

## RESEARCH

# Knockdown of NLRP3 alleviates high glucose or TGFB1-induced EMT in human renal tubular cells

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## Abstract

Tubular injury is one of the crucial determinants of progressive renal failure in diabetic nephropathy (DN), while epithelial-to-mesenchymal transition (EMT) of tubular cells contributes to the accumulation of matrix protein in the diabetic kidney. Activation of the nucleotide binding and oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome leads to the maturation of interleukin (IL)-1 $\beta$  and is involved in the pathogenic mechanisms of diabetes. In this study, we explored the role of NLRP3 inflammasome on high glucose (HG) or transforming growth factor-B1 (TGFB1)-induced EMT in HK-2 cells. We evaluated EMT through the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and E-cadherin as well as the induction of a myofibroblastic phenotype. Reactive oxygen species (ROS) was observed using the confocal microscopy. HG was shown to induce EMT at 48 h, which was blocked by *NLRP3* silencing or antioxidant N-acetyl-L-cysteine (NAC). We found that *NLRP3* interference could inhibit HG-induced ROS. Knockdown of *NLRP3* could prevent HG-induced EMT by inhibiting the phosphorylation of SMAD3, P38 MAPK and ERK1/2. In addition, P38 MAPK and ERK1/2 might be involved in HG-induced NLRP3 inflammasome activation. Besides, TGFB1 induced the activation of NLRP3 inflammasome and the generation of ROS, which were blocked by *NLRP3* interference or NAC. Tubular cells exposed to TGFB1 also underwent EMT, and this could be inhibited by *NLRP3* shRNA or NAC. These results indicated that knockdown of *NLRP3* antagonized HG-induced EMT by inhibiting ROS production, phosphorylation of SMAD3, P38MAPK and ERK1/2, highlighting NLRP3 as a potential therapy target for diabetic nephropathy.

## Key Words

- ▶ diabetic nephropathy
- ▶ NLRP3
- ▶ ROS
- ▶ EMT
- ▶ TGFB1

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## Introduction

Diabetic nephropathy (DN) is a worldwide predominant cause of chronic kidney disease (CKD) and the end-stage kidney failure (ESRD) (Fan *et al.* 2015). It is featured by an accumulation of extracellular matrix (ECM), a thickening of the glomerular basement membrane (GBM) and an increased mesangial matrix, ended up with the

glomerulosclerosis and tubulointerstitial fibrosis (Loeffler *et al.* 2012). Tubulointerstitial fibrosis is the crucial pathological alteration underlying the progressive CKD in diabetes, while the transdifferentiation of renal tubular epithelial cells into myofibroblasts, namely, epithelial to mesenchymal transition (EMT), is momentous in

tubulointerstitial fibrosis (Wei *et al.* 2013). Characterized by loss of epithelial phenotype and gain of profibrotic features, which is the feature of mesenchymal cells, EMT has been implicated in the accelerated fibrogenesis of renal tubular cells that is relevant with diabetic nephropathy (Morcos *et al.* 2002, Burns *et al.* 2006).

It has been reported that high glucose (HG) may be associated with lots of structural or functional alterations of renal tubular cells (Sun *et al.* 2008, Sun *et al.* 2011), and that EMT is one of the potential mechanisms of HG-induced nephrotic dysfunctions (Simonson 2007). Accumulating evidence implicates that HG-induced EMT contributes to the generation of renal fibrosis in diabetic nephropathy (Forbes *et al.* 2008). Further, renal tubular EMT can also be induced by transforming growth factor B1 (TGFB1) (Rhyu *et al.* 2005, Wei *et al.* 2013), and TGFB1 might be the key inducer of EMT since TGFB1 signaling is sufficient to induce EMT in cultured epithelial cells (Piek *et al.* 1999, Bottinger & Bitzer 2002). Wang *et al.* (2017) demonstrated that IL17 could induce A549 alveolar epithelial cells to undergo EMT through the TGFB1-mediated extracellular regulated protein kinases1/2 (ERK1/2) and *Drosophila* mothers against decapentaplegic protein2/3 (SMAD2/3) activation. Another study suggested that P38 mitogen-activated protein kinases (P38 MAPK) might play an important role in the HG-induced EMT in tubular epithelial cells (Lv *et al.* 2011).

Reactive oxygen species (ROS) are considered as critical signaling molecules modulating renal lesions in diabetes (Nishikawa *et al.* 2000). Besides, the generation of ROS by damaged or dysfunctional mitochondria, particularly O<sub>2</sub><sup>-</sup>, has been well recognized as the principal initial event in the development of diabetic complications (Ha *et al.* 2002). High glucose, advanced glycation end products (AGEs), angiotensin II (Ang II), along with TGFB1, all could increase the intracellular ROS in renal cells and contribute to the progression of diabetic renal injuries (Jaimes *et al.* 1998, Yamagishi *et al.* 2003, Rhyu *et al.* 2005, Lee & Han 2010). In addition, previous studies have shown that ROS-mediated HG, Ang II and TGFB1-induced EMT in renal tubular cells (Stutz *et al.* 2009, Lee & Han 2010, Chen *et al.* 2012). Thus, cutting down the production of ROS might have therapeutic effect on renal injuries related with diabetes.

Nucleotide binding and oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome, including the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC) and pro-Caspase-1 (Nakahira *et al.* 2011, Shaw *et al.* 2011), is the best-featured inflammasome up to now and serves

as a molecular platform for the maturation and secretion of interleukin-1B (IL1B) and interleukin-18 (IL18) (Shaw *et al.* 2011). Previous studies have verified that NLRP3 inflammasome was activated by a few sorts of stimuli in various cell types (Kim *et al.* 2015, Ding *et al.* 2016), especially in renal tubular epithelial cells (Vilaysane *et al.* 2010, Wang *et al.* 2015), and in the kidneys of diabetic animals (Fioretto & Mauer 2007, Wang *et al.* 2015). One hypothesis suggests that NLRP3 activators have in common that they induce ROS, which, in turn, are sensed indirectly by NLRP3 leading to its activation (Dostert *et al.* 2008, 2009). HG and TGFB1 could induce ROS (Rhyu *et al.* 2005, Lee & Han 2010), and the produced ROS, could lead to the generation of a potential ligand of NLRP3 or, alternatively, modify NLRP3 or associated proteins directly the other way round. However, the role of NLRP3 inflammasome on HG- or TGFB1-induced EMT via ROS in renal tubular epithelial cells is still unclear.

In the present study, we examined the activation of NLRP3 inflammasome in HG-treated HK-2 cells and the effects of knockdown of NLRP3 with shRNA on HG or TGFB1-induced EMT *in vitro*.

## Materials and methods

### Reagents

DMEM-F12 medium was purchased from Corning (Steuben County, NY, USA). D-glucose, mannitol, N-acetylcysteine (NAC), SB203580 and PD98059 were obtained from Sigma. FuGENE HD Transfection Reagent and the reverse transcription system were purchased from Promega and SYBR Premix Ex TaqTMII was from TaKaRa. Recombinant human TGFB1 was obtained from Abgent (San Diego, CA, USA). Antibody against NLRP3, IL18 and p-SMAD3 was purchased from Abcam. Antibodies for TGFB1,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), E-cadherin, Caspase-1 and ASC were obtained from Proteintech (Chicago, IL, USA). Antibodies for P38 MAPK, ERK1/2, SMAD3, pP38 MAPK and pERK1/2 were purchased from Cell Signaling Technology. NLRP3 shRNA plasmid and control shRNA plasmid were obtained from Yingrun Biotechnologies, Inc (Changsha, China).

### Cell culture and transfection

The HK-2 cells (ATCC) were cultured in a 95% air, 5% CO<sub>2</sub> atmosphere at 37°C in DMEM-F12 (3:1) medium supplemented with 10% fetal bovine serum, 100U/mL penicillin, and 100µg/mL streptomycin. Stable transfections of HK-2 cells with NLRP3 shRNA plasmid

or control shRNA plasmid were conducted with FuGENE HD Transfection Reagent (Promega) according to the manufacturer's instructions. The cells were cultured in serum-free DMEM-F12 medium for 6h, and then the substrate was changed into the original medium. After this period, HK-2 cells were stimulated with normal glucose (NG) (5.6mM), HG (30mM), NG plus mannitol (24.4mM) as an osmotic control, HG plus NAC (5mM), HG plus TGFB1 (4ng/mL), HG plus SB203580 (10 $\mu$ M) or HG plus PD98059 (50 $\mu$ M) at indicated time points.

### Quantitative RT-PCR analysis

Total RNA and cDNA from HK-2 cells were prepared using TRIzol reagent and RT-PCR kits according to the manufacturer's instructions. cDNA was amplified using PCR with specific primers for *NLRP3*, *ASC*, *Caspase-1*, *IL1B*, *IL18*, *E-cadherin*,  $\alpha$ -SMA and 18s rRNA (Table 1). Real-time PCR was performed in a 96-well optical reaction plate using SYBR Premix Ex TaqTMII. Real-time PCR reactions were performed on Agilent Mx3000P QPCR Systems.

### Western blot analysis

Proteins were extracted from HK-2 cells using RIPA buffer, separated by SDS-PAGE and then transferred to a PVDF membrane (Millipore). The membrane was incubated with the primary antibodies (*NLRP3*, *ASC*, *Caspase-1*, *IL1B*, *IL18*, *P38 MAPK*, *p38 MAPK*, *pERK1/2*, *ERK1/2*, *pSMAD3*, *SMAD3*,  $\alpha$ -SMA, *E-cadherin* and  $\beta$ -actin) overnight at 4°C and incubated with goat anti-rabbit or

mouse IgG horseradish peroxidase conjugate, and then scanned using the Odyssey Fc System (LI-COR, USA).

### Mitochondrial ROS detection

The mitochondrial formation of ROS was detected using MitoSox (Sigma). After culture in six-well plates under the different experimental conditions for 48h, the cells were incubated with MitoSOX at a final concentration of 5 $\mu$ M for 30min at 37°C. Cells were washed twice with PBS and images were acquired using a confocal microscope (Leica, Germany). The mitochondrial ROS intensity was quantified using the software Image-Pro Plus 6.0.

### Immunofluorescence

HK-2 cells were grown and stimulated in six-well chamber slides fixed with frozen acetone for 10min at 4°C. We punched the HK-2 cell membrane using Triton X-100. Then, the cells were incubated with anti- $\alpha$ -SMA as well as *E-cadherin* antibody at 4°C overnight. Afterward, the HK-2 cells were exposed to goat anti-rabbit Alexa Fluor 594 conjugated secondary antibody for 1h at 37°C. Finally, the cells were examined by a fluorescence microscope (Olympus BX63).

### Statistical analysis

The results from at least three independent experiments were expressed as mean $\pm$ s.d.. Statistical analysis was performed by one-way ANOVA. Statistical significance was defined as  $P < 0.05$ .

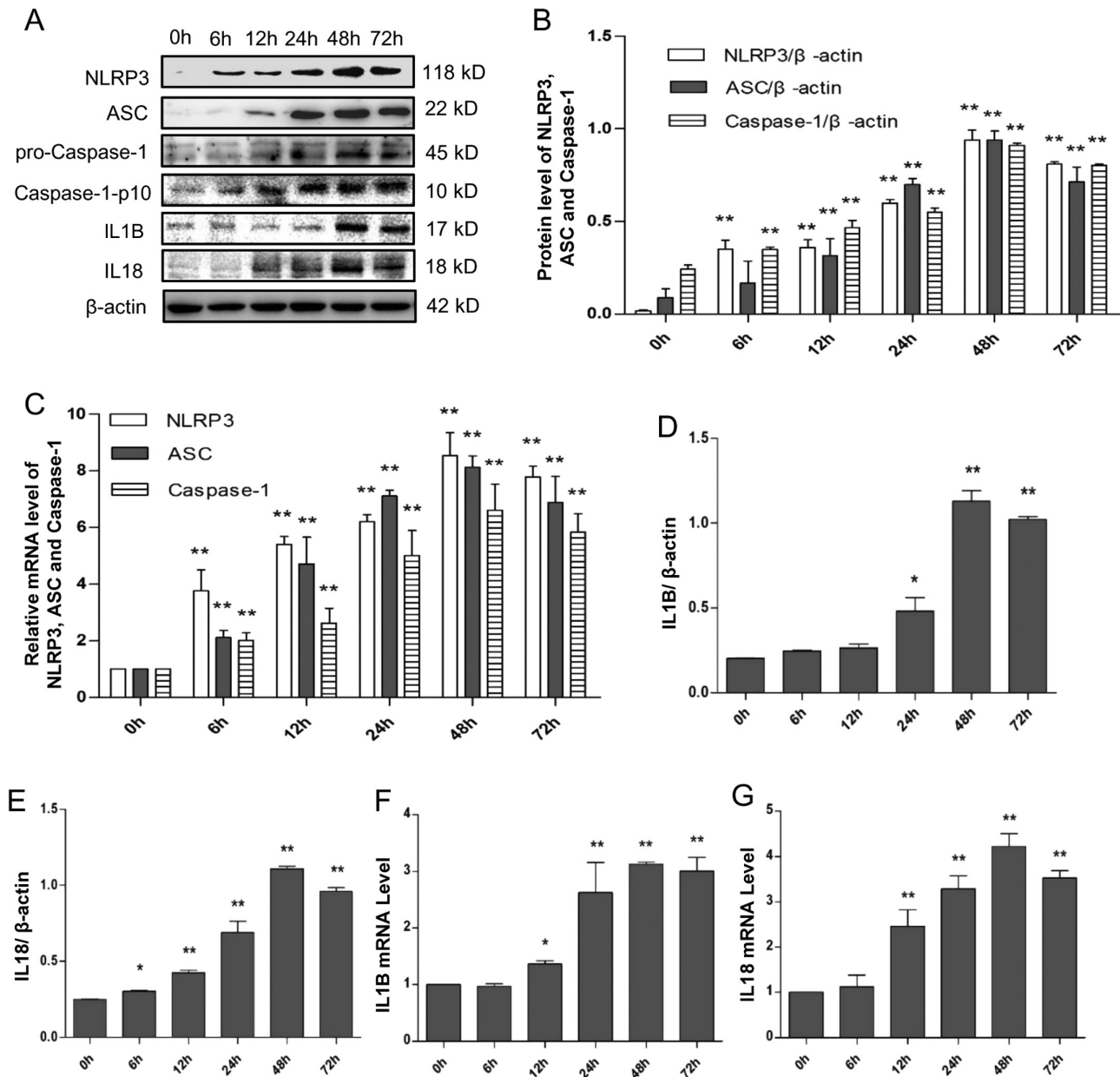
## Results

### HG induces the activation of NLRP3 inflammasome in HK-2 cells

To ascertain the role of HG in the activation of NLRP3 inflammasome *in vitro*, HK-2 cells were incubated with high concentration of glucose for varying time phases, and then, the protein and mRNA levels of NLRP3 inflammasome, *IL1B* and *IL18* were determined. As shown in Fig. 1A, B and C, the expression of NLRP3, *ASC* and *Caspase-1* significantly increased in time-dependent manner in HK-2 cells treated with HG, which appeared to reach its maximum at the time point of 48h. We also measured the expression of *IL1B* and *IL-8* of HK-2 cells

**Table 1** The sequences of the PCR primer pairs used in this study.

| Gene              | Sequences                                                                      | Product size (bp) |
|-------------------|--------------------------------------------------------------------------------|-------------------|
| <i>NLRP3</i>      | sense 5'-TGAACAGCCACCTCACTT-3'<br>antisense 5'-CAACCACAATCTCCGAAT-3'           | 170               |
| <i>ASC</i>        | sense 5'-TGGACGCCTTGGACCTCA-3'<br>antisense 5'-GGACCTTCCGTACAGAGCAT-3'         | 225               |
| <i>Caspase-1</i>  | sense 5'-ATTACAGACAAGGGTGCT-3'<br>antisense 5'-GAATAACGGAGTCAATCAAA-3'         | 101               |
| <i>IL1B</i>       | sense 5'-GTGGTGGTGGGAGATTCTGTAG-3'<br>antisense 5'-GAAATGATGGCTTATTACAGTGGC-3' | 141               |
| <i>IL18</i>       | sense 5'-CTCACCACAACCTCTACCTCC-3'<br>antisense 5'-TTCAAGACCAGCCTGACCAAC-3'     | 153               |
| <i>E-cadherin</i> | sense 5'-GAACGCATTGCCACATAC-3'<br>antisense 5'-ACCTTCCATGACAGACCC-3'           | 156               |
| $\alpha$ -SMA     | sense 5'-ATAACATCAAGCCCAAATC-3'<br>antisense 5'-ACTTCCCAAAGCATCAGC-3'          | 245               |
| 18s               | sense 5'-ACACGGACAGGATTGACAGA-3'<br>antisense 5'-GGACATCTAAGGGCATCACAG-3'      | 238               |

**Figure 1**

Activation of NLRP3 inflammasome in HG-treated HK-2 cells. (A) The activation of NLRP3 inflammasome in cultured HK-2 cells treated with HG (30 mM) at various time points were analyzed by Western blot ( $n=6$ ). (B) The quantitative analysis of NLRP3 inflammasome protein level in cultured HK-2 cells treated with HG (30 mM) at various time points ( $n=6$ ). (C) The mRNA expression of NLRP3 inflammasome was analyzed by RT-qPCR ( $n=6$ ). (D and E) The quantitative analysis of protein level of IL1B and IL18 in HG-treated HK-2 cells at various time points ( $n=6$ ). (F and G) The mRNA expression of IL1B and IL18 was analyzed by RT-qPCR ( $n=6$ ). Values are expressed as mean  $\pm$  s.d. \* $P<0.05$ , \*\* $P<0.01$  vs control (0h).

exposed to HG at six time points (0–72h) by Western blotting and RT-qPCR. The protein and mRNA levels of IL1B and IL18 were elevated within 6h and kept on increasing until 72h, which reached its peak at 48h (Fig. 1A, D, E, F and G). Besides, compared with those of the NG group, mannitol did not change the expression of any of these markers in HK-2 cells among different time points (data not shown).

### Knockdown of *NLRP3* prevents HG-induced ROS generation

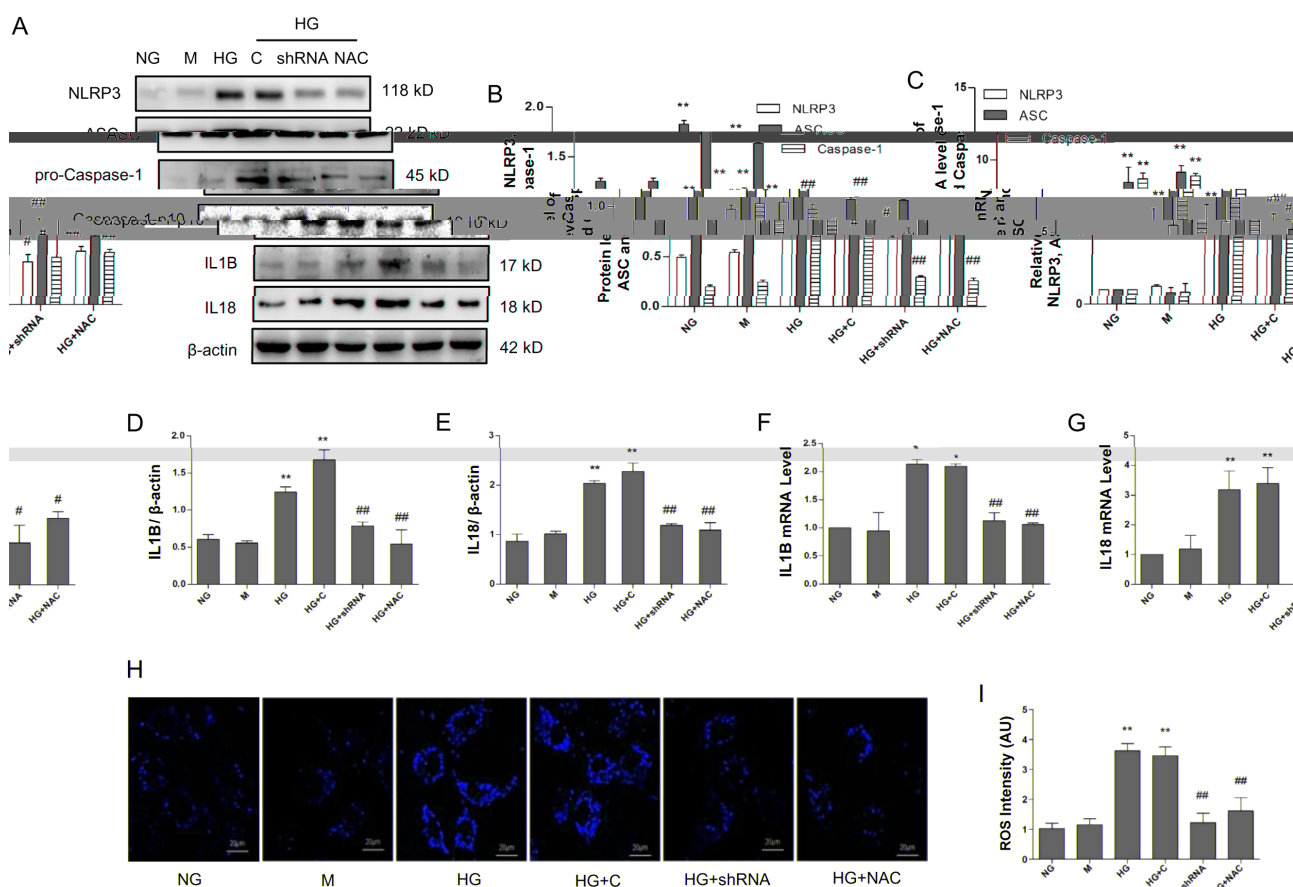
In order to explore the effect of *NLRP3* silencing on HG-induced activation of NLRP3 inflammasome, *NLRP3* shRNA plasmid or control shRNA plasmid were transfected in HK-2 cells, followed by an incubation with HG for 48h. The protein levels of NLRP3, ASC, Caspase-1, IL1B

and IL18 were significantly inhibited by transfection with *NLRP3* shRNA plasmid under HG conditions (Fig. 2A, B, D and E). RT-qPCR results were consistent with the results of Western blot (Fig. 2C, F and G). We also found that NAC, a sort of antioxidant, could prevent the activation of NLRP3 inflammasome and the expression of IL1B and IL18 efficiently (Fig. 2A, B, C, D, E, F and G). Then, ROS generation was detected using a fluorescence probe. The enhanced mitochondrial ROS levels induced by HG were obviously suppressed by transfection of *NLRP3* shRNA plasmid or NAC treatment in HK-2 cells (Fig. 2H and I).

### Knockdown of *NLRP3* prevents HG-induced EMT

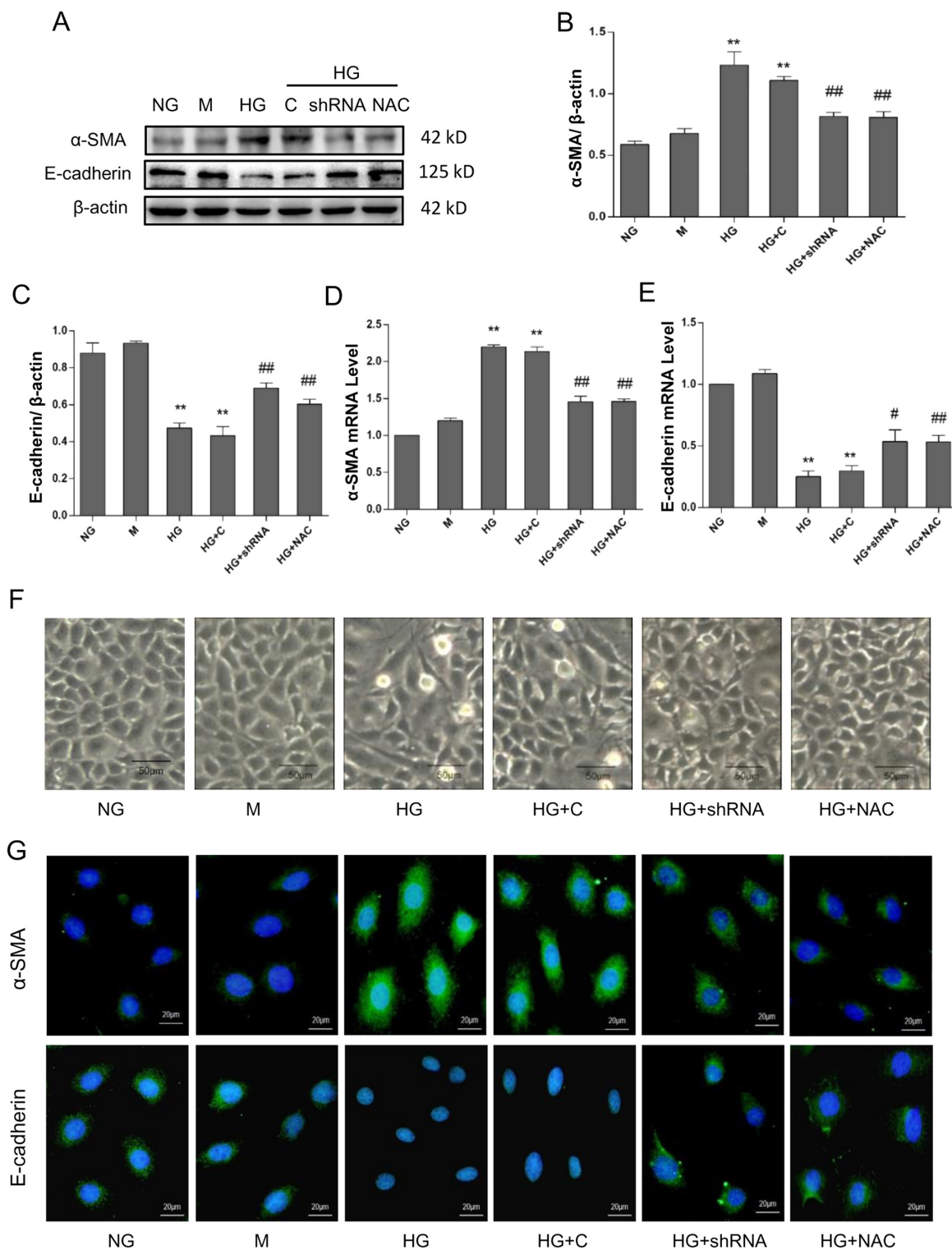
It has been reported that EMT is one of the potential mechanisms of HG-induced renal dysfunction (Wei *et al.*

2013). We investigated whether the activation of NLRP3 inflammasome could regulate HG-induced EMT *in vitro*. HK-2 cells that were cultured in NG or NG+mannitol presented a typical epithelial cuboidal shape with a featured cobblestone morphology; but the cells appeared to be more elongated, less adhered and lost their apical-to-basal polarity after incubated with HG or HG+control shRNA plasmid; while these morphological changes above induced by HG were reversed by *NLRP3* silencing or NAC (Fig. 3F). Compared with the NG group, HG obviously elevated the expression of  $\alpha$ -SMA protein and descended the expression of E-cadherin protein (Fig. 3A, B and C). However, these alterations induced by HG were significantly reversed by transfection with *NLRP3* shRNA plasmid or NAC (Fig. 3A, B and C). The relative mRNA levels of E-cadherin and  $\alpha$ -SMA presented a similar trend



**Figure 2**

Effects of *NLRP3* shRNA plasmid transfection on HG-induced activation of NLRP3 inflammasome and ROS production in HK-2 cells. (A) The protein expression of NLRP3 inflammasome, IL1B and IL18 was analyzed by Western blot ( $n=6$ ). (B) The quantitative analysis of NLRP3 inflammasome protein level ( $n=6$ ). (C) The mRNA expression of NLRP3 inflammasome was analyzed by RT-qPCR ( $n=6$ ). (D and E) The quantitative analysis of protein level of IL1B and IL18 ( $n=6$ ). (F and G) The mRNA expression of IL1B and IL18 was analyzed by RT-qPCR ( $n=6$ ). (H) Mitochondrial ROS were detected by the confocal microscope ( $n=6$ ). (I) The quantification of mitochondrial ROS intensity. NG: 5.6 mM D-glucose; HG: 30 mM D-glucose; HG+C: HG+control shRNA plasmid; HG+shRNA: HG+*NLRP3* shRNA plasmid; HG+NAC: HG+N-acetylcysteine (5 mM). Values are expressed as means  $\pm$  s.d. \* $P<0.05$ , \*\* $P<0.01$  vs NG, # $P<0.05$ , ## $P<0.01$  vs HG+C.

**Figure 3**

Effects of *NLRP3* silencing on HG-induced EMT in HK-2 cells. (A, B and C) The expression levels of  $\alpha$ -SMA and E-cadherin protein were detected by Western blot ( $n=6$ ). (D and E) The mRNA levels of  $\alpha$ -SMA and E-cadherin were analyzed by RT-qPCR ( $n=6$ ). (F) Morphological changes of HK-2 cells cultured under different conditions were analyzed by the inverted microscope. (G) The effect of *NLRP3* shRNA plasmid on the expression of  $\alpha$ -SMA and E-cadherin in HK-2 cells was detected by immunofluorescence. NG: 5.6 mM D-glucose; HG: 30 mM D-glucose; HG+C: HG+control shRNA plasmid; HG+shRNA: HG+*NLRP3* shRNA plasmid; HG+NAC: HG+N-acetylcysteine (5 mM). Values are expressed as means  $\pm$  s.d. \*\* $P<0.01$  vs NG, \* $P<0.05$ , ## $P<0.01$  vs HG+C.

with the results of Western blotting (Fig. 3D and E). In addition, immunofluorescence detection of E-cadherin and  $\alpha$ -SMA confirmed the results of Western blotting (Fig. 3G).

### Knockdown of *NLRP3* prevents HG-induced EMT through inhibiting phosphorylation of SMAD3, P38 MAPK and ERK1/2

Previous studies demonstrated that P38 MAPK, ERK1/2 and SMAD3 were involved in HG-induced EMT in renal tubular epithelial cells (Lan 2012, Wei *et al.* 2013). It remains unclear whether knockdown of *NLRP3* suppressed HG-induced activation of SMAD3, P38 MAPK or ERK1/2. The phosphorylation levels of SMAD3, P38 MAPK and ERK1/2 were markedly upregulated in the HG group, compared with those of the NG group; while the increased phosphorylation levels of SMAD3, P38 MAPK and ERK1/2 were evidently downregulated by *NLRP3* shRNA plasmid transfection or NAC treatment (Fig. 4A, B, C and D).

### HG-induced *NLRP3* inflammasome activation might be mediated by P38 MAPK and ERK1/2

In order to investigate whether P38 MAPK or ERK1/2 signal pathway affected the activation of *NLRP3* inflammasome induced by HG, we preincubated the HK-2 cells with a specific P38 MAPK inhibitor SB203580 (10  $\mu$ M) or ERK1/2 inhibitor PD98059 (50  $\mu$ M) for 0.5 h, and then the cells were incubated with HG in the presence of SB203580 or PD98059 for 48 h. Interestingly, the HG-induced activation of *NLRP3* inflammasome, as well as the secretion of IL1B and IL18 could be inhibited by SB203580 or PD98059 (Fig. 4E, F, G and H), indicating that P38 MAPK and ERK1/2 signaling pathway might be involved in HG-induced activation of *NLRP3* inflammasome in renal tubular epithelial cells.

### TGFB1 induces the activation of *NLRP3* inflammasome in ROS-dependent manner

To explore the role of TGFB1 on *NLRP3* inflammasome activation, HK-2 cells were treated with TGFB1 (4 ng/mL) for 48 h. Compared with the N group, the expression levels of *NLRP3*, ASC and Caspase-1 were upregulated in TGFB1 group; meanwhile, the increased levels of *NLRP3*, ASC and Caspase-1 were significantly alleviated through *NLRP3* silencing or NAC (Fig. 5A, B and C). Besides, the protein and mRNA levels of IL1B and IL18 also showed an elevation in TGFB1 and TGFB1+C group, which

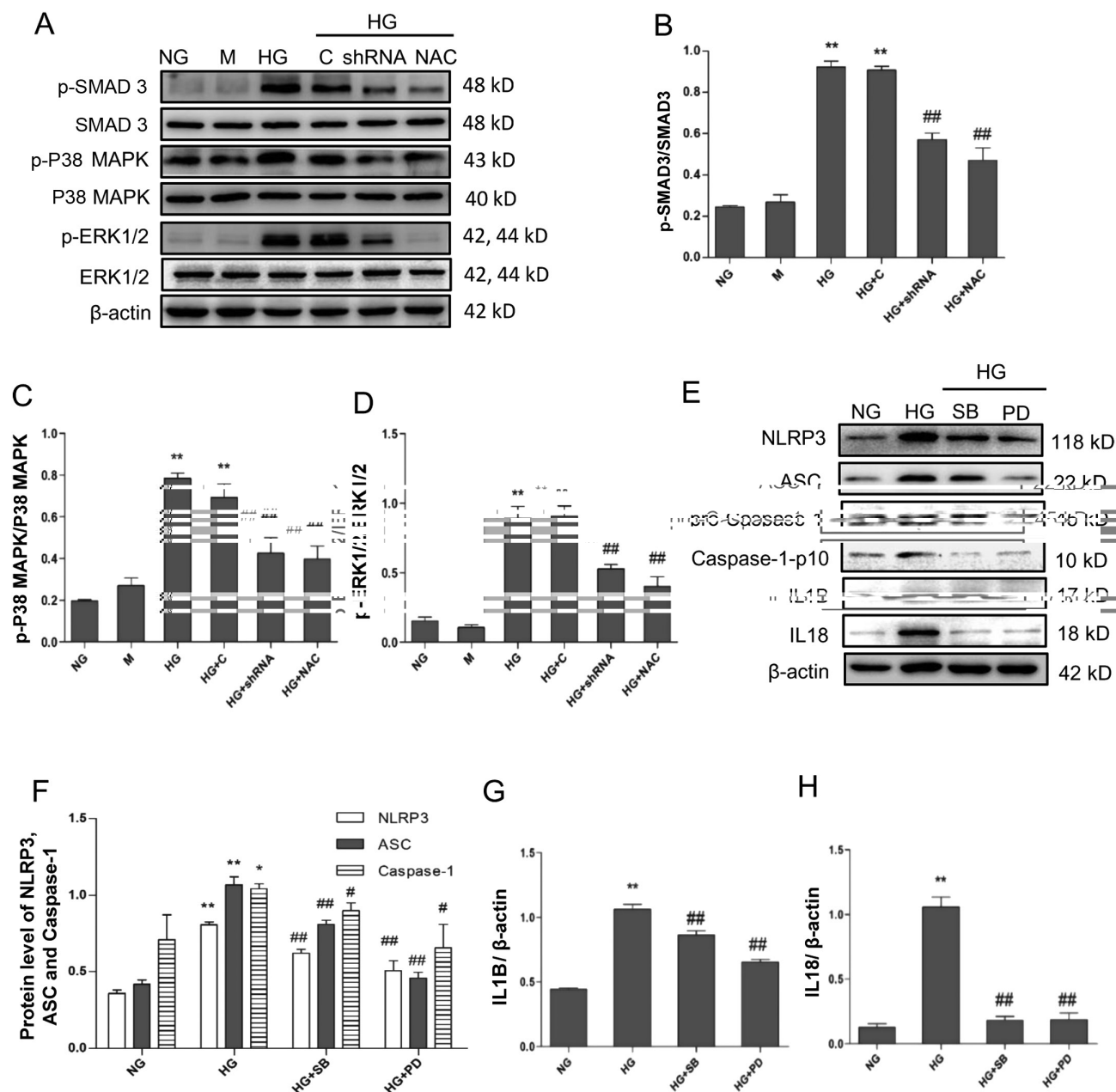
descended by *NLRP3* silencing or NAC (Fig. 5A, D, E, F and G). Next, we detected the ROS generation of different groups in HK-2 cells by the confocal microscope. TGFB1 strongly stimulated an upregulation of ROS generation, which was remarkably reversed by *NLRP3* interference or preincubation with NAC (Fig. 5H and I). Hence, these results indicate that *NLRP3* inflammasome activation induced by TGFB1 is ROS dependent.

### *NLRP3* silencing prevents TGFB1-induced EMT via ROS

Eventually, we investigated the effect of *NLRP3* interference on the TGFB1-induced EMT *in vitro*. HK-2 cells were transfected with *NLRP3* shRNA plasmid or control shRNA plasmid separately, and then cells were treated with TGFB1 for 48 h. Both protein and mRNA expression of  $\alpha$ -SMA were significantly increased in HK-2 cells exposed to TGFB1, while the levels of epithelial marker E-cadherin were blocked (Fig. 6A, B, C, D and E). However, the alterations induced by TGFB1 in HK-2 cells were significantly attenuated by *NLRP3* silencing or NAC (Fig. 6A, B, C, D and E). Besides, the results of immunofluorescence were consistent with that of Western blot above (Fig. 6G). Then, we utilized an inverted microscope to observe morphologic changes of HK-2 cells in various groups. A cuboidal shape was seen in HK-2 cells of N group, whereas a treatment with TGFB1 made their shape turn into spindle-like (Fig. 6F). However, *NLRP3* interference or NAC prevented these alterations induced by TGFB1 (Fig. 6F). Taken together, these data demonstrate that knockdown of *NLRP3* inhibited TGFB1-induced EMT in HK-2 cells via ROS.

## Discussion

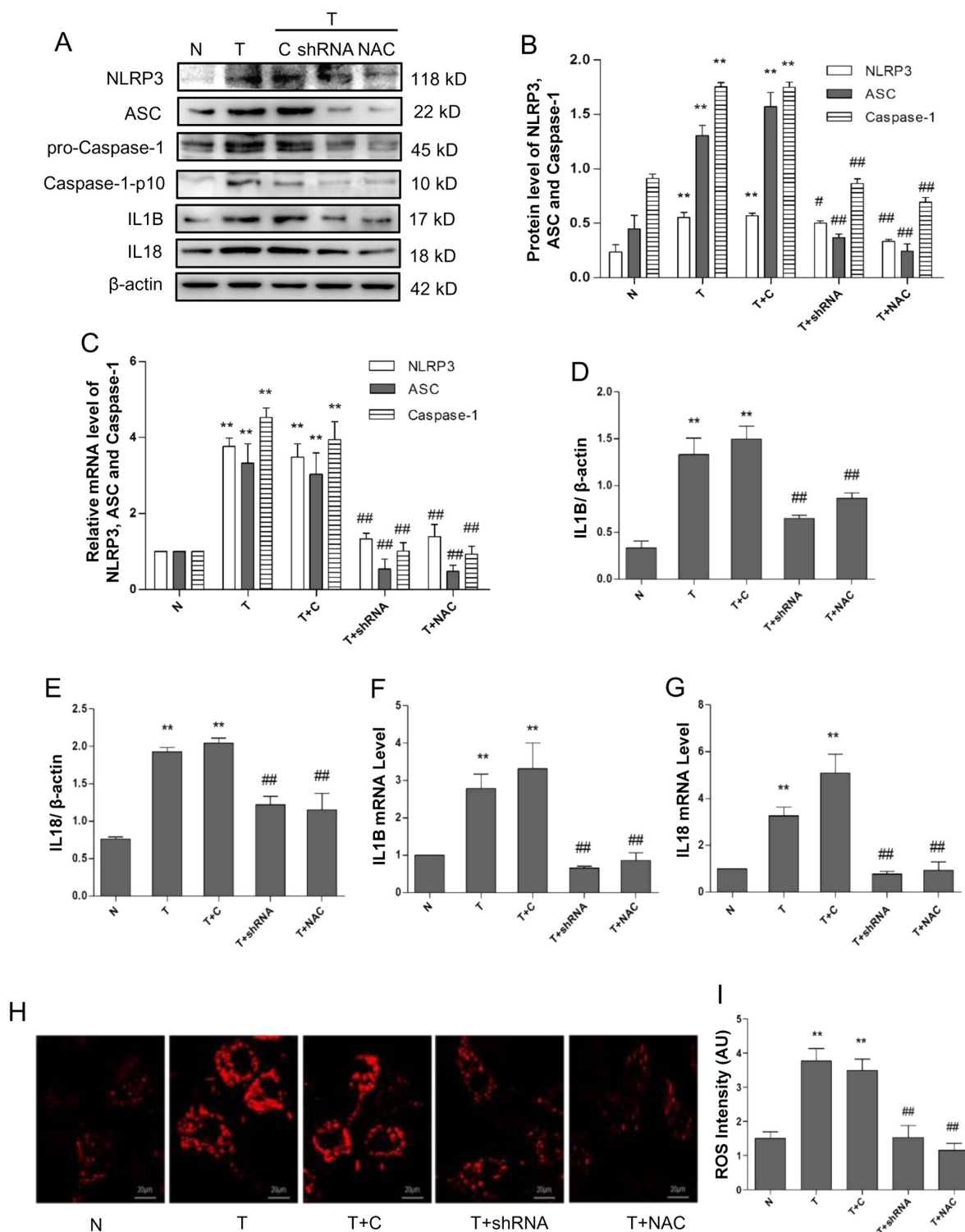
As for diabetic nephropathy, tubulointerstitial lesions were usually regarded as one of the most important elements that could lead to renal fibrosis, rather than glomerular lesions and EMT (Morcos *et al.* 2002). Excessive deposition of extracellular matrix (ECM) of the tubulointerstitium and glomerular mesangium is intimately associated with the progressive decline of renal function in diabetes (Liu 2004, Fioretto & Mauer 2007). EMT, the epithelial-mesenchymal transition, which is induced by HG or TGFB1, has been reported to be accommodated via ROS (Liu 2004, Lee *et al.* 2013). In this study, we explored the effect of *NLRP3* interference on HG-induced EMT in HK-2 cells *in vitro*. We found that  $\alpha$ -SMA was upregulated and E-cadherin was downregulated in HK-2 cells exposed to

**Figure 4**

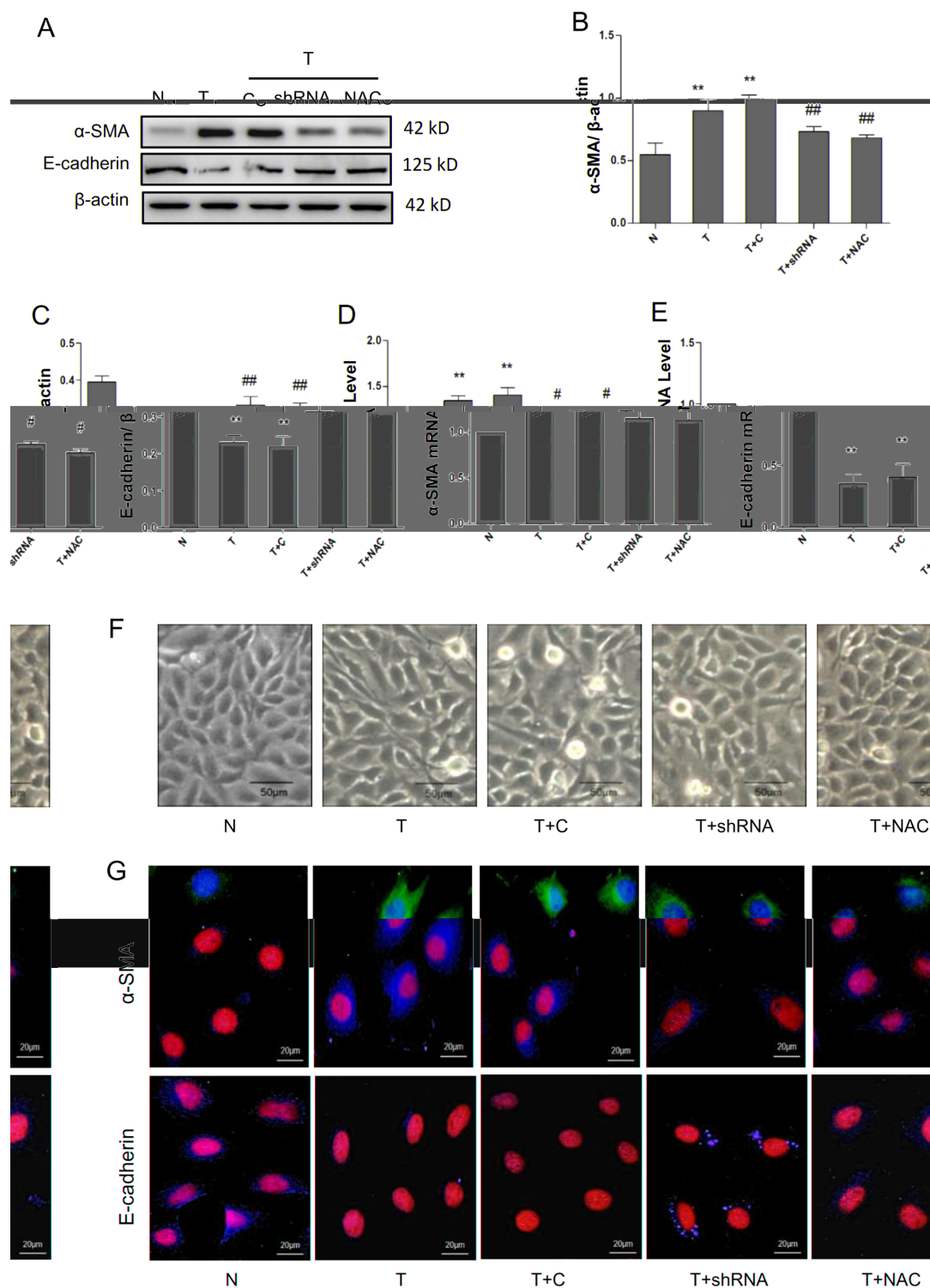
Effect of *NLRP3* interference on HG-induced activation of SMAD3, P38 MAPK and ERK1/2 as well as the effect of SB203580 and PD98059 on NLRP3 inflammasome. (A, B, C and D) The expression levels of p-SMAD3, SMAD3, p-P38 MAPK, P38 MAPK, pERK1/2 and ERK1/2 were analyzed by Western blot ( $n=6$ ). NG: 5.6 mM D-glucose; HG: 30 mM D-glucose; HG+C: HG+control shRNA plasmid; HG+shRNA: HG+NLRP3 shRNA plasmid; HG+NAC: HG+N-acetylcysteine (5 mM). (E, F, G and H) The expression level of NLRP3 inflammasome and the maturation of IL1 $\beta$  and IL18 was analyzed by Western blot ( $n=4$ ). NG: 5.6 mM D-glucose; HG: 30 mM D-glucose; HG+SB: HG+SB203580 (10  $\mu$ M) and HG+PD: HG+PD98059 (50  $\mu$ M). Values are expressed as means  $\pm$  s.d. \* $P<0.05$ , \*\* $P<0.01$  vs NG, # $P<0.05$ , ## $P<0.01$ , vs HG+C.

elevated glucose conditions within a time-dependent manner. Knockdown of *NLRP3* inhibits HG-induced EMT and activation of SMAD3, P38 MAPK and ERK1/2 in HK-2 cells. We also found that *NLRP3* interference inhibited EMT induced by TGF $\beta$ 1.

The NLRP3 inflammasome is a protein complex in cytoplasm, which is activated upon signs of cellular 'danger' to trigger innate immune defenses (Ding *et al.* 2016). When it comes to probing cellular stress, NLRP3 could recruit the adaptor protein ASC and pro-Caspase-1,

**Figure 5**

The effect of TGFB1 on the activation of NLRP3 inflammasome and ROS production in HK-2 cells. HK-2 cells were exposed to 4 ng/mL TGFB1 under different conditions for 48 h. (A) The protein expression of NLRP3 inflammasome, IL1B and IL18 was detected by Western blot ( $n=5$ ). (B) The quantitative analysis of NLRP3 inflammasome protein level ( $n=5$ ). (C) The mRNA expression of NLRP3 inflammasome was analyzed by RT-qPCR ( $n=5$ ). (D and E) The quantitative analysis of protein level of IL1B and IL18 ( $n=5$ ). (F and G) The mRNA expression of IL1B and IL18 was analyzed by RT-qPCR ( $n=5$ ). (H) Mitochondrial ROS were detected by the confocal microscope ( $n=5$ ). (I) The quantification of mitochondrial ROS intensity. N: normal control; T: TGFB1 (4 ng/mL); T+C: TGFB1 (4 ng/mL)+control shRNA plasmid; T+shRNA: TGFB1 (4 ng/mL)+NLRP3 shRNA plasmid; T+NAC: TGFB1 (4 ng/mL)+N-acetylcysteine (5 mM). Values are expressed as means  $\pm$  s.d. \*\* $P<0.01$  vs N, \* $P<0.05$ , ## $P<0.01$  vs T+C.

**Figure 6**

The effect of *NLRP3* silencing on TGFB1-induced EMT in HK-2 cells. HK-2 cells were exposed to 4 ng/mL TGFB1 under different conditions for 48 h. (A, B and C) The protein levels of α-SMA and E-cadherin were detected by Western blot ( $n=5$ ). (D and E) The mRNA expression levels of α-SMA and E-cadherin were analyzed by RT-qPCR ( $n=5$ ). (F) Morphological changes of HK-2 cells were analyzed by the inverted microscope. (G) The effect of *NLRP3* shRNA plasmid on the expression of α-SMA and E-cadherin in HK-2 cells was detected by immunofluorescence. N: normal control; T: TGFB1 (4 ng/mL); T+C: TGFB1 (4 ng/mL) + control shRNA plasmid; T+shRNA: TGFB1 (4 ng/mL) + *NLRP3* shRNA plasmid; T+NAC: TGFB1 (4 ng/mL) + N-acetylcysteine (5 mM). Values are expressed as means ± s.d. \*\* $P < 0.01$  vs N, # $P < 0.05$ , ## $P < 0.01$  vs T+C.

resulting in Caspase-1 activation and the processing of cytoplasmic targets, including IL1B and IL18 (Schroder & Tschopp 2010, Zhou *et al.* 2011). Lately, the NLRP3 inflammasome was demonstrated to participate in the pathogenesis of renal disease (Ding *et al.* 2016). Moreover, *NLRP3* deficiency was shown to mitigate renal inflammation and fibrosis in a model of renal tubular injury (Iyer *et al.* 2009, Zhuang *et al.* 2014). Existing research demonstrated that NLRP3 protein expression, cleavage of Caspase-1, along with the maturation of IL1B and IL18 significantly increased in HK-2 cells after HG stimulation (Chen *et al.* 2013). Also, it has been reported that ROS production contributes to HG-induced NLRP3 inflammasome activation in MVECs (Chen *et al.* 2016). In this article, we showed that HG promoted the expression of NLRP3 protein and mRNA in a time-dependent manner in HK-2 cells, and that treatment with the antioxidant, NAC, could significantly suppress NLRP3 expression induced by HG, suggesting that ROS may be involved in HG-induced NLRP3 expression in HK-2 cells. ROS mediated HG-induced EMT in renal tubular cells has been documented (Ha & Lee 2003, Lee *et al.* 2013). In the present study, we found that transfection with *NLRP3* shRNA plasmid or treatment with NAC notably protected the renal tubular cells from HG-induced EMT and ROS generation. These results indicate that *NLRP3* interference might prevent HG-induced EMT via the reduction of ROS.

Till now, the potential molecular mechanisms still remain sealed. Varied forms of signaling pathways are considered to be relevant with the regulation of EMT, for instance, P38 MAPK, ERK1/2 and TGFB1/SMAD (Schramek *et al.* 2003, Liu 2004, Rhyu *et al.* 2005, Hou *et al.* 2015). Previous studies demonstrated that HG-induced EMT blocked by procyanidin and zinc was associated with P38 MAPK and ERK signaling activation (Li *et al.* 2015a, Zhang *et al.* 2015). Lv *et al.* (2011) showed that P38 MAPK played really an important role in the HG-induced EMT in human renal tubular cells. In our study, we confirmed that HG not simply reduced E-cadherin expression and increased  $\alpha$ -SMA expression, but also activated the phosphorylation levels of SMAD3, P38 MAPK and ERK1/2 in HK-2 cells. In order to testify the role of NLRP3, we conducted the transfection experiment against NLRP3 in the activation of SMAD3, P38 MAPK and ERK1/2 induced by HG. An *NLRP3* shRNA plasmid was utilized to transfect with HK-2 cells, as a result, this was ultimately proven to be able to prevent HG-induced activation of SMAD3, P38 MAPK and ERK1/2. Simultaneously, HG-induced SMAD3, P38 MAPK and ERK1/2 activation could also be inhibited by NAC treatment. Interestingly, when we used P38

MAPK inhibitor (SB203580) or ERK inhibitor (PD98059) in HK-2 cells exposed to HG, which were similar to the antioxidant NAC, we found that HG-induced NLRP3 inflammasome activation could be suppressed by them, further suggesting the role of ROS-MAPK in NLRP3 inflammasome activation. In short, the results above implicated that knockdown of *NLRP3* mitigates HG-induced EMT via ROS-MAPK signaling pathway in HK-2 cells, and P38 MAPK and ERK1/2 might mediate the activation of NLRP3 inflammasome.

EMT is involved in the process of renal fibrosis, which is a major contributor in this pathogenesis, and that TGFB1 is a crucial mediator in EMT (Zeisberg & Neilson 2010). As a matter of fact, TGFB1 plays a critical role in the process of diabetic nephropathy and is well recognized as a profibrotic and inflammatory cytokine in the kidney (Qi *et al.* 2006, Zeisberg & Neilson 2010, Lan 2012). It has been reported that ROS has a great impact on tubulointerstitial fibrosis and EMT induced by TGFB1 (Rhyu *et al.* 2005, Samarakoon *et al.* 2013, Liu *et al.* 2016). Our recent study demonstrated that TGFB1 induced EMT and the generation of ROS, and treatment with NAC, the antioxidant, could effectively prevent EMT induced by TGFB1 in HK-2 cells (Wei *et al.* 2013). Analogously, we found that TGFB1 could induce EMT and ROS generation, and treatment with NAC could markedly prevent EMT induced by TGFB1 in HK-2 cells. Moreover, we also found that *NLRP3* silencing could suppress ROS generation and TGFB1-induced EMT. Thus, our data implicate that *NLRP3* silencing may ameliorate TGFB1-induced EMT via inhibition of ROS generation. Besides, we focused further exploration on the effect of TGFB1 on the expression of NLRP3 *in vitro*. In the present study, we found that both mRNA and protein levels were facilitated by TGFB1 in HK-2 cells, and the activation of NLRP3 inflammasome was significantly suppressed by pretreatment with NAC. Our results are consistent with a previous observation that the level of NLRP3 was upregulated by TGFB1 in colon cancer cells (Wang *et al.* 2016). Whereas a NLRP3 inflammasome-independent pathway has been proposed, which involves the TGFB signaling mechanism (Wang *et al.* 2013); and another article reported that inflammasome-related Caspase-1 and non-inflammasome related phospho-SMAD 2/3 pathways both associated with uric acid-induced EMT, inflammation and fibrosis in kidney (Romero *et al.* 2017).

Taken together, all these data above verify that *NLRP3* silencing withstood EMT induced by HG via suppression of ROS generation, activation of SMAD3, P38 MAPK and ERK1/2. Also, *NLRP3* interference could prevent

TGFβ1-induced EMT via ROS. Hence, we may conjecture that NLRP3 could become a promising target for diabetic nephropathy.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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