

# Epac ameliorates tubulointerstitial inflammation in diabetic nephropathy via C/EBP $\beta$ /SOCS3/STAT3 pathway

**Wenxia Yang**

Central South University

**Shumin Zhang**

Central South University

**Huafen Wang**

Central South University

**Yifei Liu**

Central South University

**Jialu Liu**

Central South University

**Lin Sun**

Central South University

**Li Xiao** (✉ [xiaolizndx@csu.edu.cn](mailto:xiaolizndx@csu.edu.cn))

Second Xiangya Hospital, Central South University

---

## Research

**Keywords:** diabetic nephropathy, Epac activation, inflammation, macrophage, tubular injury

**Posted Date:** February 26th, 2020

**DOI:** <https://doi.org/10.21203/rs.2.24571/v1>

**License:**   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

**Background:** Tubulointerstitial inflammation plays a pivotal role in the progression of diabetic nephropathy (DN), and tubular cells act as the driving force in the inflammation cascade. The aim of the study is to investigate whether activation of Epac alleviates tubulointerstitial inflammation through SOCS3/JAK/STAT3 pathway under DN. **Methods:** The kidney morphological changes and the level of oxidative stress were observed in db/db mice treated with Epac agonist (8-pCPT-2'-O-Me-cAMP). The expression of inflammatory cytokines, fibrosis markers, C/EBP $\beta$ , SOCS3 and p-STAT3 in renal tissues were assessed. In in vitro study, the changes of above genes and proteins were also detected in human proximal tubular cell line (HK-2) incubated with high glucose (HG) with and without Epac agonist. Overexpression of SOCS3 plasmid, SOCS3 siRNA and C/EBP- $\beta$  siRNA were employed and the translocation of C/EBP- $\beta$  and p-STAT3 were observed by immunofluorescence. Macrophage migration assay were detected in Transwell system. **Results:** In db/db mice, we observed that the expression of MCP-1 was up-regulated in renal tubular cells, which concurrent with increased influx of macrophages, cytokines production (MCP-1, TNF- $\alpha$ , and IL-6), and tubulointerstitial fibrosis. Moreover, we also found that these changes were accompanied by reduced C/EBP- $\beta$  expression and increased SOCS3 and phosphorylated STAT3 (p-STAT3) expression. However, after treated db/db mice with 8-O-cAMP, an Epac activator, the expression of SOCS3 further up-regulated while other protein expression profile was partially reversed. In vitro, high glucose (30mM, HG) treatment down-regulated C/EBP- $\beta$  expression while increased SOCS3, p-STAT3 and MCP-1 expression in HK-2 cells; Activation of Epac by 8-O-cAMP further increased the gene and protein expression of SOCS3 while reversed the changes of the other proteins. Mechanistically, under HG ambience, knockdown of C/EBP- $\beta$  or SOCS3 in HK-2 cells partially blocked the inhibitory effect of Epac activation on MCP-1 expression. Furthermore, 8-O-cAMP treatment enhanced the nuclear-translocation of C/EBP- $\beta$  and further increased the transcription of SOCS3 gene, which decreased MCP-1 expression through inhibiting the phosphorylation of STAT3 in HK-2 cells. **Conclusions:** These data indicate that Epac ameliorates tubulointerstitial inflammation in DN at least partially through C/EBP $\beta$ /SOCS3/STAT3 pathway. Therefore, Activation of Epac by 8-O-cAMP may be a novel therapeutic strategy for DN.

## Background

Diabetic nephropathy (DN) is one of the most serious microvascular complications of diabetes and is the major cause of end-stage renal disease (ESRD) (1). Despite current therapies of blood pressure and glycemic control have been shown to slow down the progression of DN. Unfortunately, a large portion of patients still progress to ESRD. Thus, it is important to elucidate the pathogenesis of DN and find appropriate strategies to protect patients with DN from progressive kidney disease.

DN has not been traditionally considered an inflammatory disease until recently. Emerging data have demonstrated that tubulointerstitial inflammation is strongly correlated with the development and progression of DN (2, 3). Macrophage infiltration in the kidney tissues has been postulated to be a prominent pathological feature and key inflammatory mediator during the progression of DN. Studies

from human biopsies and animal models have identified the presence of macrophages in diabetic kidneys, even in the early stage of the disease(4). Moreover, the degree of tubulointerstitial macrophage infiltration is closely associated with the renal function decline and interstitial fibrosis (5, 6). Depletion of macrophage by silencing macrophage scavenger receptor-A expression in animal models alleviated STZ-induced diabetic renal damage(7). Therefore, diminishing macrophage recruitment and activation may be a novel therapeutic approach for the treatment of DN.

Notably, it was found that tubular damage may be the primary event in DN and may be strongly associated with macrophage infiltration(8, 9). High glucose induced renal tubular epithelial cells secreting many inflammatory cytokines, including MCP-1, IL-6 and TNF- $\alpha$ (10–12). Among them, MCP-1 is one of the well-documented and important chemotactic factors(13, 14). Recently, Kuehn and his colleagues have found that the integrity of cilia of tubular cells modulated macrophages recruitment via an MCP-1 dependent way(15). Downregulated MCP-1 expression alleviated STZ-induced diabetic renal damage and macrophage infiltration(16). Therefore, MCP-1 signaling cascade plays a key role in the cross-talk between injured tubular cells and infiltrating macrophages in the progression of DN.

Exchange protein activated by cAMP (Epac) is a cyclic AMP-regulated guanine nucleotide exchange factor (GEFs) that activates the small GTPase Rap(17, 18). Epac1 and Epac2 are two isoforms of Epac and they have different tissue distribution and expression levels (19). Generally, Epac1 is higher expression in the kidney than Epac2, especially in renal tubular epithelial cells(20, 21). Studies have shown that activation of Epac might exert a renoprotective effect in various CKD model. For instance, activation of Epac decreased the Ang II-induced ROS generation and inflammatory cytokines (IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$ ) production in renal tubular cells(22). Subsequent study further indicated that Ang II-induced expression of inflammatory cytokine were regulated by Epac1-Rap1a-NHE3 pathway in tubular cells(23), indicating that Epac may play an inhibitory effector in response to renal inflammatory. However, whether Epac is involved in the anti-inflammatory signaling by targeting MCP-1 in the proximal renal cells expose to hyperglycemia remains unclear.

Previous studies have suggested that hyperglycemic stimulus enhanced the activation of the JAK/STAT signaling cascade in renal tubular cells, which contributed to renal tubular damage in DKD(24, 25). Overexpression of SOCS3 by renal delivery adenovirus, an important endogenous suppressor of JAK/STAT3 pathway(26), significantly reversed STAT3 activation-induced MCP-1 upregulation and macrophage infiltration in DN (10), suggesting a renoprotective role of SOCS3. Therefore, the manipulation of SOCS3 expression would be imperative and is a therapeutic target for DN. Additionally, it has been reported that Epac1 induces SOCS3 gene expression in various types of cells, including cardiocyte(27), vascular endothelial cells(28) and retinal ganglion cells(29). Based on these perspectives, we hypothesized that activation of Epac may protect against the inflammation injury by targeting SOCS3 gene expression in the context of hyperglycemia.

In the present study, we investigated the role of pharmacological activation of Epac by 8-pCPT-2'-O-Me-cAMP (8-O-cAMP) in both in vitro and in vivo models of DN. We found that activation of Epac decreased

macrophage infiltration and subsequent inflammatory cytokines production in the kidney of db/db mice, and significantly ameliorated renal tubulointerstitial fibrosis. Mechanistically, selective activation of Epac reduced HG-induced STAT3 activation and MCP-1 expression via increased SOCS3 gene expression in proximal tubular cells. These results revealed that the beneficial effect of activation of Epac by attenuating macrophage-mediated renal injury in DN.

## Methods

### Animal experimental design

The Animal Care and Use Committee of Second Xiangya Hospital of Central South University approved all animal procedures. C57BL/KS db/db and C57BL/KS db/m mice were used for the animal experiments. These mice were purchased from the Aier Matt Experimental Animal Company (Suzhou, China) and then housed in Second Xiangya Hospital Animal Center, Central South University. All mice access to standard chow and filtered tap water *ad libitum*. At 8 weeks of age, they were organized into the following three groups for animal experiments: a db/m group (control, n=5), a db/db group (n=5) and a db/db group receiving an intraperitoneal injection (i.p.) of 8-pCPT-2'-O-Me-cAMP (Sigma, 1.47mg/kg, once every other day for 8 weeks, n=5). Every two weekly body weight and fasting blood glucose were monitored throughout the study and at 16 weeks of age, all the mice were euthanized under intraperitoneal pentobarbital sodium anesthesia (50 mg/kg body weight).

### Assessment of physiological features and renal function

Body weights and blood glucose levels were measured every two weeks. Blood glucose levels were detected by tail vein blood samples using a blood glucose monitor (Boehringer Mannheim, Mannheim, Germany). Urine albumin concentrations were measured using a mouse urine albumin ELISA kit (Bethyl Laboratories, USA), and serum creatinine levels were tested by HPLC, liquid chromatography/tandem mass spectrometry.

### Morphological analysis of kidneys

Kidney tissues were fixed in 4% paraformaldehyde and four-micron thick paraffin-embedded kidney sections obtained from aforementioned groups were stained with hematoxylin-eosin (HE), periodic acid-Schiff (PAS) and Masson's trichrome staining. Glomerular or tubular damage was scored as previously described(30): (0), no glomerular/tubular damage; (1) 25% of the glomerular/tubular area affected; (2) 25–50% of the glomerular/tubular area affected; (3) 50% of the glomerular/tubular area affected.

### Immunohistochemistry

Four-micron thick paraffin-embedded kidney sections were utilized for immunohistochemistry (IHC) studies as previously described(31). In brief, the sections were dewaxed, rehydrated, and incubated with primary antibodies overnight at 4 °C and later incubated with horseradish peroxidase-conjugated

secondary antibody and diaminobenzidine (DAB) substrate. After counterstaining with hematoxylin and dehydration, the sections were mounted and analyzed with a Nikon microscope.

### **Measurement of superoxide generation and immunofluorescence assay**

Dihydroethidium (1  $\mu$ M, DHE, Sigma-Aldrich) were used to assess intracellular ROS production in 4-mm-thick kidney cryostat sections as described previously(32). The mean fluorescence intensity (MFI) of DHE was calculated by ImageJ. For immunofluorescence assay, the cells or frozen sections were fixed with 4% paraformaldehyde for 10 min at room temperature. Then, the sections were blocked with 0.1% TritonX-100 and 5% BSA mixture for 60 min at room temperature and then incubated with primary antibody overnight at 4°C. Later incubated secondary antibodies conjugated with Alexa Fluor 488 (green) or 594 (red) for 1 hour. These cells or slides were counterstained with 4',6-diamidino-2 phenylindole (DAPI) and their fluorescent signals were visualized using an LSM 780 META laser scanning microscope (Zeiss, Thornwood, NY). The MFI of p-STAT3 and C/EBP- $\beta$  was calculated by ImageJ.

### **Cell culture studies**

The human proximal tubular cell line HK-2 (ATCC, Rockville, USA) was used for our in vitro studies. Time-dependent experiments were performed using 30 mM D-glucose for 0-48 h. The HK-2 cells were pretreated with 8-pCPT-2'-O-Me-cAMP for 5 hours before exposure to 30mM glucose. C/EBP- $\beta$  or SOCS3 siRNA were transfected into the HK-2 cells using Lipofectamine 2000 reagent (Life Technologies, USA) for in vitro studies.

### **Real -Time PCR analysis**

Total RNA from renal sections and HK-2 cells was prepared using TRIzol (Takara). First-strand cDNA was prepared by two-step RT-qPCR (Takara). Relative gene expression was determined using SYBR Green quantitative real-time PCR assays on Light Cycler 96 System (Roche). The cycling conditions were as follows: denaturing at 50 °C for 2 min, 95 °C for 10 min. Preincubation at 95 °C for 30 sec, then 40 cycles of 95 °C for 5 sec, 60 °C for 30 sec and 97 °C for 1 sec. All reactions were carried out in triplicate with a control. Expression was calculated using the  $2^{-\Delta\Delta C_t}$  method and normalized to ACTB.

### **Western blot assay**

The protein extraction of kidney cortex from db/db mice, HK-2 cells and controls were lysed in radio immunoprecipitation assay (RIPA) buffer containing protease inhibitors and phosphatase inhibitors. BCA Protein Assay kit (CoWin Biosciences, China) was used to determine protein concentration. Proteins were separated using 8% and 12% SDS-PAGE and transferred to PVDF membranes, which were probed with primary antibodies against C/EBP- $\beta$ , MCP-1, TNF- $\alpha$  (Abcam, USA), p-STAT3 (Cell Signaling Technology, USA), STAT3, and SOCS3 (Proteintech, CHINA), respectively.  $\beta$ -Actin (Proteintech, CHINA) was used as a loading control. Following incubation with an appropriate secondary antibody, the protein expression was detected by western blot analysis, as described previously(31).

## Macrophage migration assay

HK-2 cells pretreated with or without 8-O-cAMP were incubated with 30 mM D-glucose, and HK-2 cells under basal conditions served as a control. The cell supernatants were collected after 24 hours. THP-1 cells cultured in the upper chambers of Transwell plates treated with 100ng/ml PMA for 48h, and then cultured in fresh complete medium that in absence of PMA for 24h. While the cell supernatants derived from HK-2 cells exposed to HG were added to the lower chambers. After 24h incubation, a cotton swab was used to remove the cells on the upper surface of the membrane; Cells that migrated to the bottom surface of the membrane were fixed and stained with DAPI, and then observed under a Nikon microscope.

## Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad, San Diego, CA, USA). Statistical different between two groups were analyzed by unpaired t test. One-way analysis of variance (ANOVA) test was used for multiple independent sample analysis. Comparisons between groups were performed with Tukey's test post hoc analysis. Values are shown as the Mean  $\pm$  SD;  $p < 0.05$  was considered to be statistically significant.

## Results

### **Activation of Epac by 8-O-cAMP improves renal function and pathologic changes in the kidneys of db/db mice.**

To test the role of Epac in the tubular damage in the kidney of db/db mouse, we treated db/db mice with Epac specific activator 8-pCPT-2'-O-Me-cAMP (8-O-cAMP) by intraperitoneal injection for 8 weeks. Db/db mice exhibited higher body weights, increased blood glucose, serum creatinine and urine albumin levels compared to db/m mice (Fig. 1a-d). Slightly but non-significantly increase in body weight was seen in db/db mice treated with 8-O-cAMP. By contrast, notable decreases in blood glucose (Fig. 1b), serum creatinine (Fig. 1c) and urine albumin levels (Fig. 1d) were observed in db/db mice after 8-O-cAMP treatment.

HE and PAS staining revealed mesangial expansion and tubular atrophy in the kidneys of db/db mice compared to db/m mice (Fig. 1e); Masson's trichrome staining showed increased interstitial fibrosis in the kidney of db/db mice (Fig. 1e). However, Activation of Epac with 8-O-cAMP dramatically ameliorated these abnormal changes (Fig. 1e). Quantitative analysis of tubular and glomerular damage score confirmed this tendency (Fig. 1f, g). These data indicated that Epac activator 8-O-cAMP may exert a therapeutic role in the diabetic kidney injury.

### **Activation of Epac alleviates the inflammation response, oxidative stress, and interstitial fibrosis in the kidney of db/db mice.**

Inflammation and oxidative stress contribute critically to the initiation and extension of DN. We therefore evaluated renal inflammatory cytokines and reactive oxidative stress (ROS) production the kidneys of

db/db mice receiving 8-O-cAMP treatment. By IHC and Western blot assay, TNF- $\alpha$ , IL-6 expression was found to be up-regulated in the tubules of db/db mice compared to those of controls (Fig. 2a (e, f vs b, a)); DHE staining demonstrated increased ROS production in the tubular cells of the kidneys of db/db mice (Fig. 2b (b vs a)). Finally, the expression of FN and Col-1 were also detected, as one can see, a notable increase in FN and Col-1 expression in the tubulointerstitium of db/db mice (Fig. 2a (g, h vs c, d)) and similar result was demonstrated by Western blot assays (Fig. 2c). Of note, these above abnormal changes were reversed after treated with 8-O-cAMP (Fig. 2a (i, j, k, l) and Fig. 2c). These results provided compelling evidence supporting a renoprotective role of activation of Epac in DN.

### **Activation of Epac attenuates renal macrophage infiltration in db/db mice.**

To further understand the role of Epac agonist 8-O-cAMP in the inflammation response in the kidney of db/db mice, we detect the expression of macrophage chemotactic factor MCP-1 and macrophage accumulation in the kidney. IHC assay showed that the expression of MCP-1 significantly increased in the tubules of db/db mice compared to the db/m mice (Fig. 3a (b vs a)). Similar results of MCP-1 protein and gene expression were demonstrated by western blot assays (Fig. 3b) and qPCR (Fig. 3c), respectively. Moreover, immunofluorescence staining results showed that the expression of F4/80 was significantly up-regulated in renal interstitium of db/db mice, indicating the increased macrophage infiltration, which were significantly inhibited by 8-O-cAMP treatment (Fig. 3a and a2). Besides, M1 macrophages promote the inflammation in kidney of DN. Next, we detected the effect of 8-O-cAMP on the changes of macrophages polarity in kidney of DN by iNOS staining. The result showed that 8-O-cAMP significantly reduced the expression of iNOS (Marker of M1 macrophage) in macrophages (Fig.3a and a3) in db/db mice. These above results indicated that activation of Epac may exert anti-inflammatory effects by reducing macrophage accumulation and activation.

### **Activation of Epac increases C/EBP- $\beta$ , SOCS3 expression while blunts STAT3 activation.**

The transcription factor, C/EBP- $\beta$ , has been reported to bind directly to the SOCS3 promoter region(33). Here, we found that the expression of C/EBP- $\beta$  in db/db mice was significantly reduced compared with db/m mice (Fig. 4a (b vs a)). Interestingly, the expression C/EBP- $\beta$  was significantly increased by the treatment of 8-O-cAMP (Fig. 4a (c) ). Considering that the MCP-1 is a classical STAT-responsive inflammatory gene and SOCS3 is known as a negative regulator of STAT3 signaling pathway. Next, we examined the effect of 8-O-cAMP on the expression of SOCS3 and p-STAT (Tyr705). Immunohistochemistry revealed a lower level of SOCS3 and p-STAT3 expression in db/m mice (Fig. 4a (d, g)), and broad distribution of them in db/db mice (Fig. 4a (e, h)). Moreover, activation of Epac with 8-O-cAMP further up-regulated SOCS3 expression (Fig. 4a (f)), while inhibited p-STAT3 expression in db/db mice (Fig. 4a (i)). Similar results were observed with respect to expression of C/EBP- $\beta$ , SOCS3 and p-STAT3, as detected by western blot assays (Fig. 4c). These data suggested that activation of Epac might increase SOCS3 expression and inhibit STAT3/MCP-1 pathway by recovering C/EBP- $\beta$  expression.

### **Sequential changes in expression of SOCS3/STAT3/MCP-1 in HK-2 cells exposed to high glucose.**

Western blot assays and real-time PCR demonstrated that HG (30 mM, D-glucose)-induced the mRNA and protein expression levels of SOCS3 was highest at 8h and then gradually decreased to normal level (Fig. 5a and b). However, the protein expression of p-STAT3 was increased in a time-dependent manner, peaking at 24-48h following treatment with HG (Fig. 5a1). In line with this, a time-dependent increase in the mRNA and protein expression of MCP-1 was observed and was maximal at 48h (Fig. 5a and c).

### **Activation of Epac reduces MCP-1 expression and decreases macrophage recruitment via SOCS3.**

In order to confirm the effect of Epac on p-STAT expression in HK-2 cells expose to HG. HK-2 cells were pretreated with 8-O-cAMP (100 $\mu$ M) for 5 hours before treated with HG. Immunofluorescence assay revealed that the p-STAT3 expression was quite low in HK-2 cells under basal conditions (5.6 mM D-glucose) and HG treatment resulted in the activation of STAT3 (Fig. 6a (a, b) and b), while it was largely reduced by the treatment with 8-O-cAMP (Fig. 6a (c) and b).

To further determine whether the inhibitory effect of Epac on the STAT3 activation was relied on SOCS3, we transfected HK-2 cells with SOCS3 siRNA contaminant with 8-O-cAMP treatment. As shown in Figure 6B, western blot results showed the inhibitory effect of Epac activation on p-STAT3 and MCP-1 expression was abolished by knockdown SOCS3 expression in HK-2 cells exposed to HG ambience. Likewise, we also observed that HG-induced increased p-STAT3 and MCP-1 expression in HK-2 cells were significantly reduced by SOCS3 overexpression, suggesting modulation of SOCS3 expression is sufficient to mimic the activation of Epac. Real-time PCR demonstrated the mRNA expression of SOCS3 and MCP-1 was in accordance with the protein expression. (Fig. 6c and d). These results suggested that Epac-induced SOCS3 expression reduced MCP-1 expression by preventing the STAT phosphorylation in HK-2 cells under HG condition.

Furthermore, macrophage migration assay was used to demonstrate activation of Epac-reduced MCP-1 expression can effectively decrease the recruitment of macrophages. The number of THP-1 cells migrated from the upper side to the bottom side of the membrane were increased by adding the hyperglycemia-stimulated HK-2 cells supernatant (Fig. 6f (b)). While supernatant from HK-2 cells treated with 8-O-cAMP under HG condition, the number of migrated-macrophages were significantly decreased (Fig. 6f (c)).

### **Activation of Epac increases SOCS3 expression via C/EBP- $\beta$ .**

By IF staining, C/EBP- $\beta$  was found to be highly expressed in the nuclear in HK-2 cells under basal conditions (Fig. 7a (a)). The HG exposure reduced nuclear fluorescence of C/EBP- $\beta$  (Fig. 7a (b)), which was restored by 8-O-cAMP treatment (Fig. 7a (c)). The transcription factor, C/EBP- $\beta$ , has been reported to bind directly to the SOCS3 promoter region(33), we next determined whether Epac modulates the expression of SOCS3 by C/EBP- $\beta$ . HK-2 cells were transfected with C/EBP- $\beta$  siRNA before HG treatment. Western blot analysis showed that the expression of C/EBP- $\beta$  significantly decreased in HK-2 cells exposure to HG, and activation of Epac by 8-O-cAMP restored C/EBP- $\beta$  and SOCS3 expression, while this effect was neutralized by C/EBP- $\beta$  siRNA transfection (Fig. 7b). Similar results of SOCS3 mRNA

expression were demonstrated using real-time PCR (Fig. 7c). These data confirmed the role of C/EBP- $\beta$  in controlling the Epac-dependent induction of the SOCS3 gene in HK-2 cells.

## Discussion

In the present study, we demonstrated that Epac activation by 8-O-cAMP could alleviate inflammatory damage by down-regulated MCP-1 expression in kidney tissues of diabetic mice. We also observed that SOCS3 contributed to this process by suppression the phosphorylation of STAT3 *in vivo* and *in vitro* studies. At the molecular level, activation of Epac induced SOCS3 transcription by recovering C/EBP- $\beta$  expression in tubular cells. These results indicated that Epac may prevent macrophage-mediated inflammation by reducing MCP-1 expression via C/EBP- $\beta$ / SOCS3/STAT3 signaling cascade in DN. Therefore, activation of Epac may be a potential therapeutic target for dampening the progression of the DN.

Emerging evidence has identified that inflammation plays a central role in the pathogenesis of DN, and macrophage infiltration in the interstitial compartment is closely related to the progression of DN(34, 35). The monocyte chemokine, MCP-1, was increased in the glomerular and tubular epithelium, and as a major contributor to macrophage infiltration in the kidney tissues of DN is well established (16, 34, 36, 37). MCP-1, a member of the CC chemokine family acting through its receptor CCR2 (38), is a critical factor in recruiting monocytes/macrophages into inflammation site(39). The expression of MCP-1 in tubular cells and urinary excretion of the protein were both upregulated in human and experimental diabetes, which coincided with macrophage infiltration in both glomeruli and interstitial and correlated with levels of albuminuria and renal function decline(34, 37, 39, 40). Pharmacologic inhibition of MCP-1 or CCR2 effectively reduced the recruitment of macrophage into the interstitial compartment and improved tubulointerstitial damage(41, 42). In diabetic animal models treated with STZ, deficiency of MCP-1 decreased the number of macrophage accumulation and prevented early diabetic renal injury(34, 40). In agreement with these previous studies, we observed that upregulated renal MCP-1 expression was accompanied by increased macrophage infiltration in tubular interstitial in diabetic mice. However, administration of Epac activator 8-O-cAMP dramatically reversed these changes (Fig. 1 and Fig. 3). These data indicated that Epac reduced macrophage-mediated renal injury by inhibiting the expression of MCP-1 in DN. But the specific mechanism by which Epac reduced MCP-1 expression is still not known.

JAK/STAT pathway has been shown to participate in various inflammation response, including in DN(10). Phosphorylated STAT3 translocates to the nucleus and initiates inflammatory-related gene transcription, such as CCL2 (the gene name of MCP-1) (43). Hyperglycemia induces the activation of JAK/STAT3 signaling in glomerular mesangial cells, resulting in cell proliferation and synthesis of extracellular matrix molecules(25). Giving STAT3 inhibitor dramatically decreased renal macrophage infiltration and attenuated inflammation and fibrosis in diabetic rats(44). Moreover, Lu's group constructed streptozocin-induced STAT3 knockdown diabetic mice and found that STAT3 deficiency dramatically reduced the expression of inflammation markers, like IL-6, MCP-1(45). Indicating that activation of STAT3 may play a crucial role in the pathogenesis of DN. However, studies of the relationship of STAT3 and DN are mostly focus on mesangial cells, the role of phosphorylated STAT3 in

the proximal tubular cells exposed to high glucose is under elucidated yet. In agreement with previous studies in renal disease, we observed that the expression of p-STAT3 in the proximal tubule was up-regulated in diabetic mice (Fig. 4). Activation of Epac with 8-O-cAMP inhibited p-STAT3 expression which dramatically reduced the expression of MCP-1 (Fig. 3 and Fig. 4). Thus, suggesting that Epac activation may inhibit MCP-1 expression by preventing STAT3 phosphorylation.

We also investigated the molecular mechanisms by which Epac regulated the STAT3 inactivation under hyperglycemic conditions. SOCS proteins are natural inhibitor family for JAK/STAT signaling pathway and have been demonstrated participating in the regulation of inflammatory signaling (26, 46). Overexpression of SOCS1 or SOCS3 has been shown effectively prevented JAK/STAT activation in cultured renal cells and subsequent inflammatory response (10) (47). Besides, it has been reported that activation of Epac1 induces SOCS3 gene expression in various types of cells, i.e. cardiocyte (27), vascular endothelial cell (28) and retinal ganglion cell (29). In this study, we observed that Epac activation significantly up-regulated the expression of SOCS3 while inhibited the expression of p-STAT3 and MCP-1 in kidney tissues (Fig. 4) and in HK-2 cells (Fig. 6); These effects were imitated by transfection with SOCS3 overexpression plasmid in HK-2 cells exposed to HG; More importantly, knockdown of SOCS3 expression could reverse the inhibitory effect of Epac on STAT3 activation and MCP-1 expression (Fig. 6). These data indicated that Epac attenuated renal inflammation may be associated with the suppression of STAT3 activation at least by SOCS3. Nonetheless, we cannot exclude the other mediators for Epac targeting STAT3 pathway. For example, in our study, we also found that activation of Epac ameliorated renal tubular oxidative stress in db/db mice (Fig. 2). Previous studies have demonstrated that hyperglycemia-induced the production of ROS could also activate JAK/STAT pathway (48). Therefore, the suppression of JAK/STAT activation by Epac might be by inhibiting ROS production in DN, but it needs further investigation.

Next, we attested the role of transcription factor C/EBP- $\beta$  in Epac-induced SOCS3 expression in HK-2 cells. Since our previous study found that Rap1b, a downstream molecule of Epac, could modulate C/EBP- $\beta$  transcription activity in DN, and studies in other cell types also shown that Epac regulated SOCS3 gene expression in a C/EBP- $\beta$  dependent manner (33, 43). As expected, in this study, activation of Epac by 8-O-cAMP could restore C/EBP- $\beta$  expression and promote its translocation to the nuclear in HK-2 cells (Fig. 7). A loss-of function assay on C/EBP- $\beta$  shown that knockdown C/EBP- $\beta$  expression would inhibit the Epac activation-induced SOCS3 gene expression. Taken together, these data suggested C/EBP- $\beta$  as Epac-activated transcription factors that mediate the induction of the SOCS-3 gene in tubular cells. However, future study like luciferase reporter assay would be taken to the evaluate whether stimulation of Epac in tubular cells leads to enhanced binding of C/EBP- $\beta$  to the SOCS-3 promoter.

## Conclusion

In summary, we use *in vivo* and *in vitro* diabetic models to demonstrate the beneficial effects of activation of Epac on macrophage-mediated inflammatory injury and the mechanisms underlying its effects. Epac regulates C/EBP- $\beta$ -mediated SOCS3 transcription and inhibits the expression of MCP-1,

thereby improving renal macrophage infiltration and inflammatory response, ultimately alleviating interstitial fibrosis (Fig. 8). This finding suggests that activation of Epac may provide a new therapeutic target for preventing renal inflammation injury in DN.

## Abbreviations

Epac, Exchange protein activated by cAMP; DN, diabetic nephropathy; ESRD, end-stage renal disease; p-STAT3, phosphorylated STAT3; 8-O-cAMP, 8-pCPT-2'-O-Me-cAMP; HG, high glucose; SOCS, suppressor of cytokine signaling; HE, hematoxylin-eosin; PAS, periodic acid-Schiff; IHC, immunohistochemistry; DHE dihydroethidium; MFI, mean fluorescence intensity; ROS, reactive oxygen species; PMA, phorbol 12-myristate 13-acetate; DAPI, 4',6-diamidino-2 phenylindole; MCP-1, monocyte chemoattractant protein-1; CCL2, chemokine ligand 2.

## Declarations

### Acknowledgements

We thank all who contributed to the present study for their time and effort.

### Funding

This work is supported by the Chinese National Natural Sciences Foundation (No. 81570658).

### Availability of data and materials

All data generated or analyzed during this study are included in this published article.

### Authors' contributions

WY, SZ, JL, YL, and HW performed experiments and analysed data. WY wrote the manuscript. WY, LS and LX conceived the ideas. LX edited the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

### Author details

Department of Nephrology, The Second Xiangya Hospital, Central South University; Hunan Key Laboratory of Kidney Disease and Blood Purification, No.139 Renmin Middle Road, Changsha, 410011, Hunan, China.

## References

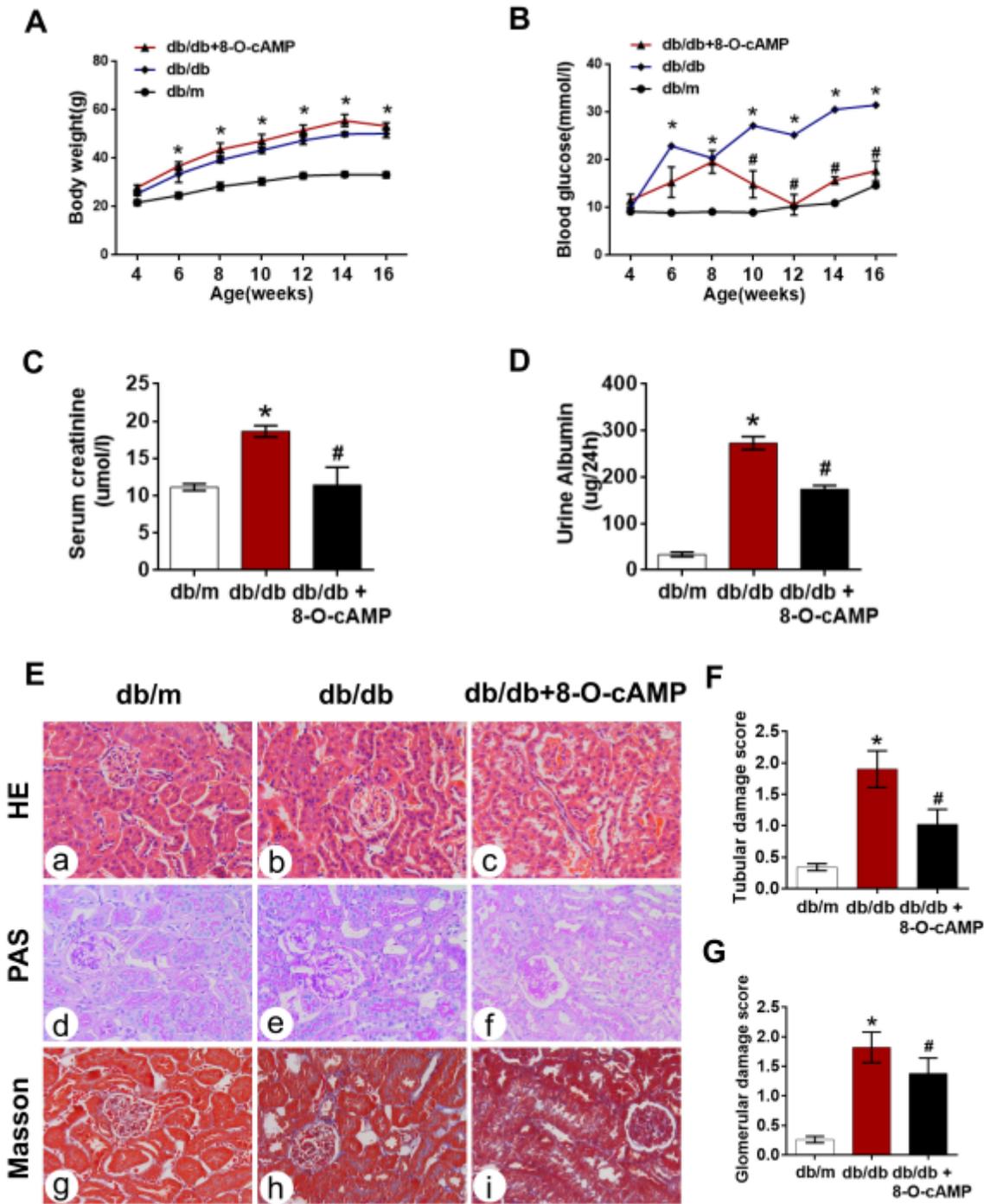
1. Zhang L, Long J, Jiang W, Shi Y, He X, Zhou Z, et al. Trends in Chronic Kidney Disease in China. *The New England journal of medicine*. 2016;375(9):905-6.
2. Hickey FB, Martin F. Role of the Immune System in Diabetic Kidney Disease. *Current diabetes reports*. 2018;18(4):20.
3. Tesch GH. Diabetic nephropathy - is this an immune disorder? *Clinical science (London, England : 1979)*. 2017;131(16):2183-99.
4. Lim AK, Tesch GH. Inflammation in diabetic nephropathy. *Mediators of inflammation*. 2012;2012:146154.
5. Klessens CQF, Zandbergen M, Wolterbeek R, Bruijn JA, Rabelink TJ, Bajema IM, et al. Macrophages in diabetic nephropathy in patients with type 2 diabetes. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association*. 2017;32(8):1322-9.
6. Niu S, Bian Z, Tremblay A. Broad Infiltration of Macrophages Leads to a Proinflammatory State in Streptozotocin-Induced Hyperglycemic Mice. 2016;197(8):3293-301.
7. Usui HK, Shikata K, Sasaki M, Okada S, Matsuda M, Shikata Y, et al. Macrophage scavenger receptor-a-deficient mice are resistant against diabetic nephropathy through amelioration of microinflammation. *Diabetes*. 2007;56(2):363-72.
8. Tesch GH. Macrophages and diabetic nephropathy. *Semin Nephrol*. 2010;30(3):290-301.
9. Gilbert RE. Proximal Tubulopathy: Prime Mover and Key Therapeutic Target in Diabetic Kidney Disease. *Diabetes*. 2017;66(4):791-800.
10. Ortiz-Munoz G, Lopez-Parra V, Lopez-Franco O, Fernandez-Vizarra P, Mallavia B, Flores C, et al. Suppressors of cytokine signaling abrogate diabetic nephropathy. *Journal of the American Society of Nephrology : JASN*. 2010;21(5):763-72.
11. Yang W, Luo Y, Yang S, Zeng M, Zhang S, Liu J, et al. Ectopic lipid accumulation: potential role in tubular injury and inflammation in diabetic kidney disease. 2018;132(22):2407-22.
12. Liu W, Chen X, Wang Y, Chen Y, Chen S, Gong W, et al. Micheliolide ameliorates diabetic kidney disease by inhibiting Mtdh-mediated renal inflammation in type 2 diabetic db/db mice. *Pharmacological research*. 2019;150:104506.
13. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research*. 2009;29(6):313-26.
14. Haller H, Bertram A, Nadrowitz F, Menne J. Monocyte chemoattractant protein-1 and the kidney. *Current opinion in nephrology and hypertension*. 2016;25(1):42-9.
15. Viau A, Bienaime F, Lukas K, Todkar AP, Knoll M, Yakulov TA, et al. Cilia-localized LKB1 regulates chemokine signaling, macrophage recruitment, and tissue homeostasis in the kidney. 2018;37(15).

16. Tesch GH. MCP-1/CCL2: a new diagnostic marker and therapeutic target for progressive renal injury in diabetic nephropathy. *American journal of physiology Renal physiology*. 2008;294(4):F697-701.
17. de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, et al. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature*. 1998;396(6710):474-7.
18. de Rooij J, Rehmann H, van Triest M, Cool RH, Wittinghofer A, Bos JL. Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs. *The Journal of biological chemistry*. 2000;275(27):20829-36.
19. Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, et al. A family of cAMP-binding proteins that directly activate Rap1. *Science (New York, NY)*. 1998;282(5397):2275-9.
20. Robichaux WG, 3rd, Cheng X. Intracellular cAMP Sensor EPAC: Physiology, Pathophysiology, and Therapeutics Development. 2018;98(2):919-1053.
21. Qin Y, Stokman G, Yan K, Ramaiahgari S, Verbeek F, de Graauw M, et al. cAMP signalling protects proximal tubular epithelial cells from cisplatin-induced apoptosis via activation of Epac. *British journal of pharmacology*. 2012;165(4b):1137-50.
22. Fang F, Liu GC, Kim C, Yassa R, Zhou J, Scholey JW. Adiponectin attenuates angiotensin II-induced oxidative stress in renal tubular cells through AMPK and cAMP-Epac signal transduction pathways. *American journal of physiology Renal physiology*. 2013;304(11):F1366-74.
23. Xie P, Joladarashi D, Dudeja P, Sun L, Kanwar YS. Modulation of angiotensin II-induced inflammatory cytokines by the Epac1-Rap1A-NHE3 pathway: implications in renal tubular pathobiology. *American journal of physiology Renal physiology*. 2014;306(11):F1260-74.
24. Huang JS, Chuang LY, Guh JY, Huang YJ, Hsu MS. Antioxidants attenuate high glucose-induced hypertrophic growth in renal tubular epithelial cells. *American journal of physiology Renal physiology*. 2007;293(4):F1072-82.
25. Wang X, Shaw S, Amiri F, Eaton DC, Marrero MB. Inhibition of the Jak/STAT signaling pathway prevents the high glucose-induced increase in tgf-beta and fibronectin synthesis in mesangial cells. *Diabetes*. 2002;51(12):3505-9.
26. Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. *Nature reviews Immunology*. 2007;7(6):454-65.
27. Jin H, Fujita T, Jin M, Kurotani R, Hidaka Y, Cai W, et al. Epac activation inhibits IL-6-induced cardiac myocyte dysfunction. *The journal of physiological sciences : JPS*. 2018;68(1):77-87.
28. Sands WA, Woolson HD, Yarwood SJ, Palmer TM. Exchange protein directly activated by cyclic AMP-1-regulated recruitment of CCAAT/enhancer-binding proteins to the suppressor of cytokine signaling-3 promoter. *Methods in molecular biology (Clifton, NJ)*. 2012;809:201-14.
29. Hellstrom M, Harvey AR. Cyclic AMP and the regeneration of retinal ganglion cell axons. *The international journal of biochemistry & cell biology*. 2014;56:66-73.
30. Zhan M, Usman IM, Sun L, Kanwar YS. Disruption of renal tubular mitochondrial quality control by Myo-inositol oxygenase in diabetic kidney disease. *Journal of the American Society of Nephrology : JASN*. 2015;26(6):1304-21.

31. Xiao L, Xu X, Zhang F, Wang M, Xu Y, Tang D, et al. The mitochondria-targeted antioxidant MitoQ ameliorated tubular injury mediated by mitophagy in diabetic kidney disease via Nrf2/PINK1. *Redox biology*. 2017;11:297-311.
32. Xie P, Sun L, Nayak B, Haruna Y, Liu FY, Kashihara N, et al. C/EBP-beta modulates transcription of tubulointerstitial nephritis antigen in obstructive uropathy. *Journal of the American Society of Nephrology : JASN*. 2009;20(4):807-19.
33. Yarwood SJ, Borland G, Sands WA, Palmer TM. Identification of CCAAT/enhancer-binding proteins as exchange protein activated by cAMP-activated transcription factors that mediate the induction of the SOCS-3 gene. *The Journal of biological chemistry*. 2008;283(11):6843-53.
34. Chow FY, Nikolic-Paterson DJ, Ozols E, Atkins RC, Rollin BJ, Tesch GH. Monocyte chemoattractant protein-1 promotes the development of diabetic renal injury in streptozotocin-treated mice. *Kidney international*. 2006;69(1):73-80.
35. Zhang MH, Feng L, Zhu MM, Gu JF, Jiang J, Cheng XD, et al. The anti-inflammation effect of Moutan Cortex on advanced glycation end products-induced rat mesangial cells dysfunction and High-glucose-fat diet and streptozotocin-induced diabetic nephropathy rats. *Journal of ethnopharmacology*. 2014;151(1):591-600.
36. Wada T, Furuichi K, Sakai N, Iwata Y, Yoshimoto K, Shimizu M, et al. Up-regulation of monocyte chemoattractant protein-1 in tubulointerstitial lesions of human diabetic nephropathy. *Kidney international*. 2000;58(4):1492-9.
37. Banba N, Nakamura T, Matsumura M, Kuroda H, Hattori Y, Kasai K. Possible relationship of monocyte chemoattractant protein-1 with diabetic nephropathy. *Kidney international*. 2000;58(2):684-90.
38. Boring L, Gosling J, Chensue SW, Kunkel SL, Farese RV, Jr., Broxmeyer HE, et al. Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *The Journal of clinical investigation*. 1997;100(10):2552-61.
39. Eardley KS, Zehnder D, Quinkler M, Lepenies J, Bates RL, Savage CO, et al. The relationship between albuminuria, MCP-1/CCL2, and interstitial macrophages in chronic kidney disease. *Kidney international*. 2006;69(7):1189-97.
40. Klueh U, Czajkowski C, Ludzinska I, Qiao Y, Frailey J, Kreutzer DL. Impact of CCL2 and CCR2 chemokine/receptor deficiencies on macrophage recruitment and continuous glucose monitoring in vivo. *Biosensors & bioelectronics*. 2016;86:262-9.
41. Boels MGS, Koudijs A, Avramut MC, Sol W, Wang G, van Oeveren-Rietdijk AM, et al. Systemic Monocyte Chemotactic Protein-1 Inhibition Modifies Renal Macrophages and Restores Glomerular Endothelial Glycocalyx and Barrier Function in Diabetic Nephropathy. *The American journal of pathology*. 2017;187(11):2430-40.
42. de Zeeuw D, Bekker P, Henkel E, Hasslacher C, Gouni-Berthold I, Mehling H, et al. The effect of CCR2 inhibitor CCX140-B on residual albuminuria in patients with type 2 diabetes and nephropathy: a randomised trial. *The lancet Diabetes & endocrinology*. 2015;3(9):687-96.

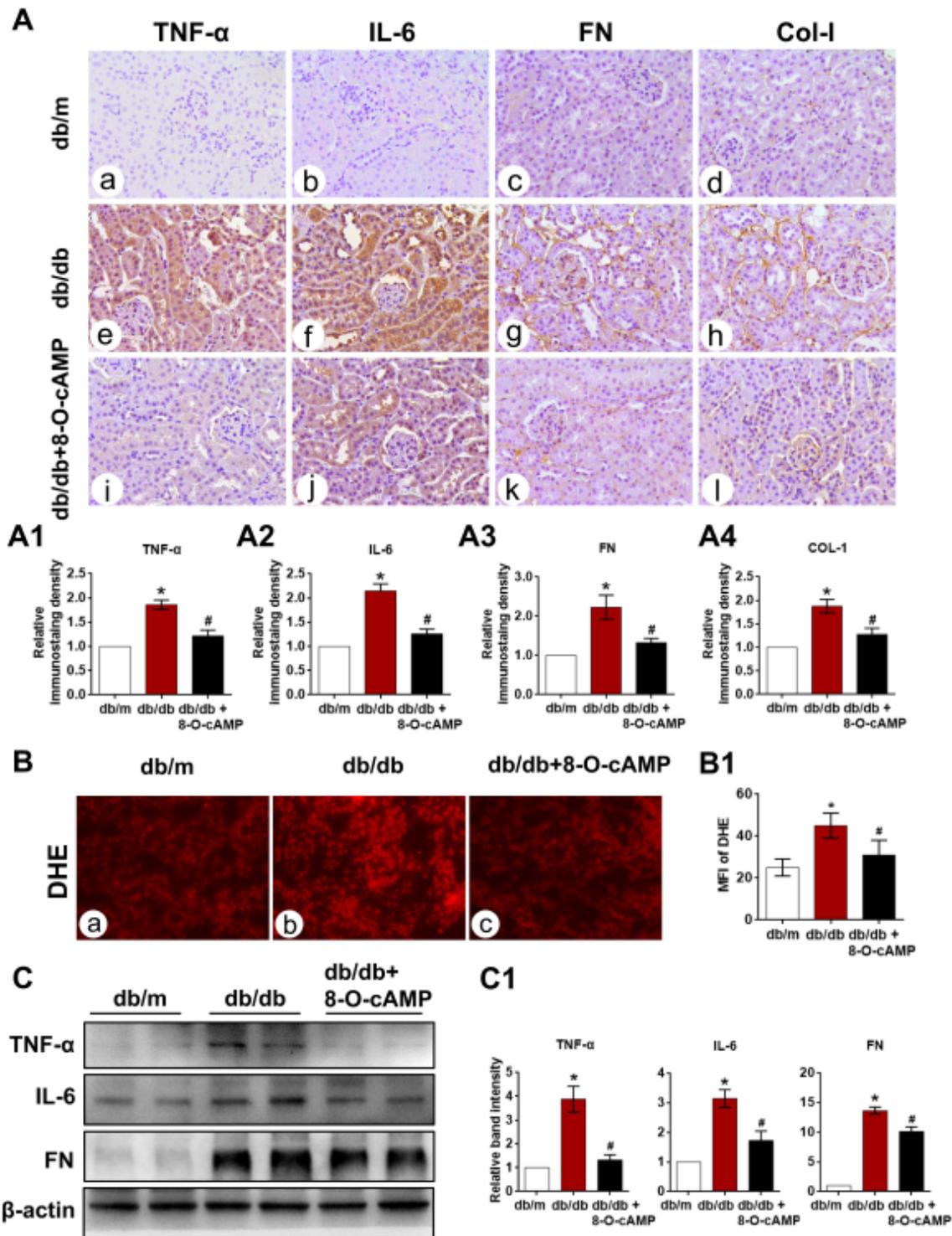
43. Parnell E, Palmer TM, Yarwood SJ. The future of EPAC-targeted therapies: agonism versus antagonism. *Trends in pharmacological sciences*. 2015;36(4):203-14.
44. Said E, Zaitone SA, Eldosoky M, Elsherbiny NM. Nifuroxazide, a STAT3 inhibitor, mitigates inflammatory burden and protects against diabetes-induced nephropathy in rats. *Chemico-biological interactions*. 2018;281:111-20.
45. Lu TC, Wang ZH, Feng X, Chuang PY, Fang W, Shen Y, et al. Knockdown of Stat3 activity in vivo prevents diabetic glomerulopathy. *Kidney international*. 2009;76(1):63-71.
46. Tan JC, Rabkin R. Suppressors of cytokine signaling in health and disease. *Pediatric nephrology (Berlin, Germany)*. 2005;20(5):567-75.
47. Sands WA, Woolson HD, Milne GR, Rutherford C, Palmer TM. Exchange protein activated by cyclic AMP (Epac)-mediated induction of suppressor of cytokine signaling 3 (SOCS-3) in vascular endothelial cells. *Molecular and cellular biology*. 2006;26(17):6333-46.
48. Marrero MB, Banes-Berceli AK, Stern DM, Eaton DC. Role of the JAK/STAT signaling pathway in diabetic nephropathy. *American journal of physiology Renal physiology*. 2006;290(4):F762-8.

## Figures



**Figure 1**

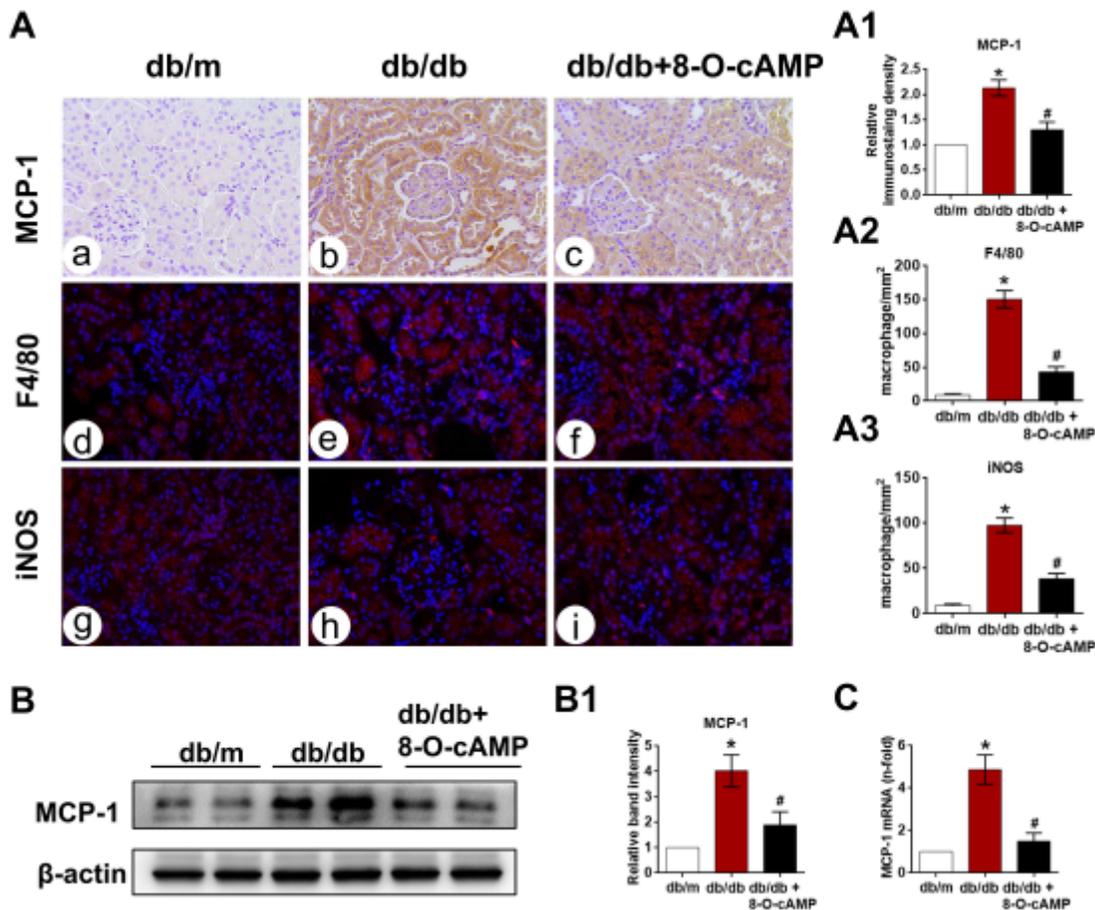
Effect of Epac-selective analogue, 8-pCPT-2'-O-Me-AMP, on renal functional and morphological characteristics in db/db mice. a Body weight changes in db/m, db/db and db/db mice receiving 8-pCPT-2'-O-Me-cAMP (8-O-cAMP) treatment for 8 weeks; b Blood glucose concentration in each group; (c-d) The levels of serum creatinine and urine albumin. e Morphologic analysis was assessed by HE (a-c), PAS (d-f) and Masson's trichrome staining (g-i); (f-g) Quantitative analysis of tubular and glomerular damage scores in each group. n=5; Values are the mean  $\pm$  SD; \*P < 0.05 vs. db/m, #P < 0.05 vs. db/db mice.



**Figure 3**

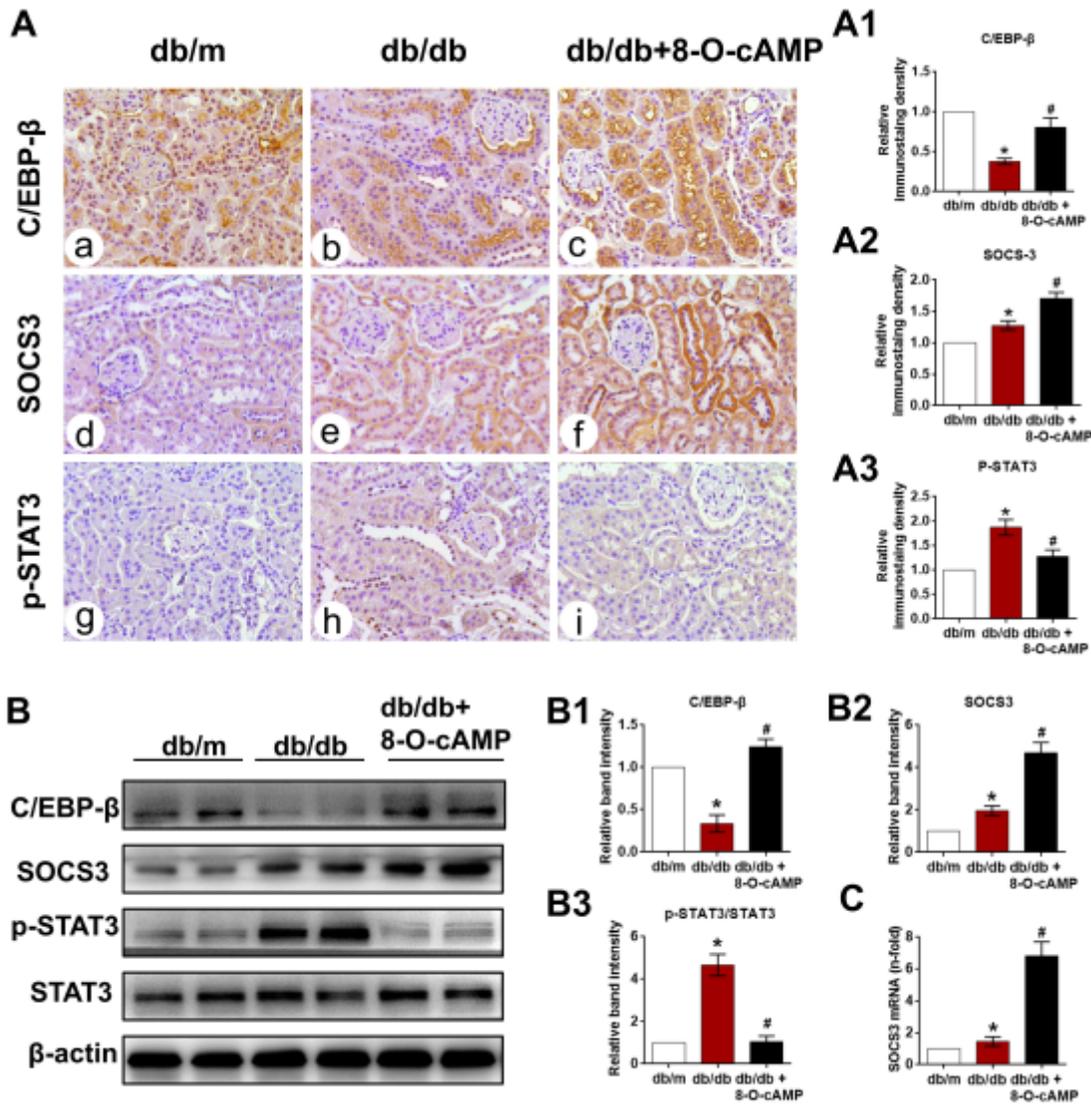
Effect of Epac-selective analogue on renal inflammation response, tubulointerstitial fibrosis and oxidative stress in db/db mice. a Kidney sections from db/m, db/db and db/db mice treated with Epac agonist (8-O-cAMP) were stained with TNF- $\alpha$ , IL-6, fibronectin (FN) and Collagen 1 (Col-I) antibodies for IHC analysis ( $\times 400$ ); (a1-a4) Quantitative analysis of immunostaining intensity of TNF- $\alpha$ , IL-6, FN and Col-I; b Oxidative stress in kidney tissues were assessed by DHE staining; b1 Quantitative analysis of mean fluorescence

intensity (MFI) of DHE; c Western blotting assays showing the changes in TNF- $\alpha$ , IL-6 and FN expression in the kidney tissues; c1 Quantification of western blot band intensity. n=5; Values are the mean  $\pm$  SD; \*P < 0.05 vs. db/m, #P < 0.05 vs. db/db mice.



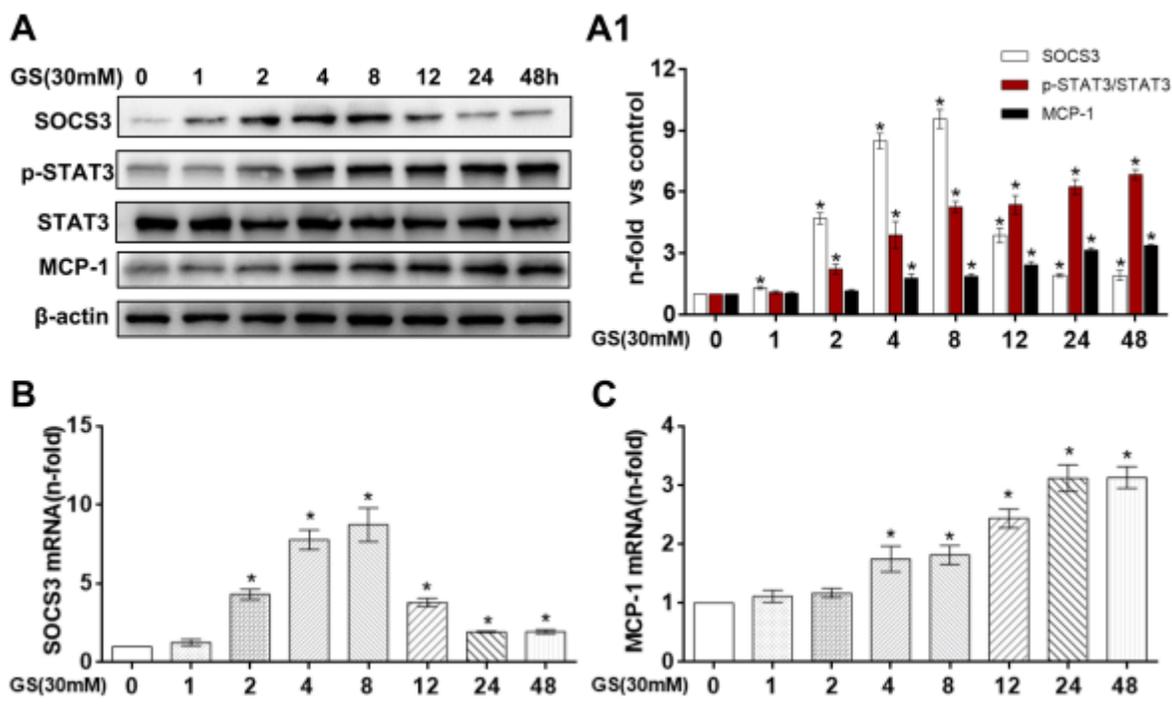
**Figure 5**

Effect of Epac agonist on the expression of MCP-1 and macrophage accumulation in kidney tissues. a IHC assay assessing the expression of MCP-1 in the kidneys of db/m, db/db and db/db mice treated with 8-O-cAMP (a-c) and macrophages infiltration was visualized by F4/80 (d-f) and iNOS (g-i) immunofluorescence staining ( $\times 400$ ); a1 Quantification analysis of MCP-1 expression; (a2-a3) Quantification of F4/80 and iNOS positive cells per mm<sup>2</sup> in kidney tissues; b The expression of MCP-1 was measured by western blot assay; b1 Quantification of western blot band intensity; c Relative MCP-1 mRNA expression in the kidney tissues. n=5; Values are the mean  $\pm$  SD; \*P < 0.05 vs. db/m, #P < 0.05 vs. db/db mice.



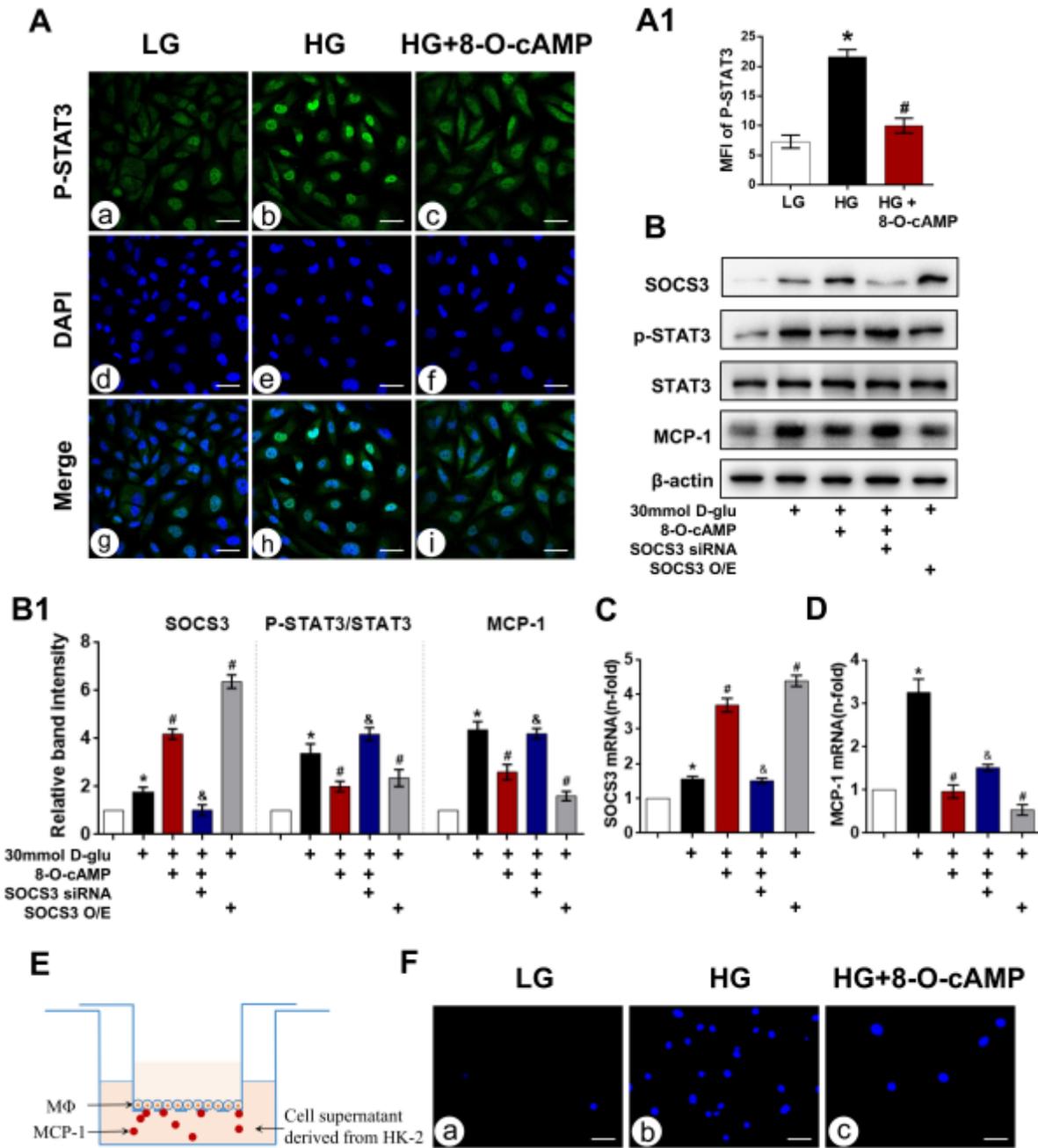
**Figure 7**

Effect of Epac agonist on the renal C/EBP-β, SOCS3 and p-STAT3 expression. a The location and expression of C/EBP-β (a-c), SOCS3 (d-f) and p-STAT (g-i) were assessed by IHC in each group (×400); (a1-a3) Quantification of C/EBP-β, SOCS3 and p-STAT expression in kidney tissues; b The expression of C/EBP-β, SOCS3 and p-STAT (Tyr705) expression were detected by western blot assay; (b1-b3) Quantification of western blot results; c Relative SOCS3 mRNA expression in the kidney tissues. n=5; Values are the mean ± SD; \*P < 0.05 vs. db/m, #P < 0.05 vs. db/db mice.



**Figure 9**

Effect of high glucose on SOCS3/STAT3/ MCP-1 expression in HK-2 cells. a Western blotting assays showed the changes in SOCS3, p-STAT3 and MCP-1 protein expression in HK-2 cells subjected to 30 mM D-glucose at different time point; a1 Quantification of western blot results; (b-c) Gene expression of SOCS3 and MCP-1 was analyzed by real-time PCR. n=3; Values are the mean  $\pm$  SD. \*P < 0.05 vs. LG; #P < 0.05 vs. HG.



**Figure 11**

SOCS3 inhibit MCP-1 expression by suppression of STAT3 activation in HK-2 cells subjected to HG ambience. a Immunofluorescence staining of phosphorylation STAT3 in HK-2 cells incubated with HG treated with or without 8-O-cAMP; a1 Quantification of MFI of p-STAT3; b The expression of SOCS3, p-STAT3 and MCP-1 were detected by western blot in HK-2 cells treated with or without 8-o-cAMP or pretransfected with SOCS3 siRNA or SOCS3 overexpression plasmid under HG condition; b1 Quantification of western blot results; (c-d) SOCS3 and MCP-1 mRNA was analyzed by real-time PCR; e Macrophage migration assay was conducted as depicted schematically; f Macrophage migration assay showing the migration capability of THP-1 macrophage incubated with media from HG stimulated HK-2

cells and detected by fluorescence microscopy after DAPI staining. Scare bar=40μm; n=3; Values are the mean ± SD. \*P < 0.05 vs LG; #P <0.05 vs. HG; & p<0.05 vs HG+8-O-cAMP.

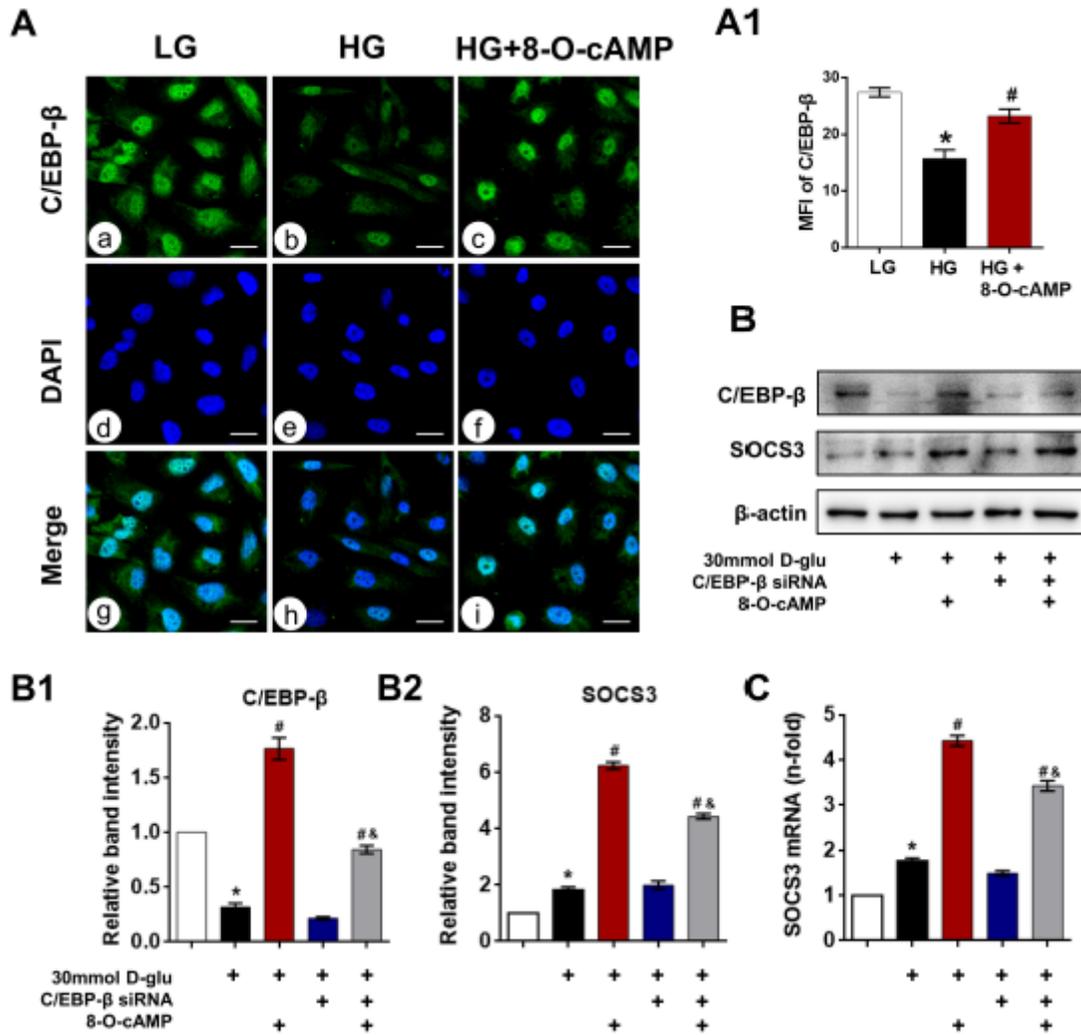


Figure 13

Effect of Epac activation on SOCS3 expression was mediated by C/EBP-β. a Immunofluorescence staining of C/EBP-β in HK-2 cells incubated with HG treated with or without 8-O-cAMP (Scare bar=20μm); a1 Quantification of MFI of C/EBP-β; b Western blot assays of C/EBP-β and SOCS3 in HK-2 cells under HG treated with 8-O-cAMP or transfected with C/EBP-β siRNA; (b1-b2) Quantification of western blot results; c SOCS3 mRNA expression in HK-2 cells as cultured in b. n=3; Values are the mean ± SD. \*P < 0.05 vs. LG; #P <0.05 vs. HG; & p<0.05 vs HG+C/EBP-β siRNA.

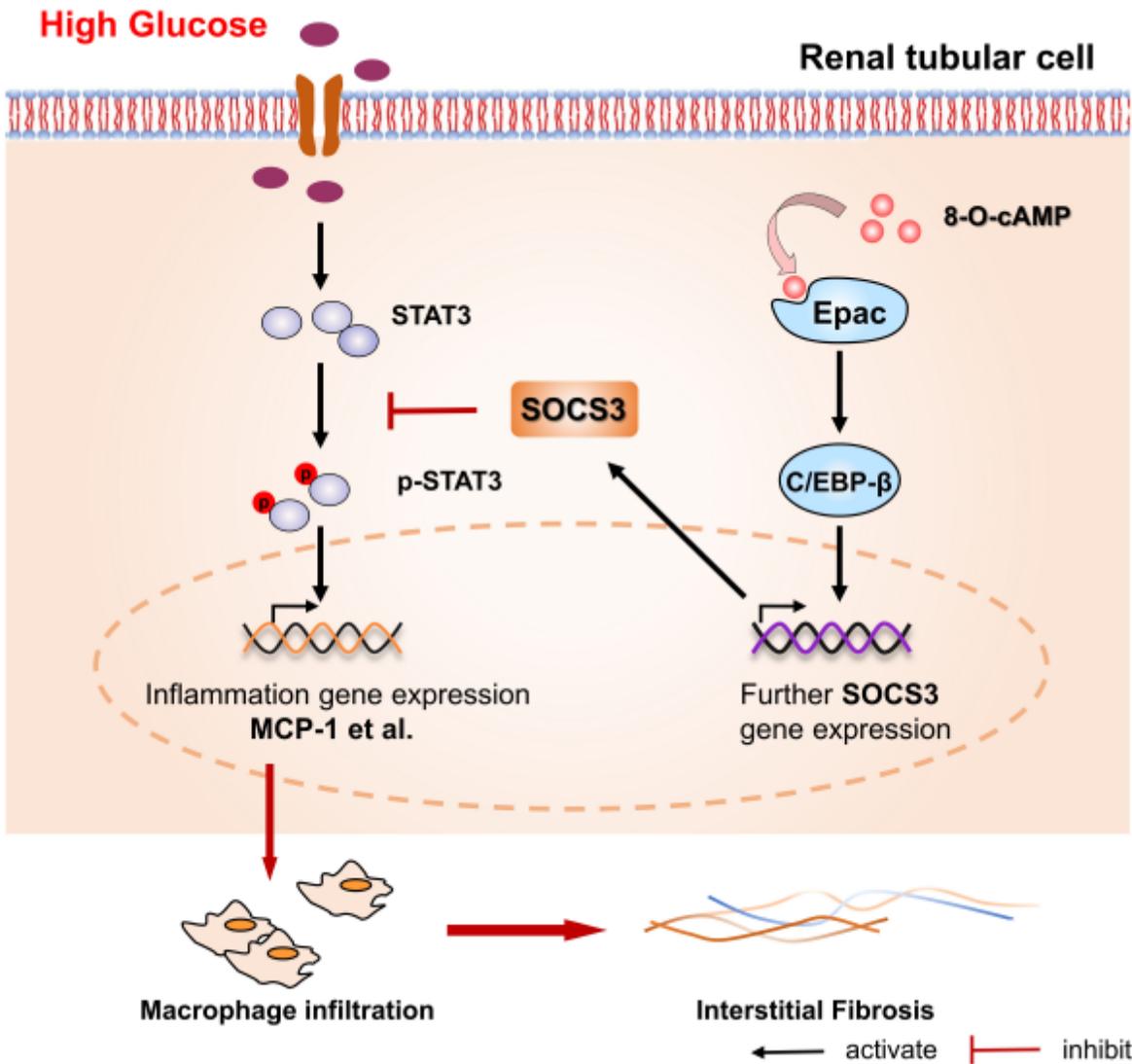


Figure 15

Protective signaling pathways activated by Epac in HK-2 cells subjected to HG ambience. Under HG conditions, selective activation of Epac promotes C/EBP-β nuclear translocation and binds to SOCS3 promoter initiating SOCS3 gene expression, resulting in suppression of STAT3 phosphorylation and subsequently decreasing STAT3-responsive inflammatory genes transcription, MCP-1, etc., thereby attenuating hyperglycemia-induced macrophage infiltration and subsequent tubulointerstitial fibrosis.