

STIM promotes the epithelial-mesenchymal transition of podocytes through regulation of FcγRII activity in diabetic nephropathy

Juan Jin^{1,2*}, Meiyu Ye^{1,3*}, Kang Hu^{1,3}, Jianguang Gong^{1,2} and Qiang He^{1,2}

¹Department of Nephrology, Zhejiang Provincial People's Hospital, ²People's Hospital of Hangzhou Medical College and ³The Second Clinical Medical College, Zhejiang Chinese Medical University, Zhejiang, PR China

*Juan Jin and Meiyu Ye contributed equally to this work

Summary. Background. Diabetic nephropathy (DN) is a serious complication in diabetic patients and has been considered as the main cause of end-stage renal disease. However, there are no studies on the role of stromal interaction molecule (STIM) and its two subtypes, STIM1 and STIM2, in the epithelial-to-mesenchymal transition (EMT) of podocytes induced by diabetic kidney disease (DKD). The present study suggests for the first time that STIM inhibition decreases DKD-induced EMT.

Methods. All DKD patients were diagnosed based on renal biopsies carried out at the Department of Nephrology, Zhejiang Provincial People's Hospital and selected using the Mayo Clinic/Renal Pathology Society Consensus Report on Pathologic Classification, Diagnosis, and Reporting of GN. Images were taken and the number of positive puncta in cells was analyzed using software equipped for immunofluorescence microscopy. STIM1, STIM2, FcγRIIa, FcγRIIb, Nephlin, CTGF, and α-SMA protein levels were detected by Western blotting analysis using the corresponding antibodies. The viability of cells was measured using CCK-8 assays. Absorbance at 450 nm was measured with a Multiskan FC Microplate Reader (Thermo Scientific, USA) and the results were normalized to those of untreated cells. All statistical analyses were performed using SPSS 19.0 software (Stanford University, Stanford, CA, USA).

Results. A total of 30 DKD patients and 30 control patients were enrolled in the study. We found that the level of urine protein in patients and db/db diabetic mice is higher than control group and the levels of STIM1 and 2 significantly increased in DKD groups. We also demonstrated that STIM is upregulated during DKD injury. Next, we discovered that DKD-induced podocyte EMT is related to STIM overexpression in vivo and in vitro. Further research demonstrated that STIM siRNA reverses podocytes from DKD-induced injury and EMT and reverses FcγRII activity in HG-treated podocytes.

Conclusion. Our study suggests that STIM and FcγRII play an essential role in the regulation of DKD-induced podocyte EMT. STIM is an essential component of FcγR activation and inhibition of STIM-mediated signaling pathway might be a new strategy to treat IgG-dependent renal diseases.

Key words: Diabetic Nephropathy, STIM, Epithelial-Mesenchymal Transition, FcγR

Introduction

DN is a serious complication in diabetic patients and has been considered as the main cause of end-stage renal disease. It develops in 30-40% of patients with type 1 or 2 diabetes mellitus (Loeffler and Wolf, 2015). The pathophysiology of diabetic nephropathy is complex and not fully elucidated. A typical hallmark of DN is the excessive deposition of extracellular matrix (ECM) proteins in the glomerulus and diffuse thickening of the glomerular basement membrane (GBM) (Zeisberg and

Neilson, 2010; Loeffler et al., 2012; Hu et al., 2015). In clinical practice, a major feature of DN is the gradual increase of urinary albumin excretion, which ultimately results in irreversible renal failure (Gross et al., 2005; Jerums et al., 2009). Therefore, early intervention to diabetes-induced renal injury may delay DN progression.

Podocytes form the filtration slit structure that prevents the escape of plasma proteins from the glomerular circulation. The regenerative capacity of podocytes is limited. Injury, and subsequent loss of podocytes, can seriously affect the integrity of the glomerular filtration barrier, which eventually leads to glomerulosclerosis (Li et al., 2008; Yamaguchi et al., 2009; Herman-Edelstein et al., 2011). Recent reports suggest that podocyte injury is critical in DN progression. The hypothesis that podocytes undergo EMT, leading to podocyte dysfunction and defective glomerular filtration, is supported by several studies in animals and observations in humans (May et al., 2014). Recent studies demonstrate that podocytes change phenotypically in the early stages of DN in STZ-induced rodents, exhibiting increased mesenchymal markers (desmin) and decreased epithelial markers (nephrin). In addition to animal studies, the extensive reduction of nephrin and ZO-1 expression has also been observed in the glomeruli of patients with diabetes (Itoh et al., 2014; Li and He, 2015).

Accumulating evidence suggests that the immune system is involved in the pathophysiology of diabetes complications (Nilsson et al., 2008). Hypercholesterolemia itself triggers proinflammatory events through the activation of pathways associated with innate and adaptive immunity. At sites of injury, IgG immune complexes are recognized by infiltrating and resident cells through specific receptors for the Fc gamma region (Fc γ Rs) (Takai, 2005; Nimmerjahn and Ravetch, 2006).

The family of Fc receptors for IgG (Fc γ R) are involved in the activation of innate effector cells, antigen presentation, immune-complex-mediated maturation of dendritic cells, and regulation of B cell activation (Anthony et al., 2008; Guillemins et al., 2014). Fc γ RIa are receptors which activate inflammation, while Fc γ RIIb are inhibitory (Lux et al., 2010). Altered Fc γ R expression has been reported in both patients and experimental models (Suzuki et al., 1998; Calverley et al., 2006; Hernandez-Vargas et al., 2006; Inoue et al., 2007). Furthermore, clinical studies have shown an association between different Fc γ R genotypes, diabetes, and cardiovascular risk (Alizadeh et al., 2007; Raaz et al., 2009).

Calcium signals are crucial for diverse cellular functions including adhesion, differentiation, proliferation, effector functions, and gene expression. Following the engagement of the B cell receptor, the intracellular calcium ion (Ca²⁺) concentration is increased promoting the activation of various signaling cascades. While elevated Ca²⁺ in the cytosol initially comes from the endoplasmic reticulum (ER), a

continuous influx of extracellular Ca²⁺ is required to maintain the increased level of cytosolic Ca²⁺. Store-operated Ca²⁺ entry manages this process, which is regulated by an ER calcium sensor, stromal interaction molecule (STIM). Previous studies have revealed the importance of the Ca²⁺ sensor STIM1 and store-operated Ca²⁺-entry (SOCE) for Fc γ -receptor activation (Braun et al., 2009). STIM2 also contributes in part to Fc γ R activation (Sogkas et al., 2015). Blockade of STIM-related functions limits mortality in experimental models of LPS-sepsis in WT mice. Nevertheless, there is no study on the role of STIM, or its two subtypes, STIM1 and STIM2, in the EMT of podocytes induced by DKD.

In the present research, we demonstrate that DKD-induced podocyte EMT is accompanied by STIM and Fc γ RIIa upregulation and Fc γ RIIb downregulation, both in DKD patients and cultured podocytes. We hypothesize that STIM and/or Fc γ RII regulates DKD-induced podocyte EMT. Our present study suggests for the first time that STIM inhibition decreases DKD-induced EMT. Furthermore, we report that DKD-induced STIM overexpression was inhibited by RNAi. Interestingly, we demonstrate that DKD-induced podocyte EMT is mediated by STIM through Fc γ RII activity.

Materials and methods

Patient selection and renal biopsies

All DKD patients were diagnosed based on renal biopsies carried out at the Department of Nephrology, Zhejiang Provincial People's Hospital, Zhejiang, China. The patients were selected using the Mayo Clinic/Renal Pathology Society Consensus Report on Pathologic Classification, Diagnosis, and Reporting of GN (Sethi et al., 2016). A total of 30 DKD patients and 30 control patients were enrolled in the study. All protocols concerning the use of patient samples in this study were approved by the Human Subjects Committee of Zhejiang Provincial People's Hospital. Informed written consent was obtained from all donors.

Reagents and antibodies

STIM1, STIM2, Fc γ RIIa, and Fc γ RIIb kits were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following primary antibodies were used: anti-STIM1, anti-STIM2, anti-Fc γ RIIa, anti-Fc γ RIIb, anti- β -actin (Abcam, England; anti-nephrin, anti-CTGF (connective tissue growth factor), anti- α -SMA (smooth muscle actin) (Proteintech Group, USA) .

Experimental animals

C57BL/KsJ mice (control) and db/db mice (DKD) (aged 8 weeks; body weight 18-20 g) were purchased from the Experimental Animal Center of Zhejiang University. Prior to the study, the animals were

STIM and FcγRII activity in diabetic nephropathy

maintained under standard conditions with a 12/12 h day/night cycle and access to food and water ad libitum. All the mice were sacrificed after 5 weeks. The experiments were carried out in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All protocols using animals were approved by the Institutional Animal Care and Use Committee of Zhejiang Provincial People's Hospital.

Cell culture, RNA interference, and cell transfection

Mouse MPC5 podocytes were maintained in RPMI 1640 medium (Hyclone CA, USA,) supplemented with 10% fetal calf serum (Hyclone), 100 U/mL penicillin G, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Podocytes were propagated at 33°C in culture medium supplemented with 10 U/mL recombinant mouse interferon-γ (IFN-γ; PeproTech EC, London, UK) to enhance the expression of a thermosensitive T-antigen (permissive conditions). Subsequently, the cells were incubated for 7 days at 37°C in the absence of IFN-γ (non-permissive conditions) to induce differentiation. In all experiments, podocytes were used following 7-14 days in culture. To silence STIM1 and STIM2 expression by RNA interference, podocytes were transfected with siRNA using Lipofectamine 2000 in accordance with the manufacturer's instructions. A non-specific scramble siRNA sequence (siRNA-Scr) was used as a negative control.

Periodic acid Schiff-methenamine (MPAS) staining

Kidney samples were fixed with 10% buffered formalin overnight and embedded in paraffin. For histological assessments, sections (3 μm) were deparaffinized, hydrated, and stained with Periodic acid Schiff-methenamine (MPAS).

Immunocytochemical staining

Cells were fixed in 4% paraformaldehyde on ice for 30 min, washed, and permeabilized using PBS containing 0.02% Triton X-100. Cells were then blocked with 5% BSA in PBS. Primary antibodies (1:200 dilution) were added to the cells and incubated for 2 h at room temperature. After extensive washing, cells were incubated with FITC-conjugated secondary antibodies (10 μg/mL) for 1 h at room temperature and washed. Images were acquired by an immunofluorescent microscope (Olympus FV1000, Tokyo, Japan). The number of positive puncta in the cells was analyzed using the software equipped for immunofluorescence microscopy (FV1000, Olympus, Tokyo, Japan).

Western blotting

STIM1, STIM2, FcγRIIa, FcγRIIb, Nephlin, CTGF, and α-SMA protein levels were measured by western

blotting analysis with the corresponding antibodies.

The cells were lysed in RIPA buffer (50 mM Tris-Cl [pH 7.6], 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, and 0.5% Triton X-100) containing 1 mg/mL protease inhibitors (leupeptin, aprotinin, and antipain), 1 mM sodium orthovanadate, and 0.5 mM phenylmethylsulfonyl fluoride. The protein concentration was quantified using the Bradford assay. Proteins (total 50 μg) were separated by a 6-15% SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane. Following blocking with 5% skimmed milk, the membranes were probed with primary antibodies overnight at 4°C, and then incubated with a horseradish peroxidase-conjugated secondary antibody. Band intensities were quantified using WCIF Image J software, and the results were expressed relative to control (Cao and Klionsky, 2007; Zhu et al., 2009).

Cell viability assay

The viability of cells was measured using CCK-8 assays. Briefly, cells were seeded in 96-well plates (1×10⁴/well) and incubated overnight. The cells were then treated with growth medium and conditioned media for specified time-periods. Following treatment, the medium was replaced with 10% CCK-8 reagent dissolved in PBS and cells were incubated for a further 3 h at 37°C in the dark. Absorbance at 450 nm was measured with a Multiskan FC Microplate Reader (Thermo Scientific, USA) and the results were normalized to those of untreated cells.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 software (Stanford University, Stanford, CA, USA). Results are expressed as mean ± standard deviation. Group means were compared using Student's t-test for independent data. All P-values are two-tailed and P<0.05 is considered to indicate statistical significance.

Results

Demographic and clinical characteristics of patients with DKD

The mean age of the 30 DKD patients (13 males and 17 females) was 61.2±8.5 years. The demographic and clinical data for these patients are listed in Table 1. Of the 30 patients, the median BMI was 21.9 kg/m², the median serum TG and TC was 1.52 mmol/L (interquartile range 1.10-2.35 mmol/L) and 4.79±0.69 mmol/L, respectively, the median serum P and Ca²⁺ was 1.39 mmol/L (interquartile range 1.04-1.59 mmol/L) and 2.22 mmol/L (interquartile range 2.01-2.43 mmol/L), respectively, the median serum CRP and Hb was 2.87 mg/L (interquartile range 1.24-5.97 mg/L) and 101.64±13.9 g/L, respectively, and the mean serum albumin was 32.92±2.9 g/L. The mean serum creatinine

STIM and Fc γ RII activity in diabetic nephropathy

and eGFR was 184.89 ± 26.1 $\mu\text{mol/L}$ and 68.7 ± 7.6 ml/min/1.73 m^2 , respectively, and the mean albumin/creatinine rate was 2398.52 ± 196.3 mg/g . All patients included were biopsy-approved (Table 1).

STIM is upregulated in DKD patients and mice

As shown in Fig. 1A, we measured the level of urine protein in patients and demonstrate that DKD significantly increased the level of urine protein ($P < 0.01$). Further, histological changes were observed using MPAS staining. As shown in Fig. 1B, the renal hypertrophy index of the DKD group was significantly higher than the control group. Furthermore, mesangial cells and matrix proliferation were observed in the DKD group compared to the control group, which indicates that DKD damaged the kidney tissues (Fig. 1B).

To investigate whether STIM is involved in the pathogenesis of DKD, we first examined STIM1 and STIM2 levels in the serum of DKD patients and controls. We found that the expression of STIM1 and STIM2 were significantly increased in the DKD group compared to that in the control group ($P < 0.01$; Fig. 1C,D), which suggests that STIM1 and STIM2 levels are associated with the pathogenesis of DKD.

We next assessed the injury role of STIM using the db/db diabetic mouse model. Similar increases in urine

protein (Fig. 2A) and histological changes were observed (Fig. 2B). Furthermore, STIM1 and STIM2 significantly increased in DKD groups compared to the control group ($P < 0.01$; Fig. 2C,D).

Table 1. Demographic and clinical parameters of patients with DKD.

Parameter	N=30
Age (years)	61.2 \pm 8.5
Gender (male/female)	13/17
BMI (kg/m ²)	21.9 (20.2, 24.0)
TG (mmol/L)	1.52 (1.10, 2.35)
TC ($\mu\text{mol/L}$)	4.79 \pm 0.69
P (mmol/L)	1.39 (1.04, 1.59)
Ca ²⁺ (mmol/L)	2.22 (2.01, 2.43)
CRP (mg/L)	2.87 (1.24, 5.97)
Hb (g/L)	101.64 \pm 13.9
Albumin (g/L)	32.92 \pm 2.9
Serum creatinine ($\mu\text{mol/L}$)	184.89 \pm 26.1
eGFR (ml/min/1.73m ²)	68.7 \pm 7.6
ACR (mg/g)	2398.52 \pm 196.3

BMI, body mass index; TG, triglycerides; TC, total cholesterol; P, serum phosphorus; Ca²⁺, serum calcium; CRP, serum C-reactive protein; Hb, serum hemoglobin; eGFR, estimated Glomerular Filtration Rate; ACR, albumin/creatinine rate; Data were expressed as mean \pm SD, median (interquartile range).

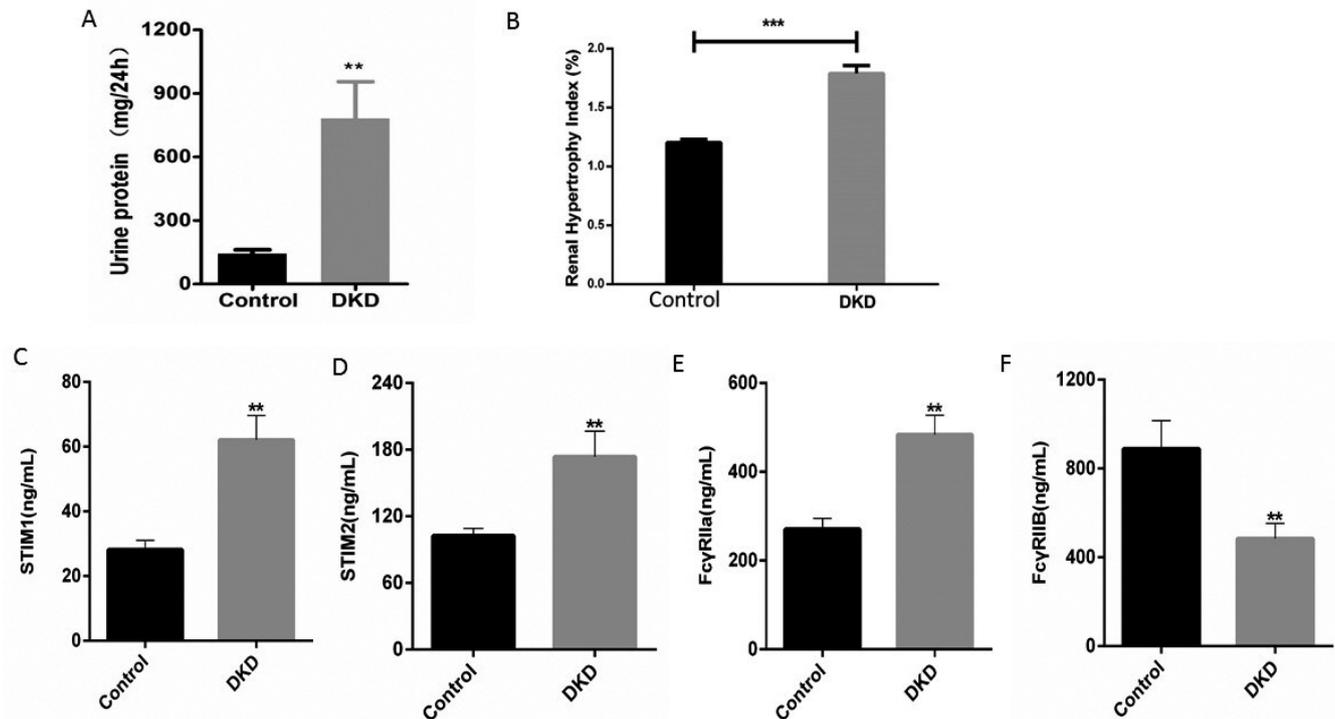


Fig. 1. STIM is upregulated and activates Fc γ RIIa levels but inhibits Fc γ RIIb levels in DKD patients. **A.** The level of urine protein in DKD patients and control group. **B.** The renal hypertrophy index of the DKD mice and control. **C-F.** The expression of STIM1, STIM2, Fc γ RIIa, Fc γ RIIb in DKD patients and control by ELISA. STIM, stromal interaction molecule; Fc γ R, Fc receptors for IgG; (***: $P < 0.001$ vs. Ctrl., **: $P < 0.01$ vs. Ctrl.).

STIM and FcγRII activity in diabetic nephropathy

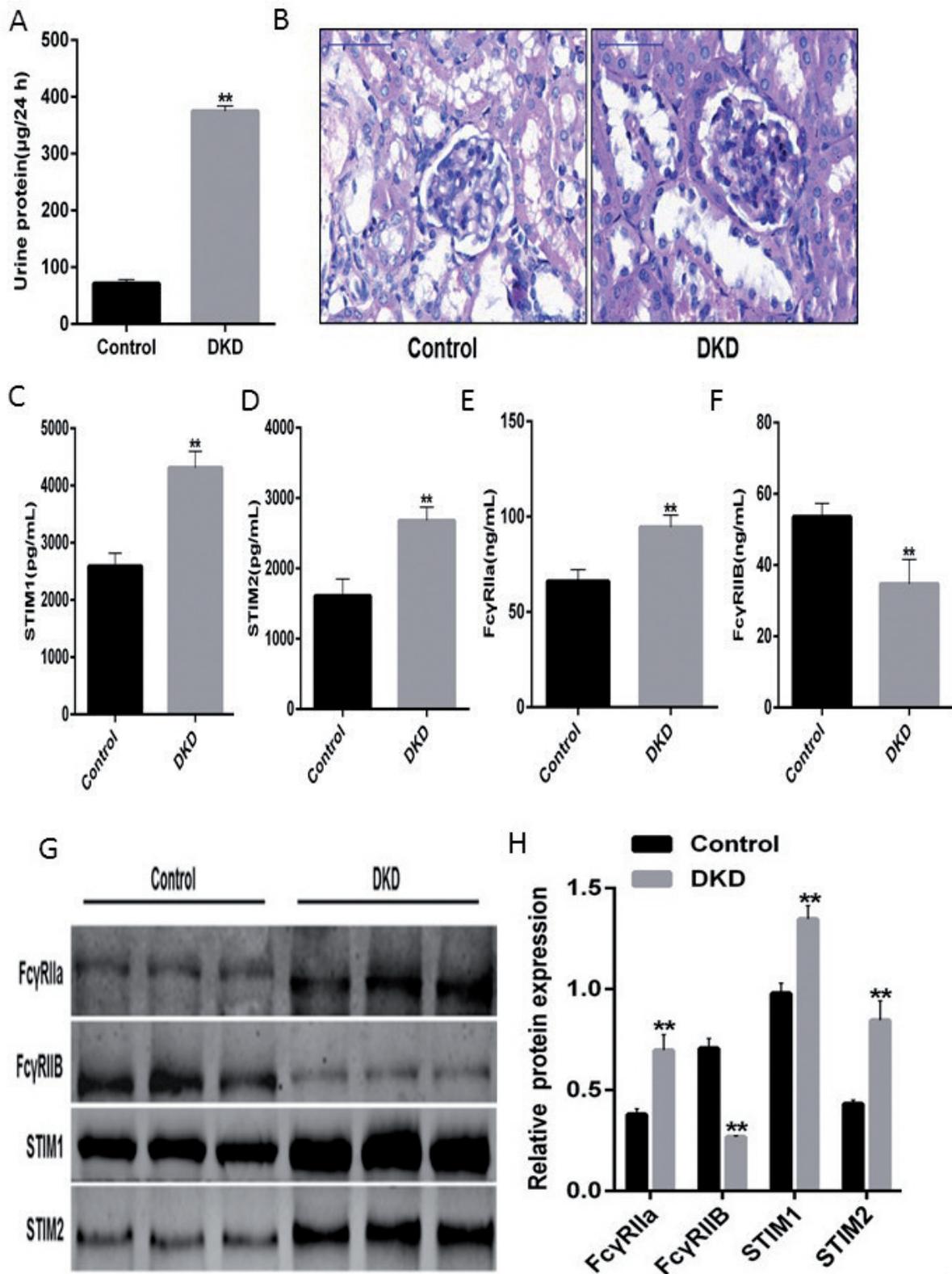


Fig. 2. STIM is upregulated and activates FcγRIIa levels but inhibits FcγRIIb levels in mouse models **A**. The level of urine protein in DKD mice and control group. **B**. The result of MPAS staining in the DKD mice and control. The magnification of the microscopic pictures was indicated in the top left corner, and the scale bars of the microscopic pictures is 50µm. **C-F**. The expression of STIM1, STIM2, FcγRIIa, FcγRIIb in the DKD mice and control by ELISA. **G, H**. The levels of STIM1, STIM2, FcγRIIa, FcγRIIb in DKD mice and control renal tissues by using western blot, band intensities were quantified using WCIF Image J software, and the results were expressed relative to control. STIM, stromal interaction molecule; FcγR, Fc receptors for IgG; (**: P<0.01 vs. Ctrl., ***: P<0.001 vs. Ctrl.).

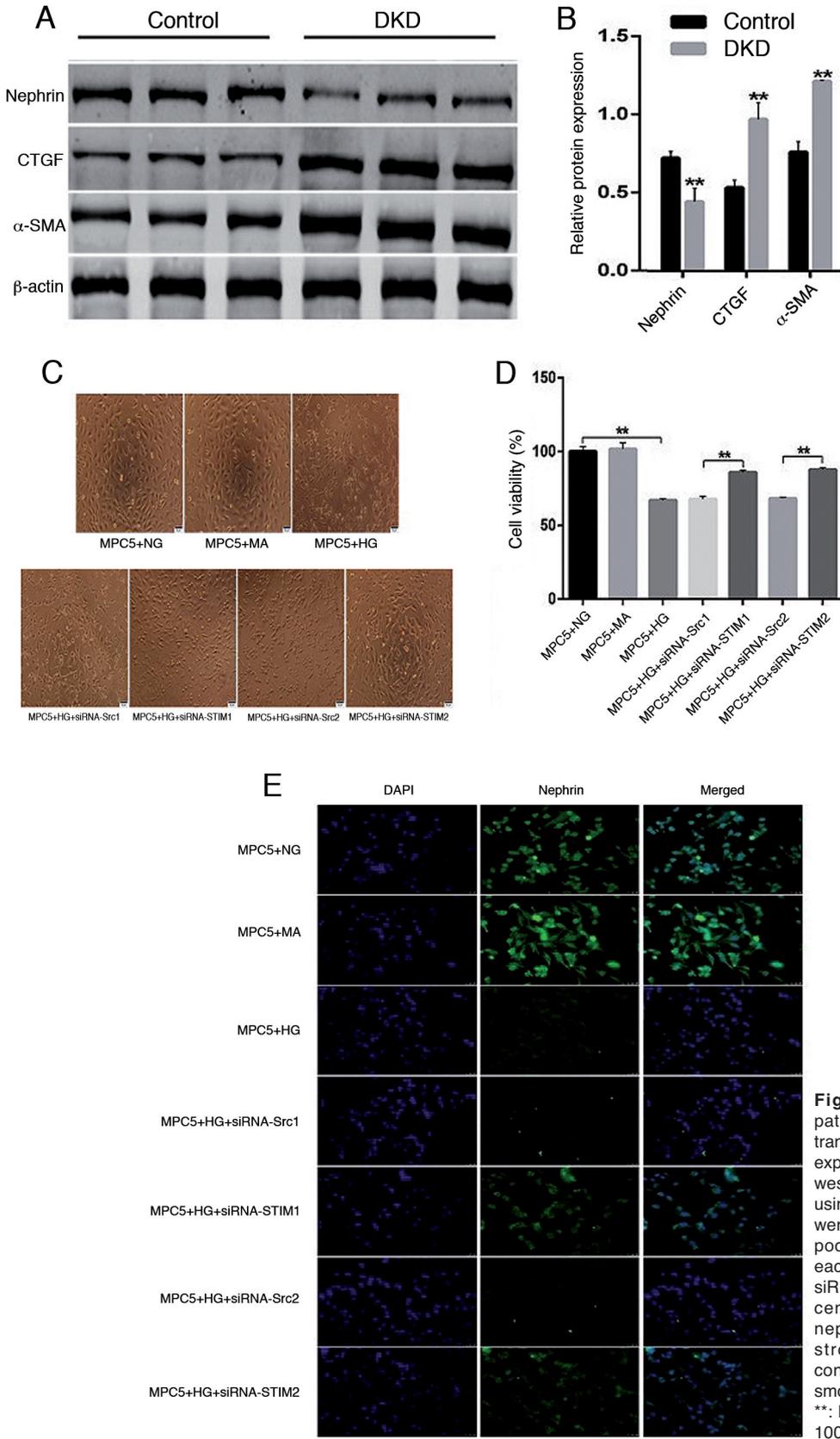
STIM and Fc γ RII activity in diabetic nephropathy

Fig. 3. STIM as a key factor in the pathogenesis of epithelial-to-mesenchymal transition in podocytes with DKD. **A, B.** The expression of Nephrin, CTGF and α -SMA by western-blot, band intensities were quantified using WCIF Image J software, and the results were expressed relative to control. **C, D.** The podocyte changes and viability of cells in each group by blocking STIM activity using siRNA-mediated silencing. **E.** Immunofluorescence staining was performed to detect nephrin proteins in different sera. STIM, stromal interaction molecule; CTGF, connective tissue growth factor; α -SMA, a-smooth muscle actin. (***: $P < 0.001$ vs. Ctrl., **: $P < 0.01$ vs. Ctrl.). Scale bars: C, 50 μ m; E, 100 μ m.

STIM and Fc γ RII activity in diabetic nephropathy

To validate the serum results, we measured levels of STIM1 and STIM2 in db/db diabetic mice and control kidney tissues by western blot. DKD mice displayed higher levels of STIM1 and STIM2 in renal tissues than controls ($P < 0.01$; Fig. 2G,H).

STIM activates Fc γ RIIa levels but inhibits Fc γ RIIb levels

We next examined whether the alteration of STIM is linked to the concomitant regulation of Fc γ RII. First, we measured the serum levels of Fc γ RIIa and Fc γ RIIb by ELISA. As expected, the expression of Fc γ RIIa is higher in the serum from DKD patients compared to controls (Fig. 1E), suggesting that STIM is upregulated during DKD injury. In contrast, the expression of Fc γ RIIb is

lower in serum from DKD patients compared to controls (Fig. 1F), suggesting that STIM is downregulated during DKD injury. Second, we measured the serum levels of Fc γ RIIa and Fc γ RIIb in DKD mice by ELISA. As expected, the results are consistent with those of DKD patients (Fig. 2E,F).

To validate the serum results we also measured levels of Fc γ RIIa and Fc γ RIIb in db/db diabetic mice and control kidney tissues via western blot. DKD mice displayed that the levels of Fc γ RIIa were higher and the levels of Fc γ RIIb were lower in renal tissues than controls ($P < 0.01$; Fig. 2G,H), which suggests that STIM regulates Fc γ RII expression at the protein level.

These results demonstrate that STIM could regulate the protein expression of Fc γ RII, suggesting that Fc γ RII

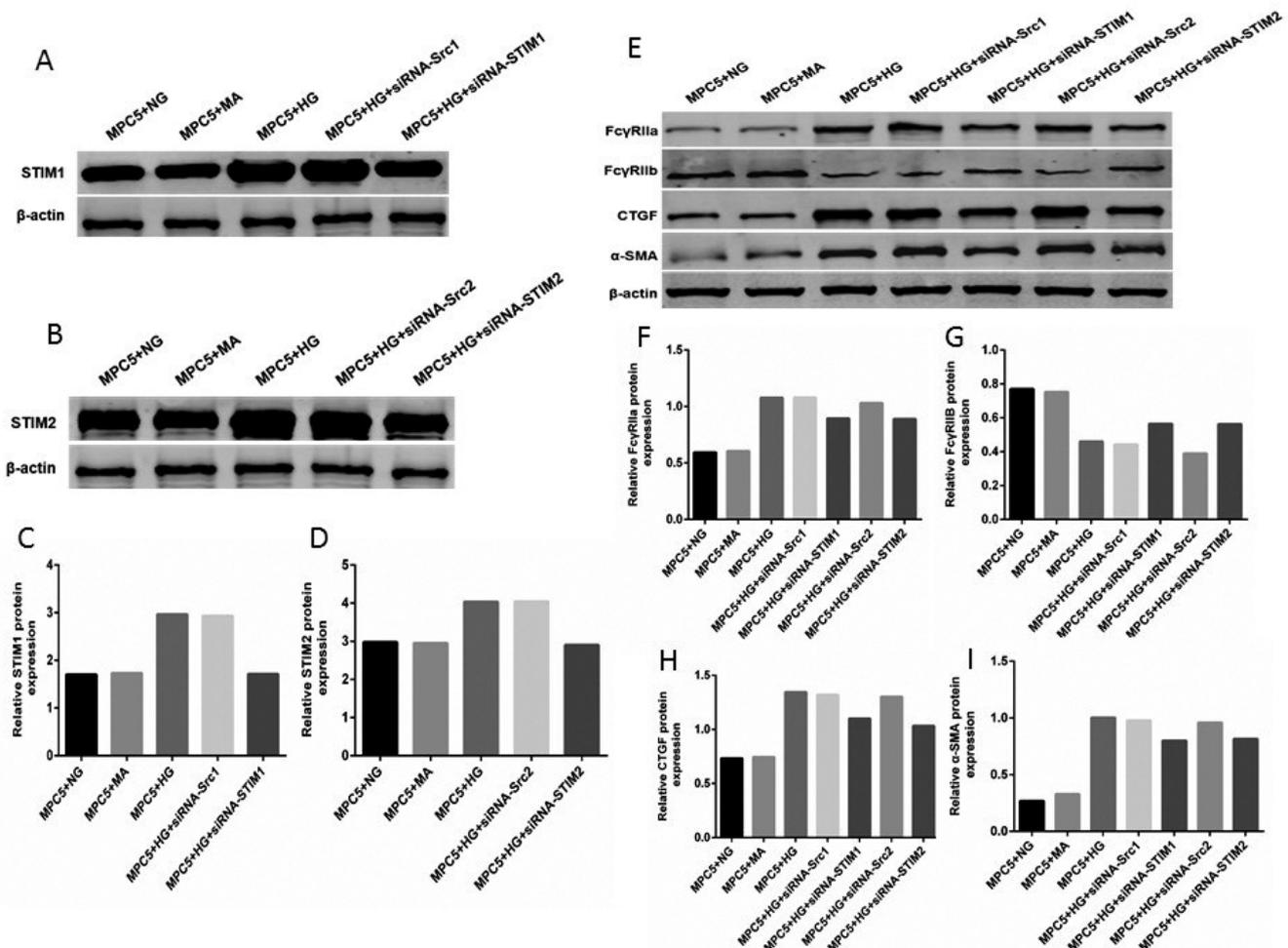


Fig. 4. Silencing STIM1 and STIM2 with siRNA could significantly inhibit podocyte epithelial-mesenchymal transition. **A, B.** The expression of STIM1 and STIM2 was standardised using β -actin, and STIM1 and STIM2 were treated with NG, MA, HG, and siRNA in MPC5 cells. **C, D.** Western blotting was used to detect the relative expression levels of STIM1 and STIM2 after the MPC5 cells were treated with NG, MA, HG, and siRNA. **E.** The expression of Fc γ RIIa, Fc γ RIIb, CTGF and α -SMA was standardised using β -actin, and Fc γ RIIa, Fc γ RIIb, CTGF and α -SMA were treated with NG, MA, HG, and siRNA in MPC5 cells. **F-I.** Western blotting was used to detect the relative expression levels of Fc γ RIIa, Fc γ RIIb, CTGF and α -SMA after the MPC5 cells were treated with NG, MA, HG, and siRNA. STIM, stromal interaction molecule; Fc γ R, Fc receptors for IgG; NG, non-glucose; HG, high glucose; (Band intensities were quantified using WCIF Image J software, and the results were expressed relative to control. ***: $P < 0.001$ vs. Ctrl., **: $P < 0.01$ vs. Ctrl).

is a target of STIM.

STIM promotes epithelial-to-mesenchymal transition in podocytes with DKD

First, we use the markers of EMT such as SNAIL and FSP1 to prove the epithelial-to-mesenchymal transition in podocytes from mouse model and cell. We hypothesized that STIM serves as a key factor in the pathogenesis of EMT and podocyte injury. To test this hypothesis, proteins related to podocyte injury were measured by western-blot. Nephrin, a podocyte marker, was significantly reduced (Fig. 3A,B). However, the expression of mesenchymal markers, CTGF and α -SMA, were increased (Fig. 3A,B).

To determine the effect of STIM levels on DKD podocyte EMT, we first assessed patterns of podocyte in DKD and control group by blocking STIM activity using siRNA-mediated silencing. The podocyte changes, including strange patterns and decreased numbers in DKD, were reversed (Fig. 3C). In addition, CCK-8 assays indicate that STIM blockade alleviates DKD podocyte damage (Fig. 3D).

To further characterize the role of STIM activity in podocyte damage in DKD-induced injury, the expression of podocyte markers, such as nephrin, was measured through immunofluorescent staining. Compared to the control group, reduced nephrin levels and increased podocyte EMT was observed in the DKD group (Fig. 3E). We silenced STIM1 or STIM2 expression in podocytes by transfecting cells with STIM1 or STIM2 inhibitors (Fig. 3E). STIM silenced podocytes displayed higher expression of nephrin (Fig. 3E) compared to DKD groups, which indicates that STIM promotes injury in podocytes. Taken together, these data indicate that STIM promotes EMT in podocytes.

Inhibition of STIM alleviates epithelial-to-mesenchymal transition in podocytes and reverses Fc γ RII levels

To determine the effect of STIM on podocytes, we blocked STIM activity and assessed HG-induced podocyte EMT and Fc γ RII levels. In this experiment, podocyte STIM activity was blocked by directly silencing STIM via STIM siRNA. Transfection with STIM1 or STIM2 siRNA led to a dramatic decrease in STIM1 or STIM2 protein levels in podocytes (Fig. 4A-D). As shown in Fig. 4E-G, knocking down STIM reversed Fc γ RII activity in HG-treated podocytes, as indicated by the decreased Fc γ RIIa and increased Fc γ RIIb activity. STIM blockade alleviates HG-induced podocyte EMT, as indicated by the decreased expression of the EMT marker proteins CTGF and α -SMA (Fig. 4E,H-I).

Discussion

Our research suggests that DKD leads to kidney damage and dysfunction with podocyte EMT in both

human patients and mice. DKD also caused proteinuria and pathological changes. We also demonstrate that DKD-induced podocyte EMT is related to STIM overexpression both in vivo and in vitro. Further research shows that STIM siRNA reversed podocytes from DKD-induced injury and EMT. Furthermore, Fc γ RII activity was found to be necessary for STIM overexpression in cultured HG-induced podocytes. Therefore, we report, for the first time, a novel molecular mechanism of DKD-induced podocyte EMT and injury, which relies on Fc γ RII activity imbalance regulated by STIM.

Kidneys are the most commonly involved organs in DKD. In the United States, >40% of the 29 million plus individuals with type 2 diabetes suffer from DKD (Bailey et al., 2014). DKD is characterized by the accumulation of extracellular matrix proteins, including predominantly various collagens, laminin, and fibronectin in the mesangium and renal tubulointerstitium, as well as by the thickening of the basement membranes (Zeisberg and Neilson, 2010; Loeffler et al., 2012; Hu et al., 2015). This increased deposition of ECM subsequently leads to renal fibrosis, which can culminate in tubulointerstitial fibrosis and glomerulosclerosis (Zeisberg and Neilson, 2010; Hills and Squires, 2011; Hu et al., 2015). It has been demonstrated that the number of myofibroblasts is inversely correlated with renal function in human DN (Pedagogos et al., 1997; Li and Bertram, 2010). It is widely accepted that these activated myofibroblasts are the principal effector cells responsible for the excess interstitial ECM deposition under pathological conditions, yet their origin is still under debate (Liu, 2004; Zeisberg and Neilson, 2010).

Many studies have confirmed that the major mechanism of Ca²⁺ entry into the cytosol of immune cells is through SOCE. STIM1 and STIM2 are ubiquitously expressed in human and animal tissues as transmembrane Ca²⁺ sensors located in the ER membrane. Following a decrease in intracellular calcium, they open plasma membrane Ca²⁺ release-activated Ca²⁺ (CRAC) channels mediated by CRAC modulators 1 through conformational change. STIM1 and STIM2 also cooperate with Orai channel proteins, ultimately permitting calcium influx from the extracellular fluid (Zhang et al., 2005; Vig et al., 2006). Ca²⁺-dependent nuclear transcription factor- κ B (NF- κ B) is then activated through multiple pathways, although the exact mechanism is complicated and remains unclear. Some studies have shown that NF- κ B signaling upstream of I κ B kinase activation in T cells is regulated by STIM1-dependent CRAC/Orai channels and Ca²⁺-dependent PKC α -mediated phosphorylation of protein p65 (Liu et al., 2016). Palkowitsch et al. (2011) demonstrated that calcineurin facilitates T cell receptor (TCR) induced NF- κ B activation by regulating the formation of a protein complex composed of Carma1-Bcl10-Malt1. Intracellular Ca²⁺ fluxes induced by Toll-like receptor (TLR) 4, 9, and 3 ligands, and activation of

STIM and FcγRII activity in diabetic nephropathy

Ca²⁺/calmodulin-dependent protein kinase II (CAMKII) in macrophages, has been linked to NF-κB activation through phosphorylation of transforming growth factor β-activated kinase 1 (TAK1) and interferon regulatory factor 3 (IRF3) (Liu et al., 2008). Furthermore, sufficient evidence supports that FcγR activation is primarily mediated by STIM1, while STIM2 appears to play a lesser role in macrophages (Sogkas et al., 2015). In DN, at the cellular level, hyperglycemia leads to activity of NF-κB and transforming growth factor β 1 (TGFβ1) with persistent advanced glycation end products. These then lead to renal podocyte impairment and inflammation. However, despite the critical role of calcium signaling in several functional processes, little is known about the role of STIM1 or STIM2 in podocytes, particularly EMT in podocyte injury. Here, in the first part of our study, we characterized the role of STIM1 and STIM2 in the pathogenesis of DKD by using the serum of DKD patients and db/db diabetic mice. The result shows that STIM1 and STIM2 levels are significantly increased among patients and mice with DKD compared to controls, accompanied by increased renal hypertrophy index, mesangial cells, and matrix proliferation.

FcγR play an important role in regulating a balanced immune response in cellular responses by coinstantaneous triggering of activating and inhibitory intracellular signaling pathways. FcγRIIa is an excitatory receptor and contains an immunoreceptor tyrosine-based activation motif (ITAM) in its intracellular region. When the receptor combines with IgG, it activates the intracellular ITAM pathway and induces the generation of tumor necrosis factor-α (TNF-α) and interferon gamma (IFN-γ). These cytokines can directly induce an inflammatory reaction and kill pathogens (Hardison et al., 2010). FcγRIIb is the only inhibitory receptor and is the most broadly expressed FcγR (Nimmerjahn and Ravetch, 2008). It can not only induce the generation of IL-10, but also co-exists with the excitatory FcγR in the surface of immune cells. The triggering of immunoreceptor tyrosine-based inhibition motif (ITIM)-containing proteins with the B cell receptor (BCR) results in the recruitment of SHIP (SH2-domain-containing inositol polyphosphate 5' phosphatase), thus interfering and inhibiting TNF-α by restraining the activation of NF-κB (Monari et al., 2006). Studies indicate that STIM1 is an essential component of FcγR activation and plays a dominant role in IgG-triggered autoimmune inflammation by activating FcγR-induced Ca²⁺ entry and phagocytosis (Braun et al., 2009). It has also been shown that SOCE is the primary mechanism of Ca²⁺ entry downstream of FcγR activation and that STIM1 is a critical mediator by interacting with store-operated Ca²⁺ (SOC) channels in this process. We also demonstrate that changes in expression of STIM1 and STIM2 are linked to the consequent increase of FcγRIIa and decrease of FcγRIIb in DKD patients and mice, suggesting that STIM is a critical regulator of FcγRII expression.

The podocyte is a terminally differentiated kidney cell located in the outer aspect of GBM. It constitutes a fundamental component of the filtration barrier and plays an important role in the prevention of protein loss through its specific charge and molecular characteristics (Mundel and Shankland, 2002). Hyperglycemia has been found to cause EMT of podocytes. This results in morphological changes by which epithelial cells lose their hallmark epithelial characteristics and gain the features of mesenchymal cells. Fundamental studies have demonstrated that high glucose induces podocyte EMT through the TGFβ/Smad pathway and several other molecular mechanisms such as the Wnt/β-catenin signaling pathway, integrins/integrin-linked kinase (ILK) signaling pathway, MAPKs signaling pathway, RTK/Ras/Erk signaling pathway, Jagged/Notch signaling pathway, PI3K/AKT/mTOR signaling pathway, and the NF-κB signaling pathway (McKay and Morrison, 2007; Neth et al., 2007; Willis and Borok, 2007; Zhang et al., 2009; Lamouille and Derynck, 2011). As a transcription factor, NF-κB plays a key role in podocyte dedifferentiation, initiation, and progression of podocyte EMT. The functional inactivation of E-cadherin, the cell-cell adhesion molecule, may be the first step of EMT. NF-κB induces and maintains EMT mainly through upregulation of transcription factors and stabilization of Snail during the loss of the epithelial phenotype (Chua et al., 2007; Julien et al., 2007; Wu et al., 2009). The NF-κB pathway is predominantly activated by pro-inflammatory cytokines. Following cellular stimulation, the activation of IκB kinase (IKK) triggers the initiation of phosphorylation and degradation of IκB proteins by proteasome (Hayden and Ghosh, 2008). Subsequently, NF-κB then translocates to the nucleus in order to regulate gene expression by recruiting transcriptional coactivators (Hoberg et al., 2006). Ultimately, NF-κB promotes the acquisition of a mesenchymal phenotype through the upregulation of several metastatic genes such as the transcription inducer Snail, cyclooxygenase-2 (COX-2), and vascular endothelial growth factor (VEGF) (Barbera et al., 2004).

Current research demonstrates that the activation of NF-κB can significantly promote EMT in podocytes, inhibit the expression of nephrin and podocin, and result in albuminuria and fibrosis in diabetic rats (Qi et al., 2016). Ghiggeri et al. indicate that complex alterations of EMT signaling are reproducible by Ca²⁺ deprivation-induced cell junction impairment (Ghiggeri et al., 2013). They also demonstrate that the activation of β-catenin pathway plays a critical role in podocyte ontogenesis, nephrin expression, and EMT regulation. The activation of β-catenin pathway is also crucial in the activity modifications of NF-κB, p53, and retinoblastoma protein (RB). Here, our study mainly certifies STIM as an important mediator in the process of EMT in podocytes injury. We demonstrate that overexpression of STIM stimulates podocyte EMT, significantly downregulates the expression of nephrin, and upregulates the expression of CTGF and α-SMA, subsequently leading

to albuminuria, mesangial cells, and matrix proliferation. Furthermore, we show that inhibition of STIM by siRNA-mediated silencing of STIM1 or STIM2 alleviates HG-induced EMT and reverses Fc γ RII activity in podocytes.

In conclusion, our study suggests that STIM and Fc γ RII play an essential role in the regulation of DKD-induced podocyte EMT. STIM signaling is a crucial trigger of EMT in podocytes and Fc γ RII is an essential mediator in this process. Therefore, STIM is an essential component of Fc γ R activation and inhibition of STIM-mediated signaling could become a new strategy to treat IgG-dependent renal diseases. However, the molecular mechanisms of EMT are complex and involve multiple signaling pathways. Further research is necessary to elucidate how STIM-dependent pathways lead to the regulation of Fc γ R activation.

Acknowledgments. We would like to thank the native English speaking scientists of Elixigen Company (Huntington Beach, California) for editing our manuscript.

Funding. This work was supported by grants from the National Natural Science Foundation of China (Grant Number: 81641026), the Major Projects of Science and Technology Department of Zhejiang Province (Grant Number: 2014C03047-2), the Natural Science Foundation of Zhejiang Province (Grant Number: LY16H050005, LZ17H050001), and the Project of Scientific Research Foundation of Chinese Medicine (Grant Number: 2017ZA010), the General Project of the Medical and Health of Zhejiang Province (Grant Number: 2016KYB007).

Availability of data and material. All the data supporting our findings is contained within the manuscript.

Authors' contributions. Qiang He and Juan Jin: designed the study. Jianguang Gong and Kang Hu: collected samples and clinical information. Meiyu Ye and Kang Hu: performed the laboratory assays. Jianguang Gong: performed the statistical analyses, Juan Jin and Meiyu Ye wrote the manuscript. The final version of the manuscript was approved by all authors.

Disclosure Statement. The authors declare no conflicts of interest.

Ethics approval and consent to participate. This study was approved through the local ethics committee of Zhejiang Provincial People's Hospital. The study has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All the enrolled patients have signed the consents of renal biopsy and researches before renal biopsy was performed.

Disclosure summary. The authors have no competing interests to declare. All authors have approved the final version of the manuscript and agreed to submit it for publication.

References

- Alizadeh B.Z., Valdigem G., Coenen M.J., Zhernakova A., Franke B., Monsuur A., van Riel P.L., Barrera P., Radstake T.R., Roep B.O., Wijmenga C. and Koeleman B.P. (2007). Association analysis of functional variants of the Fc γ RIIa and Fc γ RIIIa genes with type 1 diabetes, celiac disease and rheumatoid arthritis. *Hum. Mol. Genet.* 16, 2552-2559.
- Anthony R.M., Nimmerjahn F., Ashline D.J., Reinhold V.N., Paulson J.C. and Ravetch J.V. (2008). Recapitulation of IVIG anti-inflammatory activity with a recombinant IgG Fc. *Science* 320, 373-376.
- Bailey R.A., Wang Y., Zhu V. and Rupnow M.F. (2014). Chronic kidney disease in US adults with type 2 diabetes: an updated national estimate of prevalence based on Kidney Disease: Improving Global Outcomes (KDIGO) staging. *BMC Res. Notes* 7, 415.
- Barbera M.J., Puig I., Dominguez D., Julien-Grille S., Guaita-Esteruelas S., Peiro S., Baulida J., Franci C., Dedhar S., Larue L. and Garcia de Herreros A. (2004). Regulation of Snail transcription during epithelial to mesenchymal transition of tumor cells. *Oncogene* 23, 7345-7354.
- Braun A., Gessner J.E., Varga-Szabo D., Syed S.N., Konrad S., Stegner D., Vogtle T., Schmidt R.E. and Nieswandt B. (2009). STIM1 is essential for Fc γ receptor activation and autoimmune inflammation. *Blood* 113, 1097-1104.
- Calverley D.C., Baldermann L.V., Heldt M.L., Kinney G.L. and Hokanson J.E. (2006). Increased platelet Fc receptor expression in diabetes is limited to those with type 2 disease and low LDL cholesterol levels. *Atherosclerosis* 185, 173-176.
- Cao Y. and Klionsky D.J. (2007). Physiological functions of Atg6/Beclin 1: a unique autophagy-related protein. *Cell Res.* 17, 839-849.
- Chua H.L., Bhat-Nakshatri P., Clare S.E., Morimiya A., Badve S. and Nakshatri H. (2007). NF-kappaB represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. *Oncogene* 26, 711-724.
- Ghiggeri G.M., Gigante M. and Di Donato A. (2013). Constitutional nephrin deficiency in conditionally immortalized human podocytes induced epithelial-mesenchymal transition, supported by beta-catenin/NF-kappa B activation: A consequence of cell junction impairment? *Int. J. Nephrol.* 2013, 457490.
- Gross J.L., de Azevedo M.J., Silveiro S.P., Canani L.H., Caramori M.L. and Zelmanovitz T. (2005). Diabetic nephropathy: diagnosis, prevention, and treatment. *Diabetes Care* 28, 164-176.
- Guilliams M., Bruhns P., Saeys Y., Hammad H. and Lambrecht B.N. (2014). The function of Fc γ receptors in dendritic cells and macrophages. *Nat. Rev. Immunol.* 14, 94-108.
- Hardison S.E., Ravi S., Wozniak K.L., Young M.L., Olszewski M.A. and Wormley F.L. (2010). Pulmonary infection with an interferon-gamma-producing *Cryptococcus neoformans* strain results in classical macrophage activation and protection. *Am. J. Pathol.* 176, 774-785.
- Hayden M.S. and Ghosh S. (2008). Shared principles in NF-kappaB signaling. *Cell.* 132, 344-362.
- Herman-Edelstein M., Thomas M.C., Thallas-Bonke V., Saleem M., Cooper M.E. and Kantharidis P. (2011). Dedifferentiation of immortalized human podocytes in response to transforming growth factor-beta: a model for diabetic podocytopathy. *Diabetes* 60, 1779-1788.
- Hernandez-Vargas P., Ortiz-Munoz G., Lopez-Franco O., Suzuki Y., Gallego-Delgado J., Sanjuan G., Lazaro A., Lopez-Parra V., Ortega L., Egido J. and Gomez-Guerrero C. (2006). Fc γ receptor deficiency confers protection against atherosclerosis in apolipoprotein E knockout mice. *Circ. Res.* 99, 1188-1196.
- Hills C.E. and Squires P.E. (2011). The role of TGF-beta and epithelial-to mesenchymal transition in diabetic nephropathy. *Cytokine Growth Factor Rev.* 22, 131-139.
- Hoberg J.E., Popko A.E., Ramsey C.S. and Mayo M.W. (2006). I kappa B kinase alpha-mediated derepression of SMRT potentiates acetylation of RelA/p65 by p300. *Mol. Cell Biol.* 26, 457-471.
- Hu C., Sun L., Xiao L., Han Y., Fu X., Xiong X., Xu X., Liu Y., Yang S.,

STIM and Fc γ RII activity in diabetic nephropathy

- Liu F. and Kanwar Y.S. (2015). Insights into the mechanisms involved in the expression and regulation of extracellular matrix proteins in diabetic nephropathy. *Curr. Med. Chem.* 22, 2858-2870.
- Inoue Y., Kaifu T., Sugahara-Tobinai A., Nakamura A., Miyazaki J. and Takai T. (2007). Activating Fc gamma receptors participate in the development of autoimmune diabetes in NOD mice. *J. Immunol.* 179, 764-774.
- Itoh M., Nakadate K., Horibata Y., Matsusaka T., Xu J., Hunziker W. and Sugimoto H. (2014). The structural and functional organization of the podocyte filtration slits is regulated by Tjp1/ZO-1. *PLoS One* 9, e106621.
- Jerums G., Panagiotopoulos S., Premaratne E. and MacIsaac R.J. (2009). Integrating albuminuria and GFR in the assessment of diabetic nephropathy. *Nat. Rev. Nephrol.* 5, 397-406.
- Julien S., Puig I., Caretti E., Bonaventure J., Nelles L., van Roy F., Dargemont C., de Herreros A.G., Bellacosa A. and Larue L. (2007). Activation of NF-kappaB by Akt upregulates Snail expression and induces epithelium mesenchyme transition. *Oncogene* 26, 7445-7456.
- Lamouille S. and Derynck R. (2011). Emergence of the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin axis in transforming growth factor-beta-induced epithelial-mesenchymal transition. *Cells Tissues Organs* 193, 8-22.
- Li J. and Bertram J.F. (2010). Review: Endothelial-myofibroblast transition, a new player in diabetic renal fibrosis. *Nephrology* 15, 507-512.
- Li X. and He J.C. (2015). An update: the role of Nephhrin inside and outside the kidney. *Sci. China Life Sci.* 58, 649-657.
- Li Y., Kang Y.S., Dai C., Kiss L.P., Wen X. and Liu Y. (2008). Epithelial-to-mesenchymal transition is a potential pathway leading to podocyte dysfunction and proteinuria. *Am. J. Pathol.* 172, 299-308.
- Liu Y. (2004). Epithelial to mesenchymal transition in renal fibrogenesis: pathologic significance, molecular mechanism, and therapeutic intervention. *J. Am. Soc. Nephrol.* 15, 1-12.
- Liu X., Yao M., Li N., Wang C., Zheng Y. and Cao X. (2008). CaMKII promotes TLR-triggered proinflammatory cytokine and type I interferon production by directly binding and activating TAK1 and IRF3 in macrophages. *Blood* 112, 4961-4970.
- Liu X., Berry C.T., Ruthel G., Madara J.J., MacGillivray K., Gray C.M., Madge L.A., McCorkell K.A., Beiting D.P., Hershberg U., May M.J. and Freedman B.D. (2016). T cell receptor-induced nuclear factor kappaB (NF-kappaB) signaling and transcriptional activation are regulated by STIM1- and orai1-mediated calcium entry. *J. Biol. Chem.* 291, 8440-8452.
- Loeffler I., Liebisch M. and Wolf G. (2012). Collagen VIII influences epithelial phenotypic changes in experimental diabetic nephropathy. *Am. J. Physiol. Renal Physiol.* 303, F733-745.
- Loeffler I. and Wolf G. (2015). Epithelial-to-mesenchymal transition in diabetic nephropathy: Fact or fiction? *Cells* 4, 631-652.
- Lux A., Aschermann S., Biburger M. and Nimmerjahn F. (2010). The pro and anti-inflammatory activities of immunoglobulin G. *Ann. Rheum. Dis.* 69 Suppl. 1, i92-96.
- May C.J., Saleem M. and Welsh G.I. (2014). Podocyte dedifferentiation: a specialized process for a specialized cell. *Front. Endocrinol.* 5, 148.
- McKay M.M. and Morrison D.K. (2007). Integrating signals from RTKs to ERK/MAPK. *Oncogene* 26, 3113-3121.
- Monari C., Kozel T.R., Paganelli F., Pericolini E., Perito S., Bistoni F., Casadevall A. and Vecchiarelli A. (2006). Microbial immune suppression mediated by direct engagement of inhibitory Fc receptor. *J. Immunol.* 177, 6842-6851.
- Mundel P. and Shankland S.J. (2002). Podocyte biology and response to injury. *J. Am. Soc. Nephrol.* 13, 3005-3015.
- Neth P., Ries C., Karow M., Egea V., Ilmer M. and Jochum M. (2007). The Wnt signal transduction pathway in stem cells and cancer cells: influence on cellular invasion. *Stem Cell Rev.* 3, 18-29.
- Nilsson J., Bengtsson E., Fredrikson G.N. and Bjorkbacka H. (2008). Inflammation and immunity in diabetic vascular complications. *Curr. Opin. Lipidol.* 19, 519-524.
- Nimmerjahn F. and Ravetch J.V. (2006). Fc gamma receptors: old friends and new family members. *Immunity* 24, 19-28.
- Nimmerjahn F. and Ravetch J.V. (2008). Fc gamma receptors as regulators of immune responses. *Nat. Rev. Immunol.* 8, 34-47.
- Palkowitsch L., Marienfeld U., Brunner C., Eitelhuber A., Krappmann D. and Marienfeld R.B. (2011). The Ca²⁺-dependent phosphatase calcineurin controls the formation of the Carma1-Bcl10-Malt1 complex during T cell receptor-induced NF-kappaB activation. *J. Biol. Chem.* 286, 7522-7534.
- Pedagogos E., Hewitson T., Fraser I., Nicholls K. and Becker G. (1997). Myofibroblasts and arteriolar sclerosis in human diabetic nephropathy. *Am. J. Kidney Dis.* 29, 912-918.
- Qi X.M., Wang J., Xu X.X., Li Y.Y. and Wu Y.G. (2016). FK506 reduces albuminuria through improving podocyte nephrin and podocin expression in diabetic rats. *Inflamm. Res.* 65, 103-114.
- Raaz D., Herrmann M., Ekici A.B., Klinghammer L., Lausen B., Voll R.E., Leusen J.H., van de Winkel J.G., Daniel W.G., Reis A. and Garlachs C.D. (2009). Fc gamma RIIa genotype is associated with acute coronary syndromes as first manifestation of coronary artery disease. *Atherosclerosis* 205, 512-516.
- Sethi S., Haas M., Markowitz G.S., D'Agati V.D., Rennke H.G., Jennette J.C., Bajema I.M., Alpers C.E., Chang A., Cornell L.D., Cosio F.G., Fogo A.B., Glassock R.J., Hariharan S., Kambham N., Lager D.J., Leung N., Mengel M., Nath K.A., Roberts I.S., Rovin B.H., Seshan S.V., Smith R.J., Walker P.D., Winearls C.G., Appel G.B., Alexander M.P., Cattran D.C., Casado C.A., Cook H.T., De Vriese A.S., Radhakrishnan J., Racusen L.C., Ronco P. and Fervenza F.C. (2016). Mayo Clinic/Renal Pathology Society Consensus Report on Pathologic Classification, Diagnosis, and Reporting of GN. *J. Am. Soc. Nephrol.* 27, 1278-1287.
- Sogkas G., Stegner D., Syed S.N., Vogtle T., Rau E., Gewecke B., Schmidt R.E., Nieswandt B. and Gessner J.E. (2015). Cooperative and alternate functions for STIM1 and STIM2 in macrophage activation and in the context of inflammation. *Immun. Inflamm. Dis.* 3, 154-170.
- Suzuki Y., Shirato I., Okumura K., Ravetch J.V., Takai T., Tomino Y. and Ra C. (1998). Distinct contribution of Fc receptors and angiotensin II-dependent pathways in anti-GBM glomerulonephritis. *Kidney Int.* 54, 1166-1174.
- Takai T. (2005). Fc receptors and their role in immune regulation and autoimmunity. *J. Clin. Immunol.* 25, 1-18.
- Vig M., Peinelt C., Beck A., Koomoa D.L., Rabah D., Koblan-Huberson M., Kraft S., Turner H., Fleig A., Penner R. and Kinet J.P. (2006). CRACM1 is a plasma membrane protein essential for store-operated Ca²⁺ entry. *Science* 312, 1220-1223.
- Willis B.C. and Borok Z. (2007). TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. *Am. J. Physiol. Lung Cell Mol. Physiol.* 293, L525-534.
- Wu Y., Deng J., Rychahou P.G., Qiu S., Evers B.M. and Zhou B.P.

STIM and FcγRII activity in diabetic nephropathy

- (2009). Stabilization of snail by NF-kappaB is required for inflammation-induced cell migration and invasion. *Cancer Cell* 15, 416-428.
- Yamaguchi Y., Iwano M., Suzuki D., Nakatani K., Kimura K., Harada K., Kubo A., Akai Y., Toyoda M., Kanauchi M., Neilson E.G. and Saito Y. (2009). Epithelial-mesenchymal transition as a potential explanation for podocyte depletion in diabetic nephropathy. *Am. J. Kidney Dis.* 54, 653-664.
- Zeisberg M. and Neilson E.G. (2010). Mechanisms of tubulointerstitial fibrosis. *J. Am. Soc. Nephrol.* 21, 1819-1834.
- Zhang Q., Helfand B.T., Jang T.L., Zhu L.J., Chen L., Yang X.J., Kozlowski J., Smith N., Kundu S.D., Yang G., Raji A.A., Javonovic B., Pins M., Lindholm P., Guo Y., Catalona W.J. and Lee C. (2009). Nuclear factor-kappaB-mediated transforming growth factor-beta-induced expression of vimentin is an independent predictor of biochemical recurrence after radical prostatectomy. *Clin. Cancer Res.* 15, 3557-3567.
- Zhang S.L., Yu Y., Roos J., Kozak J.A., Deerinck T.J., Ellisman M.H., Stauderman K.A. and Cahalan M.D. (2005). STIM1 is a Ca²⁺ sensor that activates CRAC channels and migrates from the Ca²⁺ store to the plasma membrane. *Nature* 437, 902-905.
- Zhu H., Wu H., Liu X., Li B., Chen Y., Ren X., Liu C.G. and Yang J.M. (2009). Regulation of autophagy by a beclin 1-targeted microRNA, miR-30a, in cancer cells. *Autophagy* 5, 816-823.

Accepted November 26, 2018