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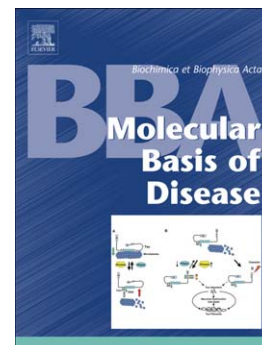
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# Expression and Regulation of A Novel Identified TNFAIP8 Family is Associated with Diabetic Nephropathy

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## Abstract

Tumor necrosis factor- $\alpha$ -inducible protein 8 (TNFAIP8) family is very recently identified proteins which share considerable sequence homology to regulate cellular and immune homeostasis. However, it is unknown whether TNFAIP8 family is expressed in the kidney and contributes to the regulation of renal functions. Therefore, the present study was designed to characterize the members of TNFAIP8 family in the kidney and to explore their possible roles in the development and progression of diabetic nephropathy. By RT-PCR and Western blot analyses, we found that all members of TNFAIP8 family were detected in the kidney. TNFAIP8 and TIPE2 expression was significantly increased in glomeruli from streptozotocin (STZ)-induced diabetic rats, and this upregulation was further confirmed in renal biopsies of diabetic patients. In *in vitro* study, TNFAIP8 was upregulated in response to high glucose in mesangial cells rather than podocytes. Moreover, a direct correlation was observed between expression of TNFAIP8 and mesangial cell proliferation and this regulation was associated with NADPH oxidase-mediated signaling pathway. However, we failed to observe the upregulation of TIPE2 in both mesangial cells and podocytes in response to high glucose. In conclusion, the present study addressed the role of TNFAIP8 family in diabetic nephropathy. These findings for the first time demonstrate that TNFAIP8 is one of critical components of a signal transduction pathway that links mesangial cell proliferation to diabetic renal injury.

**Key words:** TNFAIP8 family, NADPH oxidase, cell proliferation, immune homeostasis, diabetic nephropathy

## Introduction

Diabetic nephropathy (DN) is one of the major microvascular complications of diabetes and the most common cause of end stage of renal diseases (ESRD) [1]. DN can be characterized by both glomerular and tubulointerstitial injury. An important histological hallmark of DN is mesangial cell proliferation, resulting in the expansion of extracellular matrix (ECM) in the mesangium. Thickening of the glomerular basement membrane (GBM) is another feature of DN [2]. Although the pathogenesis of DN is multifactorial, a number of clinical and animal model studies have implicated inflammatory mechanisms as important pathogenic factors in DN [3, 4]. Inflammatory markers such as IL-1 $\beta$  and TNF- $\alpha$  are increased in the serum and tissues of patients with DN. This occurs at a very early stage of disease, and correlates with the degree of albuminuria. Inflammation is traditionally considered as a process resulting in macrophage infiltration. However, current studies have also demonstrated that resident glomerular cells such as mesangial cells and podocytes, can produce cytokines and express molecules that are part of the co-stimulatory pathway [5]. These inflammatory cytokines and other mediators may stimulate glomerular cells to enhance production or reduce degradation of ECM proteins [3, 6]. Tumor necrosis factor- $\alpha$ -inducible protein 8 (TNFAIP8) family is very recently identified proteins and induced by TNF- $\alpha$  stimulation and by activation of transcription factor NF- $\kappa$ B [7, 8], it is possible that this family may be the molecular link between TNF- $\alpha$ -mediated signaling and DN.

TNFAIP8 family consists of TNFAIP8, TIPE1 (TNFAIP8L1, TNF- $\alpha$ -induced protein 8-like 1), TIPE2 (TNFAIP8L2) and TIPE3 (TNFAIP8L3), which share considerable sequence homology to regulate cell functions [7, 8]. TNFAIP8 (also called SCC-S2/GG2-1/NDED), the first identified member in this family, is associated with enhanced cell survival and inhibition of apoptosis [9].

Overexpression of TNFAIP8 contributes to enhanced DNA synthesis, cell proliferation and inhibition of activities of the apoptotic enzymes caspase 8 and caspase 3 [10]. Knocking down TNFAIP8 expression in tumor cells reduces their tumorigenicity, suggesting that it may play a role in oncogenesis [11]. Therefore, cell survival and malignant growth-related signaling pathways are intricately linked by TNFAIP8 [12]. However, the physiological role of TNFAIP8 is not clear. In 2008, a second member of TNFAIP8 family, designated TIPE2 (TNFAIP8L2) was identified as a negative regulator to maintain immune homeostasis [8, 13]. TIPE2 deletion in mice leads to multiorgan inflammation, splenomegaly, and premature death. TIPE2 is preferentially expressed in lymphoid tissues. Interestingly, although TIPE2 is not expressed in NIH 3T3 fibroblasts cell line, following stimulation with TNF- $\alpha$ , NIH 3T3 fibroblasts expressed detectable levels of TIPE2 mRNA, suggesting that TIPE2 may be expressed in other cell types to establish equilibrium during an inflammatory response. Besides TNFAIP8 and TIPE2, two additional uncharacterized members of this family may exist, which share high degrees of sequence homology with TIPE2 and are designated as TTIPE1 and TIPE3 in the gene bank [8]. However, so far it is unknown whether these four members of TNFAIP8 family are expressed in the kidney and further contribute to the regulation of renal functions in DN. Therefore, the present study was designed to characterize TNFAIP8 family in the kidney and renal cells and further to explore the possible roles in the development and progression of DN.

## Methods

**Animal Studies:** Male Sprague-Dawley rats (150-170 g) were purchased from Laboratory Animals Center of Shandong University, China. Diabetes was induced by tail-vein injection of streptozotocin (STZ) at 50 mg/kg body wt in sodium citrate buffer (0.01 M, pH 4.5). An equivalent amount of sodium citrate buffer alone was used as a vehicle control. Blood glucose levels were monitored 48 h

later and periodically thereafter (LifeScan One Touch glucometer, Johnson & Johnson, Milpitas, CA, USA) by rat-tailed blood sampling. Rats with blood glucose levels above 18.0 mM were considered as diabetic. All rats had unrestricted access to food/water and were maintained for 12 weeks in accordance with Institutional Animal Care and Use Committee procedures of Shandong University. Rats were weighed weekly and blood pressure was determined by tail cuff plethysmography (Harvard Apparatus, Holliston, MA, USA) monthly. At the end of the study, weight, blood pressure and blood glucose were measured. Urine was collected for 24 h in a metabolic cage and urinary albumin excretion was measured using a rat albumin ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX, USA). Simultaneously, rats were killed under ketamine anesthesia. The fixed kidneys were paraffin-embedded, and sections were prepared and stained with periodic acid–Schiff stain. Glomerular damage index was calculated from 0 to 4 on the basis of the degree of glomerulosclerosis and mesangial matrix expansion as our previous studies [14]. Glomeruli from the remainder of the kidney cortex were harvested by differential sieving [15]. They were homogenized in lysis buffer or Trizol for immunoblotting and mRNA analysis, respectively.

**Human renal biopsy samples and Immunohistochemical studies:** Renal biopsies had been performed as part of routine clinical diagnostic investigation. Informed consent was obtained from patients for use of archived human biopsy material and chart review in accord with institutional research ethics board review. The patient samples were obtained from Department of Pathology, Shandong University School of Medicine with documented a pathologic diagnosis of DN. Immunohistochemistry studies were performed from an independent cohort of control subjects (histologically verified unaffected regions from tumor nephrectomies) and DN case subjects. TNFAIP8 and TIPE2 staining were used as a primary polyclonal antibody at 1:150 (ProteinTech Group, Chicago, IL, USA) in this study.

**Cell culture:** Rat renal mesangial cells were cultured in DMEM supplemented with 10% FCS

(Invitrogen, Gaithersburg, MD, USA), streptomycin (100 µg/ml), and penicillin (100 U/ml) at 37°C in 95% air/5% CO<sub>2</sub>. Medium contained 5.6 mM glucose (Control group). To study the effect of high glucose (HG), D-glucose at a final concentration of 10, 20, or 40 mM (4.4, 10.4, or 34.4 mM D-glucose was added in the medium for HG condition) were employed in this study and mannitol was added in the medium as the osmolarity control.

A clonal cell line of conditionally-immortalized murine podocytes was the generous gift from Prof. Pin-Lan Li at Department of Pharmacology, Virginia Commonwealth University, USA. Podocytes were cultured as our previous studies [16]. Treatments of podocytes with high glucose were similar as mesangial cell's treatments.

**RNA extraction and RT-PCR:** Total RNA was isolated from renal glomeruli or cells using TRIzol reagent (Invitrogen) as we described previously [16]. The mRNA levels for target genes were analyzed by RT-PCR or real-time quantitative RT-PCR using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA, USA). The specific primers for target genes used for both RT-PCR and real-time quantitative RT-PCR are list in table 1. Levels of the housekeeping gene  $\beta$ -actin were used as an internal control for the normalization of RNA quantity and quality differences among the samples.

**Western blot analysis:** Western blotting was performed as we described previously [17]. Primary antibodies anti-TNFAIP8, anti-TIPE2 (1:1000 dilution, ProteinTech Group) and secondly antibodies horseradish peroxidase-labeled anti-mouse IgG or anti-rabbit IgG (1:6000 dilution) were used in this study. To document the loading controls, the membrane was reprobed with a primary antibody against housekeeping protein  $\beta$ -actin.

**RNA interference:** Small interference RNA to TNFAIP8 (siRNA-TNFAIP8) was synthesized by Biomics Biotechnologies Co., Ltd. (Nantong, Jiangsu, China). The DNA target sequence for siRNA-TNFAIP8 (5'-CCA GCA GCG AGG TCC TGG ATG AGC T-3') was designed based on the core sequence of rat TNFAIP8 cDNA (Accession number NM\_001107387). The scrambled small



RNA (5'-AAT TCT CGA ACT GTC ACG T-3') has been confirmed as non-silencing double stranded RNA and was used as control. In these experiments, siRNA transfection was performed according to the manufacturer's instruction in Bio-Red siLentFect™ Lipid transfection kit.

**Cell proliferation:** Colorimetric assay were used in the assessment of cell proliferation. Cell proliferation was measured by DNA content using a colorimetric assay as described [18]. Cells were grown in normal or high glucose medium for 48 h. After washing the cells with ice cold Hank's balanced salt solution. DNA was precipitated with 10% trichloroacetic acid for 10 min at 4°C, and 0.5 ml of a solution containing 20 volumes of a stock (1 g of diphenylamine, 90 ml of glacial acetic acid and 2 ml of sulfuric acid), 8 volumes of H<sub>2</sub>O and 0.1 volume of 1% acetaldehyde was added to each well for 48 h. DNA content was determined in triplicate wells by reading OD 580 nm and comparing to a standard curve established with calf thymus DNA. Cell viability was determined at the end of treatments by trypan blue dye exclusion method.

**Measurements of NADPH oxidase activity:** NADPH oxidase activity was determined by measurement of O<sub>2</sub><sup>-</sup> production in isolated glomeruli or cell homogenates. Fluorescence spectrometry for O<sub>2</sub><sup>-</sup> production was performed by using a modified DHE fluorescent spectrometric assay as described previously [17].

**Statistics:** Data are expressed as means ± SE. The significance of the differences in mean values between and within multiple groups was examined by one-way ANOVA followed by Duncan's multiple range test.  $P < 0.05$  was considered statistically significant.

## Results

**Expression of TNFAIP8 family in the kidney and renal cells:** To determine the expression patterns of TNFAIP8 family, total RNA was extracted from tissues of Sprague-Dawley rats and

RT-PCR was performed. As shown in Figure 1A, among kidney, brain, liver and spleen, TNFAIP8 was the most abundant in the kidney, whereas it was hard to detect in the liver. TIPE1 was detected in these tissues with relative high levels in the spleen and liver. TIPE2 was rich in spleen and brain, whereas expression level in the kidney was very low. TIPE3 was detected in brain and kidney. We further determined the expression levels of TNFAIP8 family in glomerular cells including mesangial cells and podocytes by real time RT-PCR analysis. We found that four members of the TNFAIP8 family were present in these cell lines. TIPE1 and TNFAIP8 were widely expressed; TIPE3 was preferentially expressed in mesangial cells; TIPE2 was very low expressed in both mesangial cells and podocytes. Figure 1B and 1C are summarized data showing the distributions of TNFAIP8 family in tissues and some glomerular cells, respectively.

**Renal function changes in experimental diabetic rats:** As shown in table 2, diabetic rats had hyperglycemia, lower body weight than their non-diabetic counterparts. No difference in blood pressure was observed between these groups. Glomerular damages by morphological analysis (Figure 2A) and increased urinary albumin excretion were observed in the diabetic rats (Figure 2B). In addition, the cytokine TNF- $\alpha$  and NADPH oxidase-mediated oxidative stress have also been implicated in the pathogenesis of DN. Therefore, we further measured glomerular mRNA level of TNF- $\alpha$  and NADPH oxidase activity. It was found that the TNF- $\alpha$  level was 2.1-fold higher than in that of non-diabetic rats (Figure 2C). NADPH oxidase activity was significantly increased in glomeruli from diabetic rats (Figure 2D).

**Enhanced TNFAIP8 and TIPE2 expression in the glomeruli from diabetic rats and patients with DN:** By real time RT-PCR analysis, we found that, among the TNFAIP8 family, TNFAIP8 and TIPE2 mRNA levels were markedly enhanced in glomeruli isolated from diabetic rats. Both TIPE1 and TIPE3 mRNA levels had no significant difference compared with those from normal rats (Figure 3A). We then assessed glomerular TNFAIP8 and TIPE2 protein levels in diabetic rats. In

consistent with the changes in mRNA levels, both TNFAIP8 and TIPE2 protein levels were significantly increased (Figure 3B). By immunochemical analysis, we further observed that TNFAIP8 was mainly expressed in mesangium of glomeruli from diabetic rats. TIPE2 was relatively widely expressed in the glomeruli and renal interstitium from diabetic rats (Figure 3C). Finally, by real time RT-PCR and immunochemical analyses of human renal biopsy samples, we also confirmed the upregulation of TNFAIP8 and TIPE2 in the kidney (Figure 3D and 3E).

**TNFAIP8 upregulation in response to high glucose in mesangial cells rather than podocytes:**

Two cells lines including mesangial cells and podocytes which mainly contribute to glomerulosclerosis were selected to investigate the changes of TNFAIP8 and TIPE2 in response to high glucose *in vitro*. As shown in Figure 4A, we found that D-glucose concentration dependently enhanced TNFAIP8 levels in mesangial cells with a 260% increase at a final glucose concentration of 40 mM. To ensure these effects were not osmotically mediated, we incubated mesangial cells with equivalent concentrations of mannitol, no effects on TNFAIP8 expression level. In mesangial cells treated with TNF- $\alpha$  (10 to 50 ng/mL), as documented in Figure 4B, TNF- $\alpha$  increased TNFAIP8 expression in mesangial cells. However, when podocytes were exposed to the same concentrations of high glucose, TNFAIP8 expression levels had no changes (Figure 4C), suggesting that TNFAIP8 may mainly act on mesangial cells and regulate their functions.

Next, we detected the TIPE2 expression under high glucose conditions in both mesangial cells and podocytes. Interestingly, TIPE2 was not upregulated in either mesangial cells or podocytes (data not shown).

**Regulation of TNFAIP8 expression is associated with NADPH oxidase activity:** Given the role of NADPH oxidase-mediated NF- $\kappa$ B activity and the fact that TNFAIP8 can be induced by NF- $\kappa$ B, we wondered whether NADPH oxidase activity is associated with the upregulation of TNFAIP8 in high glucose-treated mesangial cells. Therefore, the activity of NADPH oxidase was measured by

fluorescence spectrometric assay. Summarized data showed that NADPH oxidase activity was increased by high glucose treatment in a concentration dependent manner, which was blocked by NADPH oxidase inhibitor apocynin (Figure 5A and B). We further found that both high glucose and TNF- $\alpha$  induced TNFAIP8 expression in mesangial cells, which was attenuated by inhibition of NADPH oxidase activity as shown in Figure 5C and 5D.

**Gene silencing of TNFAIP8 attenuated high glucose-induced mesangial cell proliferation:** To investigate the role of TNFAIP8 on the regulation of high glucose-induced mesangial cell proliferation, TNFAIP8 gene silencing by siRNA was used in this study. By real-time RT-PCR and Western blot analyses, TNFAIP8 mRNA and protein levels were decreased by 76% and 61 % in siRNA-TNFAIP8 transfected mesangial cells, respectively (Figure 6A and 6B). This siRNA-TNFAIP8 had no effect on other members of TNFAIP family expression levels, indicating the specificity of siRNA to TNFAIP8 (data not shown). Then, we determined the effect of siRNA-TNFAIP8 on mesangial cell proliferation. It was found that high glucose significantly increased mesangial cell proliferation, which was markedly blocked by siRNA-TNFAIP8 or apocynin indicating that TNFAIP8 contributes to high glucose-induced mesangial cell proliferation (Figure 6C and 6D). Similar results were also obtained in mesangial cells treated with TNF- $\alpha$  (25 ng/ml) (Figure S1).

## Discussion

This is the first report to characterize TNFAIP8 family in the kidney and investigate their potential roles in DN. We found that, among four members of this family, TNFAIP8 and TIPE2 were upregulated in DN, indicating that they may participate in the pathogenesis of DN. A direct correlation was observed between expression of TNFAIP8 and cell proliferation in high glucose-treated mesangial cells. We further demonstrated that this regulation is associated with

NADPH oxidase-mediated signaling pathways. In combination with clinical data, formalin-fixed, paraffin-embedded human kidney tissues provide important information of TNFAIP8 family in the pathogenesis of DN.

A subfamily of proteins that contribute to cellular homeostasis possesses a hexahelical bundle motif, called the death effector domain (DED) [19]. Both pro- and anti-apoptotic proteins containing DEDs have been identified. For Fas and other death receptors, homotypic DED interactions connect the Fas-associated death domain (FADD) protein to caspase-8 and caspase-10 to mediate the formation of the death-inducing signal complex (DISC), thereby initiating apoptosis [19, 20]. This complex can be inhibited by other DED-containing proteins. Accumulating evidence suggests that DED-containing proteins also play a role in controlling pathways of cellular activation and proliferation [19, 21, 22]. Cellular and viral antiapoptotic DED proteins, known as FLIPs, inhibit Fas-induced death by interfering with FADD and caspase-8 [23, 24]. A DED-containing protein PEA-15 inhibits apoptosis induced by Fas in astrocytes and some tumor cells [25]. In addition, overexpression of PEA-15 activates ERK and stimulates proliferation *in vitro* in a Ras-dependent manner [26]. Thus, the DED proteins may be pivotal to cellular homeostasis by establishing a 'cell renewal set point' that coregulates proliferation and apoptosis in parallel [19]. TNFAIP8 family is normally considered as a new subfamily of DED containing proteins by sequence analysis showing the high sequence homology with DED and experimental evidence showing the regulatory role of apoptosis. However, the high-resolution crystal structure of TIPE2 reveals a previously uncharacterized fold different from the predicted fold of a DED [13]. Strikingly, TIPE2 contains a notable feature—a large, hydrophobic central cavity that is poised for cofactor binding [13]. Therefore, it was proposed that the central cavity may have an important role in the maintenance of immune homeostasis by binding to a cofactor. Hence, considering the high degree of sequence conservation

among TNFAIP8 family, TNFAIP8 family may represent a novel class of molecules that maintain immune and cellular homeostasis through a previously uncharacterized mechanism.

In the present study, our major aim is to investigate whether TNFAIP8 family is associated with DN. We first determined the expression profile of TNFAIP8 family in the rat kidney. By mRNA and Western blot analyses, all members of TNFAIP8 family are detected in the kidney, although the TIPE2 expression level is very low. One of the most important findings of this study is that both TNFAIP8 and TIPE2 levels are markedly increased in glomeruli from diabetic rats and diabetic patients, indicating that TNFAIP8 and TIPE2 may play important roles in the development of glomerulosclerosis in DN.

In this study, two cells lines including mesangial cells and podocytes which mainly contribute to glomerulosclerosis were selected in *in vitro* study. As we know, among the pathological changes of DN, uncontrolled mesangial cell proliferation is correlated closely with progression of renal failure, which causes an increase in extracellular matrix (ECM) deposition and ultimately leads to glomerulosclerosis [1, 27]. In renal diseases, both resident renal cells and infiltrating immune cells such as macrophages are capable of secreting pro-inflammatory cytokines including IL-1 $\beta$  and TNF- $\alpha$  [28]. These cytokines may initially serve to sustain the inflammatory process, thereby promoting ongoing mesangial cell proliferation and ECM production, leading to the progressive glomerular injury in DN. Hence, we further investigated whether hyperglycemia regulates TNFAIP8 expression and whether TNFAIP8-related signaling is involved in the regulation of mesangial cell proliferation. We found that, in response to high glucose, TNFAIP8 expression was significantly enhanced in mesangial cells and this increase in TNFAIP8 expression contributed to mesangial cell proliferation. Our results are consistent with previous studies indicating that TNFAIP8 is associated

with enhanced cell survival and inhibition of apoptosis [9, 12]. However, a very recent report showed that downregulating the expression of TNFAIP8 was effectively protect thymocytes against glucocorticoid-induced apoptosis, suggesting that TNFAIP8 is crucial in regulating glucocorticoid-mediated apoptosis of thymocytes [30]. Therefore, it is necessary to further investigate the regulatory role of TNFAIP8 on cell survival and apoptosis, which may provide unusual insights into the mechanisms of maintaining cellular homeostasis. Recent studies have also correlated podocyte loss with the onset and magnitude of glomerulosclerosis [29, 30]. We then detected the TNFAIP8 expression in podocytes. However, there was no significant difference on TNFAIP8 expression in podocytes with or without high glucose treatment, indicating that TNFAIP8 may mainly act on mesangial cells and regulate their functions.

A number of reports have indicated that NADPH oxidase-derived reactive oxygen species (ROS) is central to hyperglycaemia-induced oxidative stress in DN [31, 32]. NADPH oxidase subunit Nox 4 appears to be the predominant isoform expressed in renal cells. Increases in Nox 4 mRNA levels were detected in the kidney of diabetic rats 4–8 weeks after the onset of diabetes with similar increases detected in mesangial cells exposed to high glucose [33, 34]. Therefore, it is imperative to know whether the regulation of TNFAIP8 expression is associated with NADPH oxidase activity. Our results showed that NADPH oxidase inhibitor apocynin attenuated high glucose or TNF- $\alpha$ -induced TNFAIP8 expression. Furthermore, we found that both apocynin and gene silencing of TNFAIP8 inhibited high glucose- and TNF- $\alpha$ -induced increase in mesangial cell proliferation. Since TNF- $\alpha$  can increase ROS production through c-Src/NADPH oxidase, in turn initiates the activation of NF- $\kappa$ B [35], it is possible that high glucose-induced TNF- $\alpha$  may stimulate NADPH oxidase, thereby leads to activation of NF- $\kappa$ B pathway, finally resulting in the upregulation of TNFAIP8.

Next, we detected the TIPE2 expression under high glucose conditions in both mesangial cells and podocytes. We failed to observe the upregulation of TIPE2 in both mesangial cells and podocytes, which is controversial with our *in vivo* study showing that the expression of TIPE2 was significantly increased in glomeruli from diabetes. As we know, infiltration of immune cells plays an important role in the development and progression of DN [36]. Macrophage is one of the central mediators of renal vascular inflammation, and its accumulation is a characteristic feature of diabetic nephropathy [37]. Therefore, it is possible that this upregulation of TIPE2 is likely expressed by infiltrating macrophages. Therefore, continuous slices of human renal biopsy samples were stained with CD68 (one marker of macrophage) and TIPE2 individually. We observed that CD68-positive infiltrating macrophages in the interstitium and glomeruli, which are colocalized with TIPE2 (Figure S2). These results indicate that infiltrating macrophages contribute to, at least in part to the upregulation of TIPE2 expression in the kidney. However, we also found that TIPE2 was induced in other area. Therefore, we can not exclude that some unknown mechanisms are involved in the regulation of TIPE2 expression acting on the renal resident cells *in vivo*. This regulation may be a complex network, further studies need to be addressed this issue. In addition, our research group is investigating the functional relevance of TIPE2 in DN using TIPE2 knockout mice, which are kindly gifts from Dr. Youhai Chen, Department of Pathology and Laboratory Medicine, University of Pennsylvania, USA.

In summary, the present study addressed the role of TNFAIP8 family in DN. These findings for the first time demonstrate that TNFAIP8 is one of critical components of a signal transduction pathway that links mesangial cell proliferation to diabetic renal injury. Pharmacological targeting of this pathway at multiple levels may provide a novel approach to treatment of diabetic renal disease.



Despite many investigators have been able to inhibit mesangial cell proliferation both *in vitro* and *in vivo* using different strategies, most of patients and experimental animal models continue to show progressive renal damage. It would be desirable to inhibit mechanisms that are unique to proliferating mesangial cells to avoid effects on other actively dividing cells. Therefore, TNFAIP8-inhibiting may provide promising new strategies in the treatment of mesangial proliferative renal diseases. Regarding this issue, current investigations in our laboratory are designed to use structure-based virtual screening to obtain potent inhibitors of TNFAIP8 and examine their effects in treatment of DN.

### **Acknowledgement**

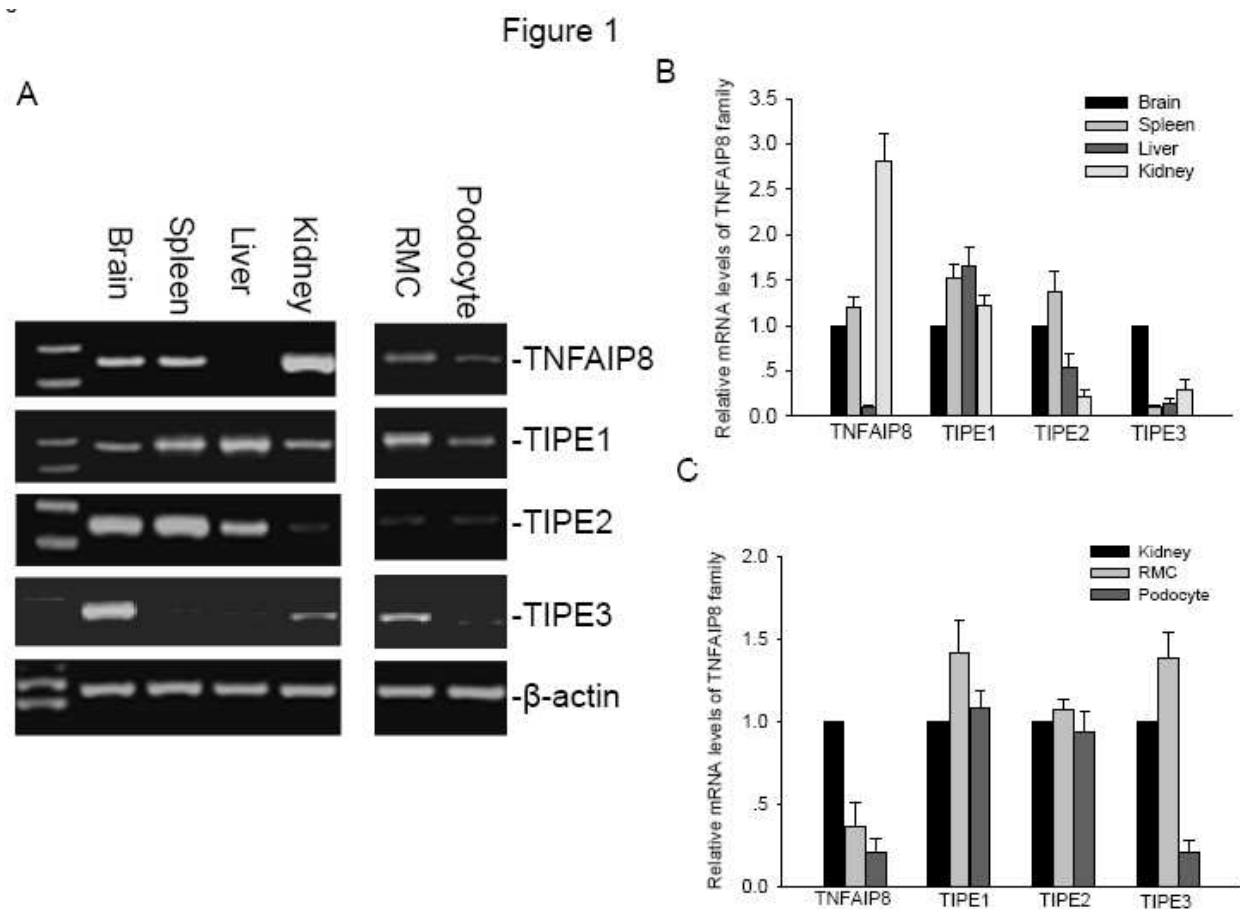
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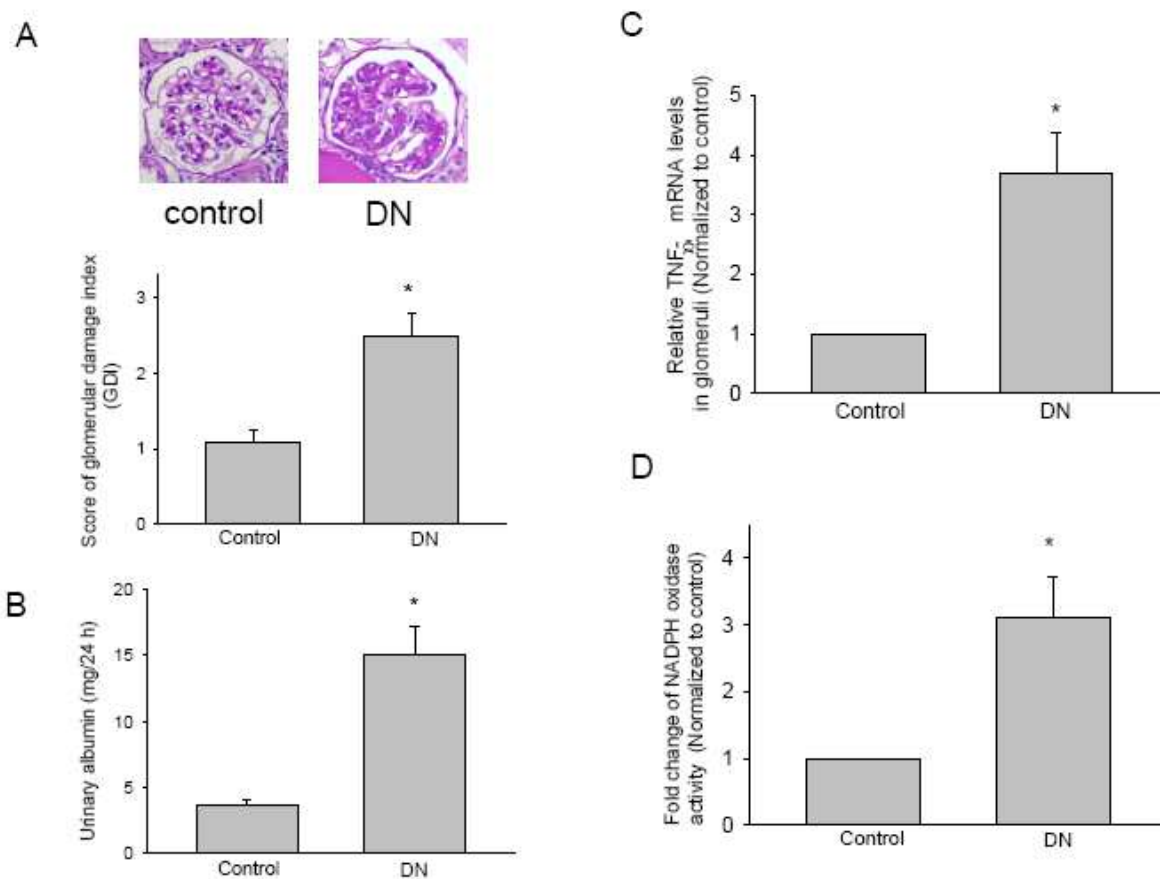
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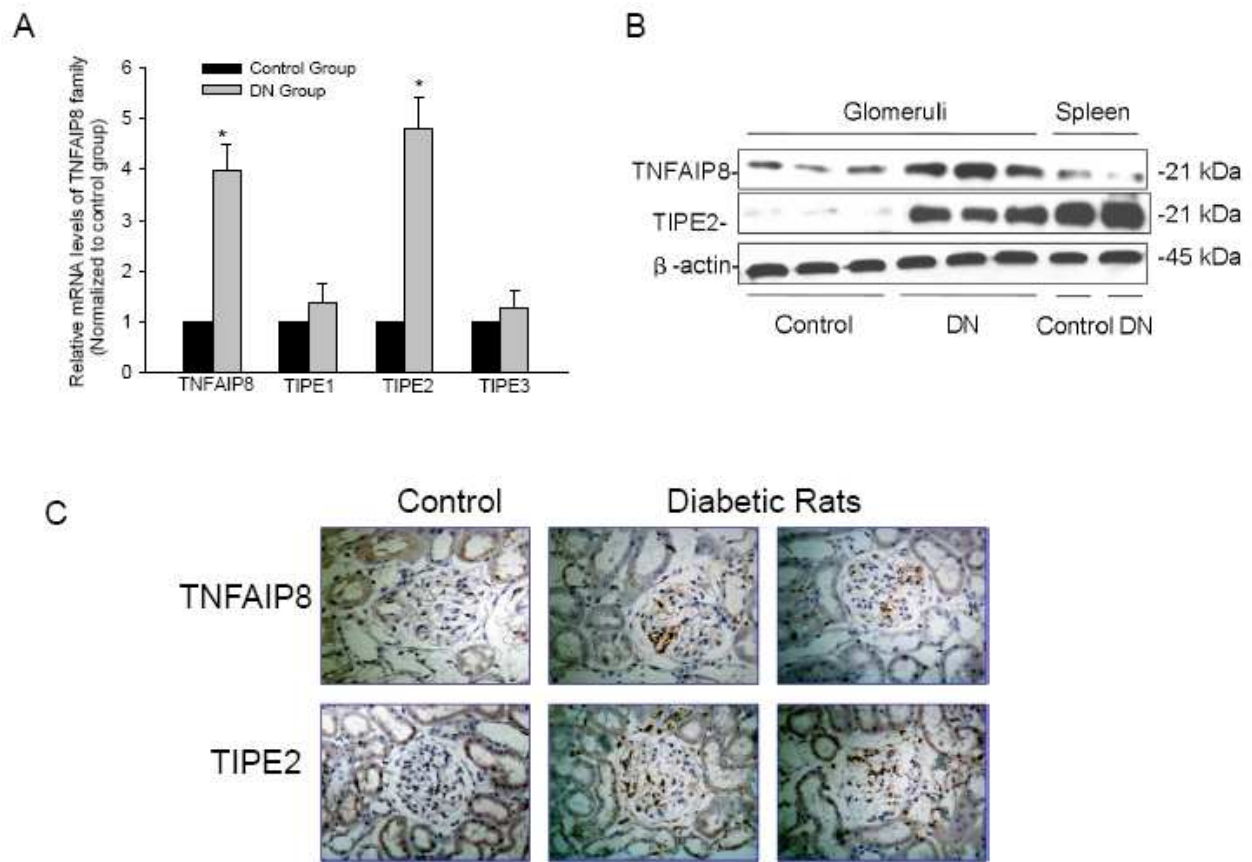
**Figure 1. Expression Patterns of TNFAIP8 family.** **A.** RT-PCR analysis of TNFAIP8 family in selected tissues and glomerular cells including renal mesangial cells (RMC) and podocytes. **B.** Summarized data showing the relative expression levels of TNFAIP family in tissues by real time RT-PCR. **C.** Summarized data showing the relative expression levels of TNFAIP family in glomerular cells by real time RT-PCR (n=5).

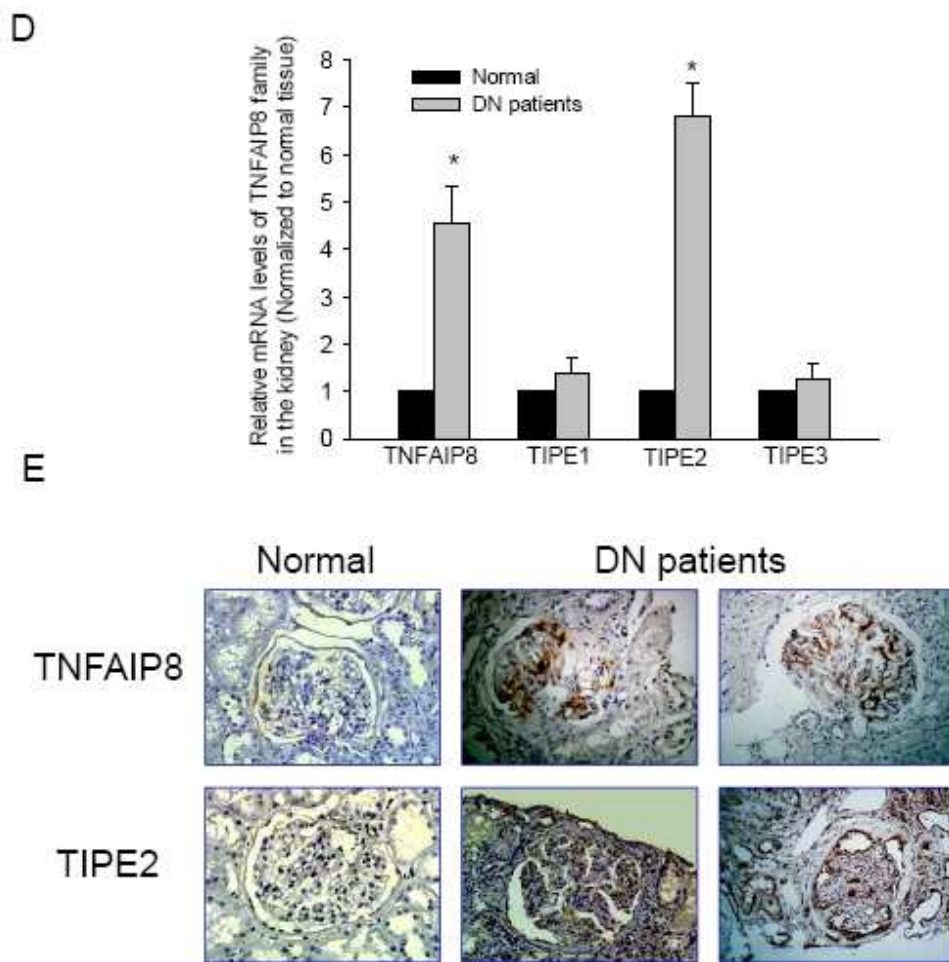
Figure 2



**Figure 2. Characterization of rat glomerular injury and related signaling molecules in streptozotocin (STZ)-induced diabetic nephropathy. A.** Photomicrographs (original magnification x250) showing typical glomerular structure and summarized glomerular damage index (GDI) by semiquantitation of scores in control and diabetic rats (DN). **B.** Urinary albumin excretion from different groups of rats. **C.** Relative mRNA levels of TNF- $\alpha$  by real-time RT-PCR analysis in glomeruli from different groups of rats. **D.** NADPH oxidase activity in glomeruli from different groups of rats. \* $P < 0.05$  compared with control (n=9).

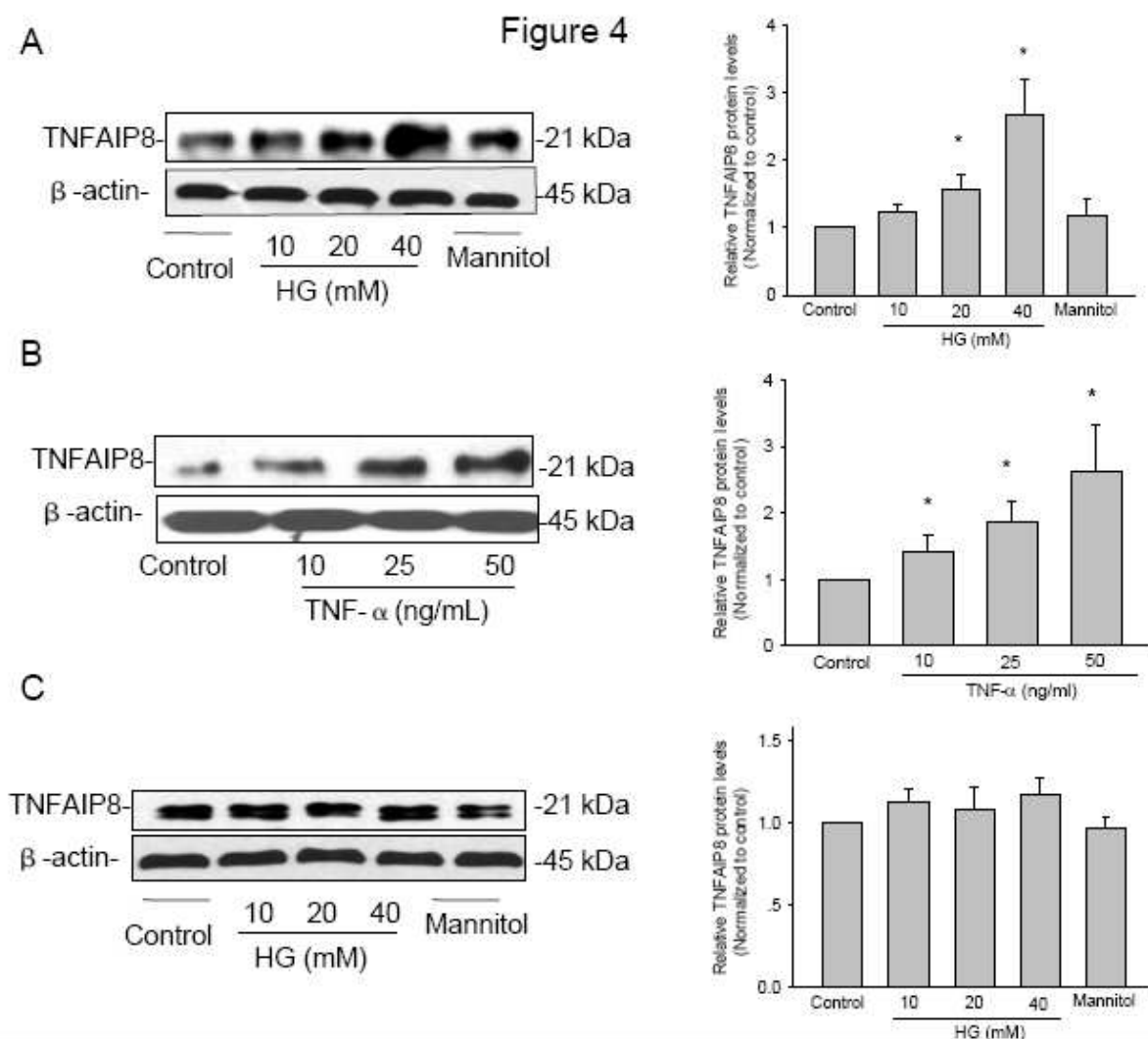
Figure 3



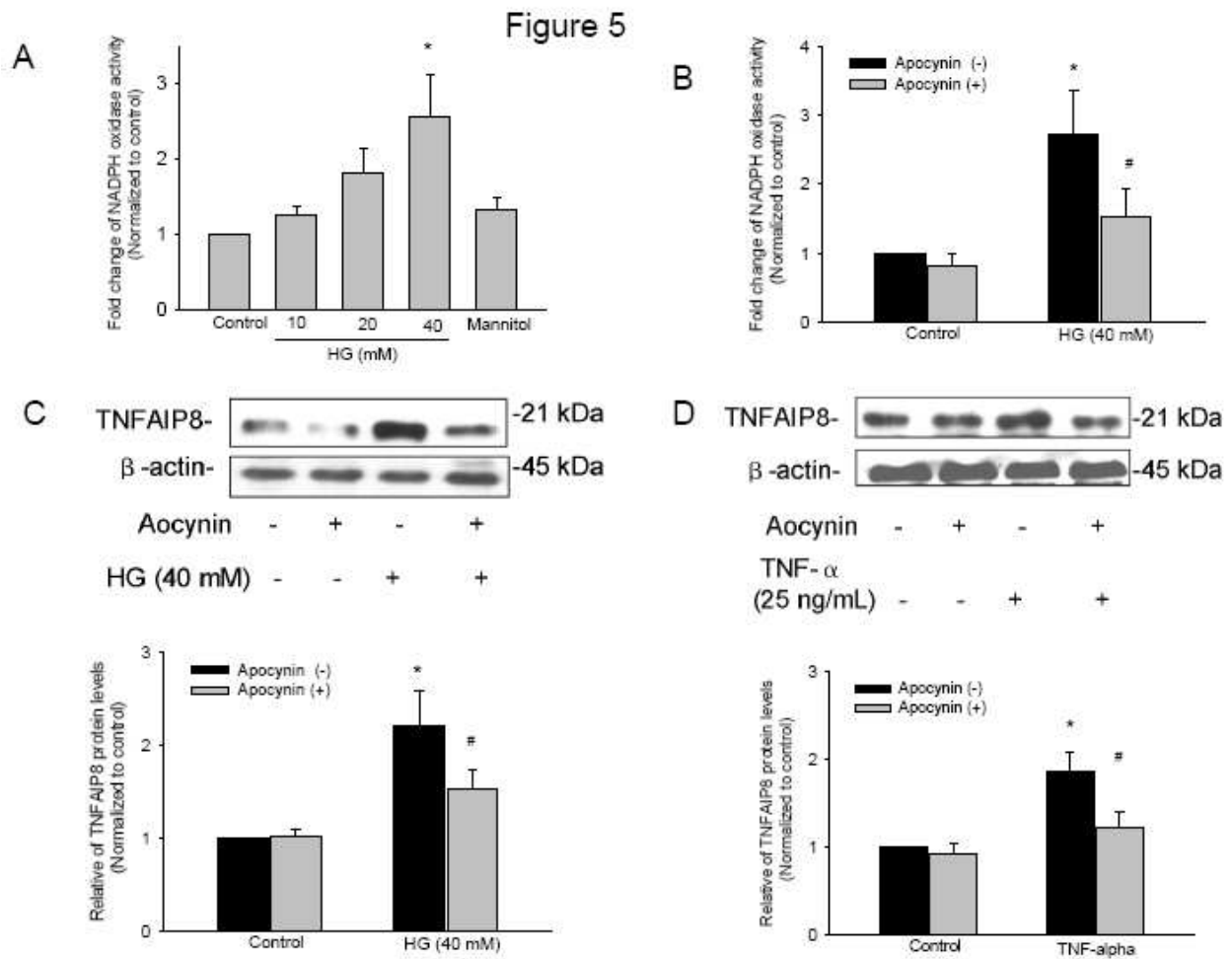


**Figure 3. The expression levels of TNFAIP8 family in glomeruli from diabetic rats. A.** Relative quantitation of mRNA levels in TNFAIP8 family by real-time RT-PCR analysis in glomeruli from different groups. **B.** Representative Western blot gel document showing the protein levels of TNFAIP8 and TIPE2 in glomeruli. **C.** Immunohistochemistry for TNFAIP8 and TIPE2 in the kidney from diabetic rats. TNFAIP8 and TIPE2 expression (brown) were observed in diabetic rats. **D.** Relative quantitation of mRNA levels in TNFAIP8 family by real-time RT-PCR analysis in human renal biopsy samples. **E.** Immunohistochemistry for TNFAIP8 and TIPE2 in human renal biopsy samples. TNFAIP8 and TIPE2 expression (brown) were observed in the kidney from diabetic patients (n=18) \* $P < 0.05$  compared with control (n=18).





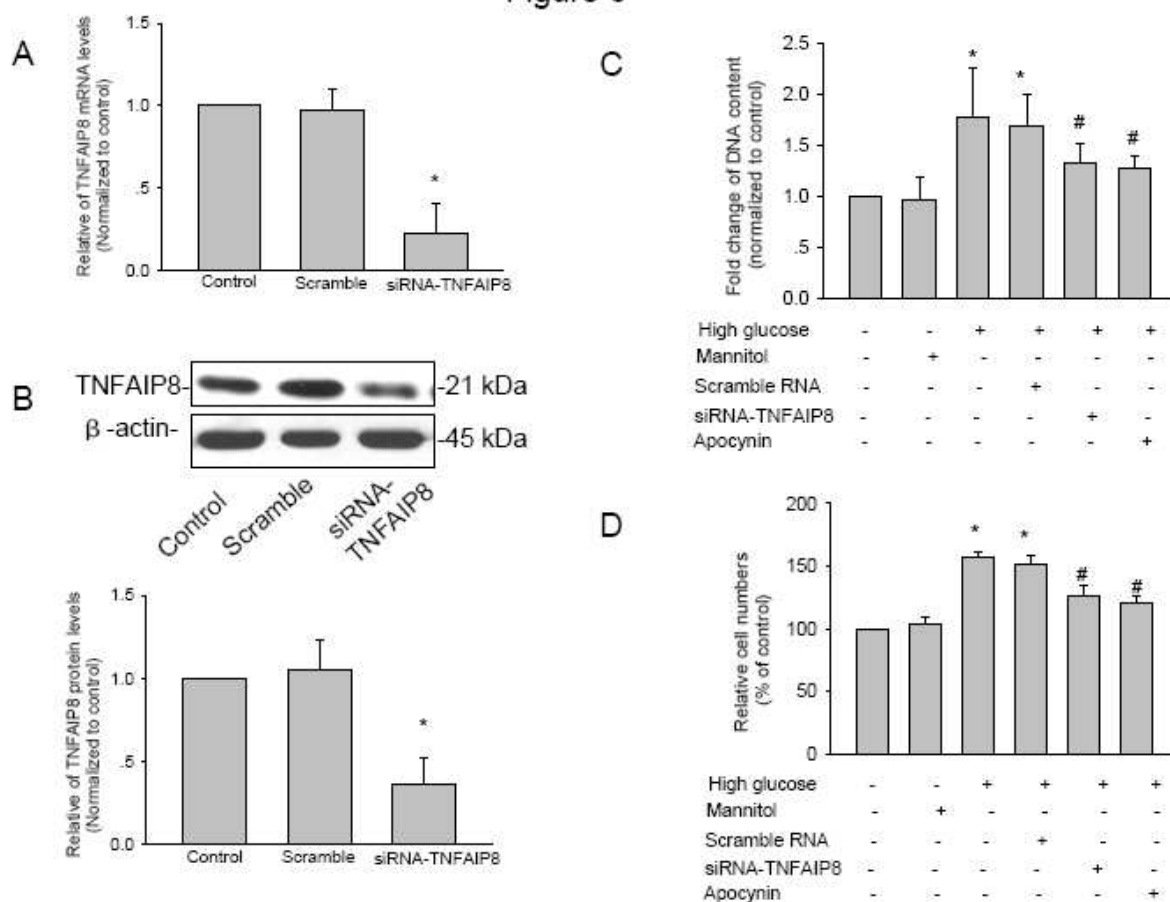
**Figure 4. Effects of high glucose (HG) on the expression of TNFAIP8 in different glomerular cells.** **A.** Representative Western blot gel documents of TNFAIP8 expression (left panel) and summarized data showing TNFAIP8 protein levels in response to high glucose in mesangial cells. **B.** Representative Western blot gel documents of TNFAIP8 expression (left panel) and summarized data showing TNFAIP8 protein levels in response to TNF- $\alpha$  in mesangial cells. **C.** Representative Western blot gel documents of TNFAIP8 expression (left panel) and summarized data showing TNFAIP8 protein levels in response to high glucose in podocytes. \*  $P < 0.05$  vs. control ( $n = 6$ ).



**Figure 5. Effects of NADPH oxidase activity on the expression of TNFAIP8 in mesangial cells.**

**A.** Summarized data showing NADPH oxidase activity in mesangial cells treated with different glucose concentrations. **B.** Summarized data showing the effect of NADPH oxidase inhibitor apocynin on NADPH oxidase activity in high glucose-treated mesangial cells. **C.** Summarized data showing the effect of apocynin on the expression of TNFAIP8 in high glucose-treated mesangial cells. **D.** Summarized data showing the effect of apocynin on the expression of TNFAIP8 in TNF- $\alpha$ -treated mesangial cells. \*  $P < 0.05$  vs. control, #  $P < 0.05$  vs. vehicle of high glucose or TNF- $\alpha$  treatment (n=6).

Figure 6



**Figure 6. Effects of TNFAIP8 on mesangial cell proliferation. A.** Quantitative RT-PCR analysis of TNFAIP8 mRNA levels in control, scrambled RNA and siRNA-TNFAIP8 transfected mesangial cells. **B.** Representative Western blot gel documents (upper panel) and summarized data showing relative of TNFAIP8 protein levels in the homogenate from transfected mesangial cells. **C.** Summarized data showing cell proliferation evaluated by the assessment of DNA content. **D.** Summarized data showing cell proliferation evaluated by the assessment of cell number. \*  $P < 0.05$  vs. control, #  $P < 0.05$  vs. vehicle of high glucose treatment (n=6).

Genes	Primers	Product (bp)
TNFAIP8	F: 5'-CCCAGGGAAGTGGCTACAGA-3' R: 5'-GCCTCCTTCTTGTTCTGGGT-3'	182
TIPE1	F: 5'-AGCAGAGGCACCTACAGAAA-3' R: 5'-AAACTCCTTAGTGGCCTGGT-3'	202
TIPE2	F: 5'-TCAGAAACATCCAAGGCCAGAC-3' R: 5'-CGGACCGACCAGCCATTTTAC-3'	147
TIPE3	F: 5'-ACTGCTGCAGGTCCTCATGT-3' R: 5'-GCCTCCTTCTTGTTGTGGGT-3'	179
TNF- $\alpha$	F: 5'-CCACCACGCTCTTCTGTCTA-3' R: 5'-TTTGCTACGACGTGGGCTAC-3'	163
$\beta$ -actin	F: 5'-AGCCATGTACGTAGCCATCC-3' R: 5'-ACCCTCATAGATGGGCACAG-3'	115

Table 1. Primer pairs used for RT-PCR and real time RT-PCR in this study.

**Table 1. Clinical characteristics of control and STZ-induced diabetic rats.** Data were expressed as means  $\pm$  SE. control = control rats, STZ= STZ- induced diabetic rats.  $P < 0.05$  was considered statistically significant.

	<b>control</b>	<b>STZ</b>	<b><i>p</i></b>
Glucose (mM)	5.59 ± 0.37	22.18 ± 2.16	< 0.05
Body weight (g)	488.2 ± 41.6	319.7 ± 22.1	< 0.05
Systolic blood pressure (mmHg)	108.5 ± 3.41	112.1 ± 4.24	N
Urine volume (ml/day)	18.28 ± 4.68	137.3 ± 22.92	< 0.05
<i>n</i>	12	12	

Table 2. Clinical characteristics of control and STZ-induced diabetic rats.

Data were expressed as means ± SE. control = control rats, STZ= STZ-induced diabetic rats.  $P < 0.05$  was considered statistically significant.