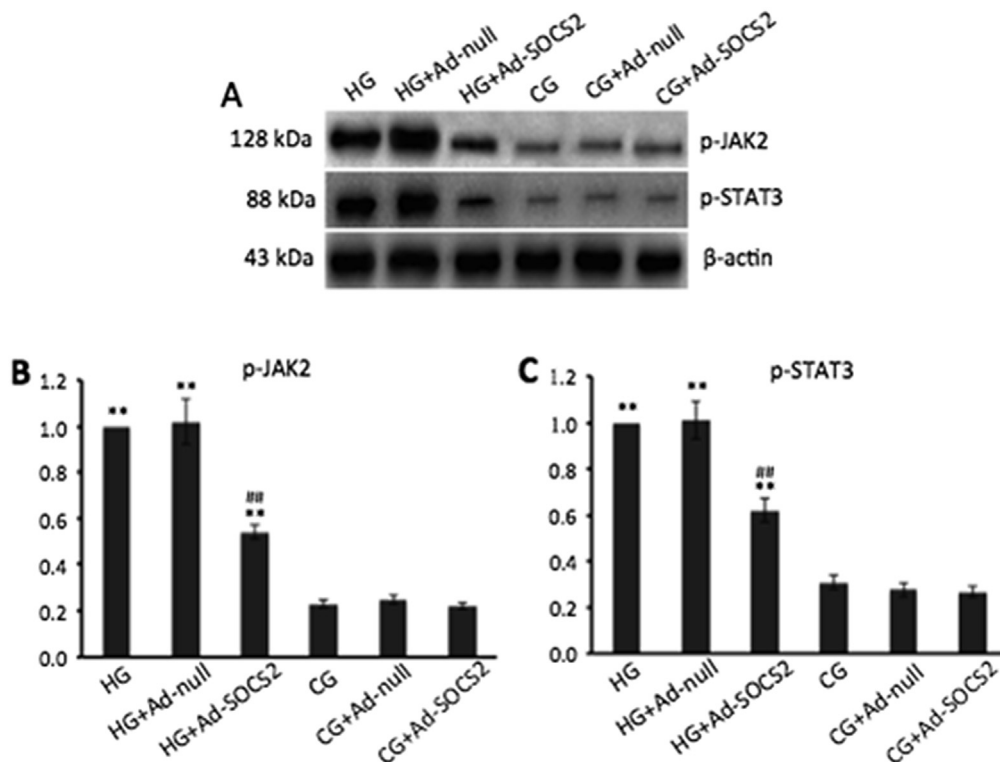


Figure 6. Expression of p-JAK2 and p-STAT3 in cells of rats by Western blot. Compared with CG group, ** $P < 0.01$; compared with HG group, ### $P < 0.01$ ($n = 3$).

which led to a series of cascade reaction and finally the kidney damage. As activated JAK/STAT signaling pathway can be suppressed by SOCS2, DN can be protected.

In conclusion, this study confirms that DN can cause the overexpression of inflammatory cytokines (MCP-1, TNF- α and IL-6) and fibrosis related proteins (FN, Collagen IV and TGF- β) *in vitro* and *in vivo* and activate JAK/STAT signal transduction pathway. SOCS2 is confirmed to conduct the function to suppress the expression of the aforesaid inflammatory cytokines and fibrosis related proteins and the activation of JAK/STAT signal transduction pathway caused by DN. It is also found that SOCS2 protects DN by suppressing the inflammatory cytokines and fibrosis related proteins, as well as by suppressing the activation of JAK/STAT signaling pathway. Based on this pathogenesis, a new clinical theory for a new DN treatment is put forward, to some extent. However, it remains unclear whether SOCS2 suppresses the expression of inflammatory cytokines and fibrosis related proteins induced by DN by suppressing the activation of JAK/STAT signal transduction pathway. That still requires further experiments to confirm so that the function of SOCS2 in the development of DN can be figured out and a foundation can be constructed for the prevention as well as treatment of DN.

Conflict of interest statement

We declare that we have no conflict of interest.

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