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Regulatory mechanism of TGF-β1/SGK1 pathway in tubulointerstitial fibrosis of diabetic nephropathy

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Abstract. – OBJECTIVE: The aim of this study was to clarify the potential function of transforming growth factor-β1/serum/glucocorticoid-regulated kinase 1 (TGF-β1/SGK1) pathway in diabetic nephropathy-induced tubulointerstitial fibrosis.

MATERIALS AND METHODS: Type 2 diabetes mellitus (T2DM) model was successfully established in rats by high-sucrose-high-fat diet combined with streptozotocin (STZ) induction. Subsequently, blood glucose level, renal function and pathological changes in kidneys of T2DM and control rats were evaluated. Western blot and quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) were conducted to determine the protein and mRNA expression levels of TGF-β1, SGK1, fibronectin (FN) and α-smooth muscle actin (α-SMA) in rat kidney tissues, respectively.

RESULTS: Blood glucose (BG), glycosylated hemoglobin (GHb), serum creatinine (Scr) and blood urea nitrogen (BUN) in T2DM rats were significantly higher than those of control rats ($p<0.05$). The morphology of glomeruli and renal tubules in rats of control group were normal. In contrast, T2DM rats showed significant lesions in glomeruli, renal tubules, and renal interstitium. Furthermore, the relative expression levels of TGF-β1, SGK1, FN, and α-SMA in kidney tissues of T2DM rats were remarkably higher than those of controls ($p<0.05$).

CONCLUSIONS: The TGF-β1/SGK1 pathway is closely related to tubulointerstitial fibrosis in T2DM rats.

Key Words:
Diabetic nephropathy, Tubulointerstitial fibrosis, TGF-β1, SGK1.

Introduction

Diabetic nephropathy (DN) is a common microvascular complication of diabetes mellitus (DM), which is the leading cause of end-stage renal disease (ESRD)[1,2]. With the increasing incidence of DM, DN has become one of the main causes of chronic renal failure. Nevertheless, the pathogenesis of DN has not been fully elucidated. Meanwhile, there is still a lack of effective treatment in clinical application[3]. Therefore, it is of great significance to clarify the mechanism of occurrence and development of DN and to improve the efficacy of therapy.

The pathological changes of DN include glomerular basement membrane thickening, mesangial expansion, glomerular sclerosis, tubular atrophy, and tubulointerstitial fibrosis (TIF)[4]. Previous studies have found that glomerular injury is the main cause of DN progression. With the progression of in-depth researches, the crucial role of TIF has been widely concerned[5]. During the transformation of renal tubular epithelial cells into mesenchymal cells, the massive extracellular matrix (ECM), including collagens and fibronectins, are abundantly deposited. This may eventually lead to TIF[6,7]. The complex pathogenesis of DN involves various influencing factors, such as inflammatory factors, cytokines, genetic factors, epigenetic modification, and autophagy[8-10]. However, the specific mechanism of DN still needs to be further explored.

Transforming growth factor-β1 (TGF-β1) is currently recognized as the strongest fibrogenic factor involved in ECM deposition, fibroblast activation, and phenotypic transformation[11]. Meanwhile, TGF-β1 serves as the key factor in regulating the progression of DN. Researchers[12,13] have demonstrated that the TGF-β1 pathway contributes to accelerating glomerular sclerosis, renal tubules, and interstitial fibrosis by regulating ECM. Therefore, the induction and activation of the

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TGF-β1 pathway are critical for triggering fibrocystic responses. Inhibition of TGF-β1 expression can reduce ECM deposition and urinary protein level by mediating the glomerular and tubular mechanisms[14,15]. Serum/glucocorticoid regulated kinase 1 (SGK1) is a novel member of the serine/threonine protein kinase family[16]. In recent years, studies have identified a wide range of physiological functions of SGK1. For example, it is closely related to the occurrence and progression of DM, hypertension, and obesity[17-19]. SGK1 expression is significantly up-regulated in DN, cirrhosis, and other fibrotic diseases. It is speculated that SGK1 may be a functional junction of cell phosphorylation cascade and cell signaling pathways. Furthermore, SGK1 can also be used as a new transcriptional target of TGF-β1, which mediates TGF-β1-induced fibrosis[20,21].

The aim of this study was to investigate the potential role of the TGF-β1/SGK1 pathway in TIF of T2DM rats. Moreover, we aimed to explore the pathogenesis of DN and provide a novel direction for clinical treatment.

Materials and Methods

Establishment of T2DM Model in vivo

Sixty healthy male Sprague Dawley (SD) rats were randomly assigned in the two groups, including control group (n=20) and model group (n=40). Rats in control group were routinely fed, whereas those in model group were fed with high-sucrose-high-fat diet for 4 weeks. After fasting for 12 h (water was permitted), rats in model group or control group were injected intraperitoneally with a single dose of streptozotocin (STZ) (45 mg/kg) or isodose 0.1 mol/L of citrate buffer (pH 4.5), respectively. After 48 h of administration, blood samples were collected from the tail vein. Subsequently, BG of each rat was measured for three consecutive days. BG ≥16.7 mmol/L was considered as a successful establishment of the DM model; otherwise, another single dose of STZ was applied. Each rat was administrated with STZ for no more than twice. After the establishment of the DM model, rats were fed for another 8 weeks. This investigation was approved by the Animal Ethics Committee of the First People's Hospital of Wujiang District Suzhou Animal Center.

Sample Collection

Rats were sacrificed after 6 h of fasting. Blood samples were first harvested from the femoral artery. After centrifugation at 4°C, serum samples were collected. Bilateral kidneys were harvested for paraffin embed (fixation in 4% paraformaldehyde) and protein and RNA extraction.

Biochemical Index Determination

The levels of blood glucose (BG), glycosylated hemoglobin (GHb), serum creatinine (Scr), and blood urea nitrogen (BUN) in rats were determined using an automatic biochemical analyzer (Hitachi, Tokyo, Japan).

Pathological Examination of Kidney Tissues

Kidney tissues were pre-fixed in 4% paraformaldehyde, paraffin embedded and sliced into 3 μm sections. Finally, pathological changes were observed under a microscope by hematoxylin and eosin (HE), periodic acid Schiff (PAS), periodic acid silver methenamine stain (PASM) and Masson staining (Boster, Wuhan, China).

Western Blot

Proteins were first extracted from kidney tissues. The concentration of the extracted protein was determined by the bicinchoninic acid (BCA) method. Subsequently, protein samples were separated by electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in 5% of skim milk for 2 h, the membranes were incubated with primary antibodies at 4°C overnight. Then, the membranes were washed with Tris-Buffered Saline and Tween 20 (TBST) and incubated with corresponding secondary antibody at room temperature. Immuno-reactive bands were exposed by enhanced chemiluminescence (ECL) and analyzed by ImageJ Software (NIH, Bethesda, MD, USA).

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA in kidney tissues was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The purity and concentration of the RNA were determined using a spectrophotometer. Subsequently, the extracted RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA), followed by amplification by PCR. The qRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. Relative levels of TGF-β1, SGK1, fibronectin (FN) and α-smooth muscle actin (α-SMA)

were calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: TGF-β1, F: 5'-CCTTGGTCTGGTAGGAGTC-3', R: 5'-CAGAGGGAAGAGTTCCCAGA-3'; SGK1, F: 5'-GTTCAACACGCAGGACATG-3', R: 5'-GATGCACCTGTACGATCCG-3'; FN, F: 5'-ACATAAAGACATACTCCACAA-3', R: 5'-CTTCTCCACAACC CTCTGCT-3'. α-SMA: F: 5'-GCTTGTCCTATAGAAGCACAAT-3', R: 5'-CGTCATTTCCACAGCCCTGTAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Data were represented as mean ± standard deviation (SD). The *t*-test was used to compare the differences between the two groups. $p<0.05$ was considered statistically significant.

Results

Changes in Biochemical Indexes

The levels of BG and GHb in T2DM rats were significantly higher than those of controls, indicating the successful establishment of the model in rats (Figure 1A and 1B). Besides, the levels of Scr and BUN in T2DM rats were significantly higher relative to controls (Figure 1C and 1D).

Pathological Changes in Kidney Tissues

HE and PAS staining showed glomerular integrity, clear structure, no enlargement, degeneration, and necrosis of kidney tubules, as well as intact basement membrane in rats of control

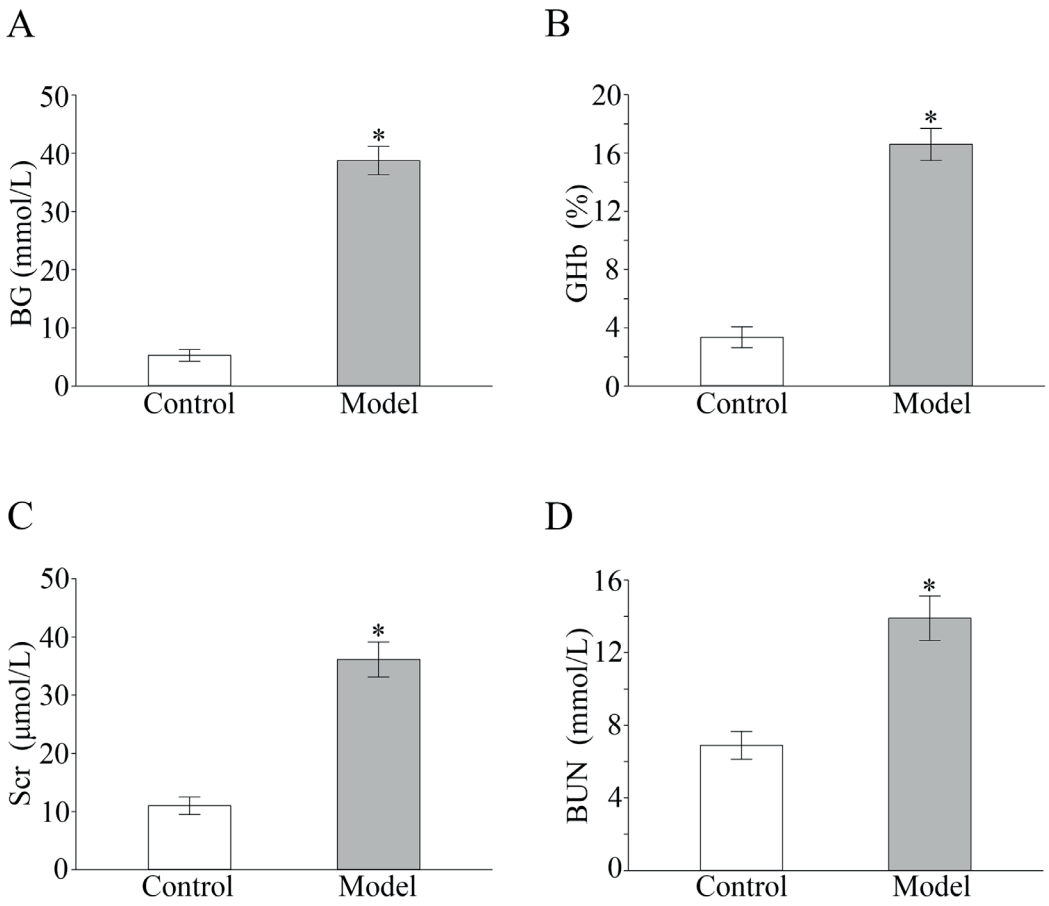

Figure 1. Changes in biochemical indexes. Sixty healthy male SD rats were randomly assigned to control group (n=20) and model group (n=40). ***A,*** BG (mmol/L) level. ***B,*** GHb (%). ***C,*** Scr (μmol/L) level. ***D,*** BUN (mmol/L) level.

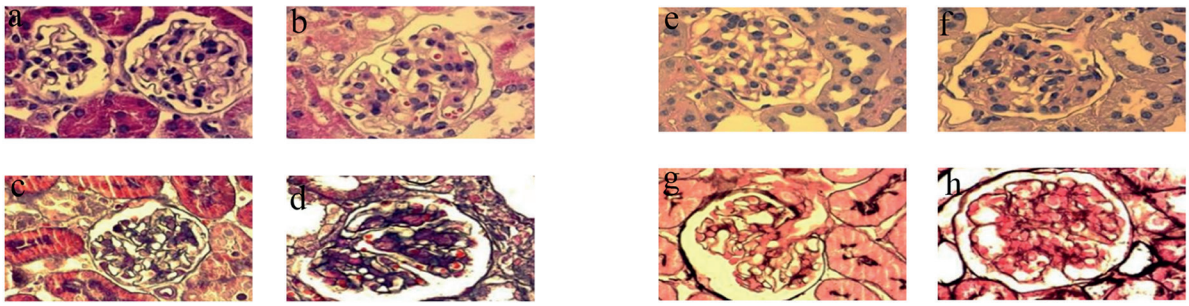

Figure 2. Pathological changes in kidney tissues (Magnification 400×). **a-b,** HE staining of kidney tissues in control (***a***) and model **(b)** group. **c-d,** Masson staining of kidney tissues in control (***c***) and model **(d)** group. **e-f,** PAS staining of kidney tissues in control **(e)** and model **(f)** group. **g-h,** PASM staining of kidney tissues in control **(g)** and model **(h)** group.

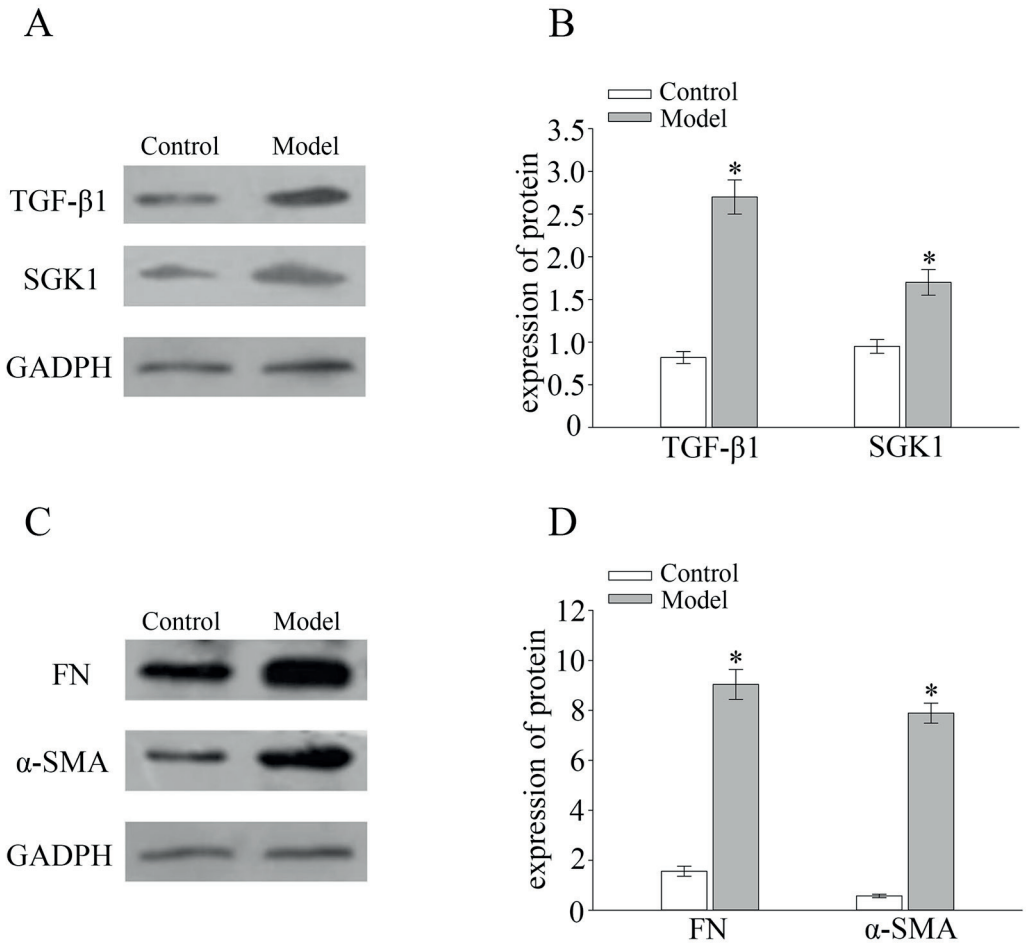

Figure 3. Protein level changes of TGF-β1, SGK1, FN, and α-SMA in control and model groups. **A,** Western blot analyses of TGF-β1 and SGK1. **B,** Relative protein levels of TGF-β1 and SGK1. **C,** Western blot analyses of FN and α-SMA. **D,** Relative protein levels of TGF-β1 and SGK1.

group. In contrast, dilated kidney tubules, epithelial cell swelling, vacuolar degeneration, irregular thickening of basement membrane, inflammatory cell infiltration and accumulation of PAS-positive staining in the interstitium were observed in rats of model group (Figure 2a, 2b, 2e, and 2f). Meanwhile, PASM staining indicated that Kimmelstiel-Wilson (KW) nodules were observed in

a concentric arrangement in rats of model group (Figure 2g and 2h). Masson staining revealed that renal tubule lumen was significantly dilated and a large amount of glomerular and renal interstitial regions were stained blue-purple in model group. Furthermore, no collagen deposition was found in the glomerular basement membrane and mesangial area of control group (Figure 2c and 2d).

The protein Level Changes of TGF-β1, SGK1, FN, and α-SMA

The protein levels of TGF-β1, SGK1, FN, and α-SMA in kidney tissues of control rats were significantly lower than those of model group (Figure 3).

The mRNA level Changes of TGF-β1, SGK1, FN, and α-SMA

Identically, the mRNA levels of TGF-β1, SGK1, FN, and α-SMA in kidney tissues of T2DM rats were markedly higher than those of control rats (Figure 4).

Discussion

With the deepening of DN research, the role of renal interstitial fibrosis in the progression of DN has been widely concerned. As a vital link in the process of TIF, the trans-differentiation of renal tubular epithelial cells is the basis for a comprehensively elucidating DN pathogenesis. Some studies[22,23] have shown that both EMT and TIF are the results of mutual restriction and antagonism between fibrotic and anti-fibrotic factors.

TGF-β1 is a key regulator of DN induction secreted by various cells. It participates in the mediation of various biological functions[24]. Elevated TGF-β1 expression and activity can also be observed in DM pa-

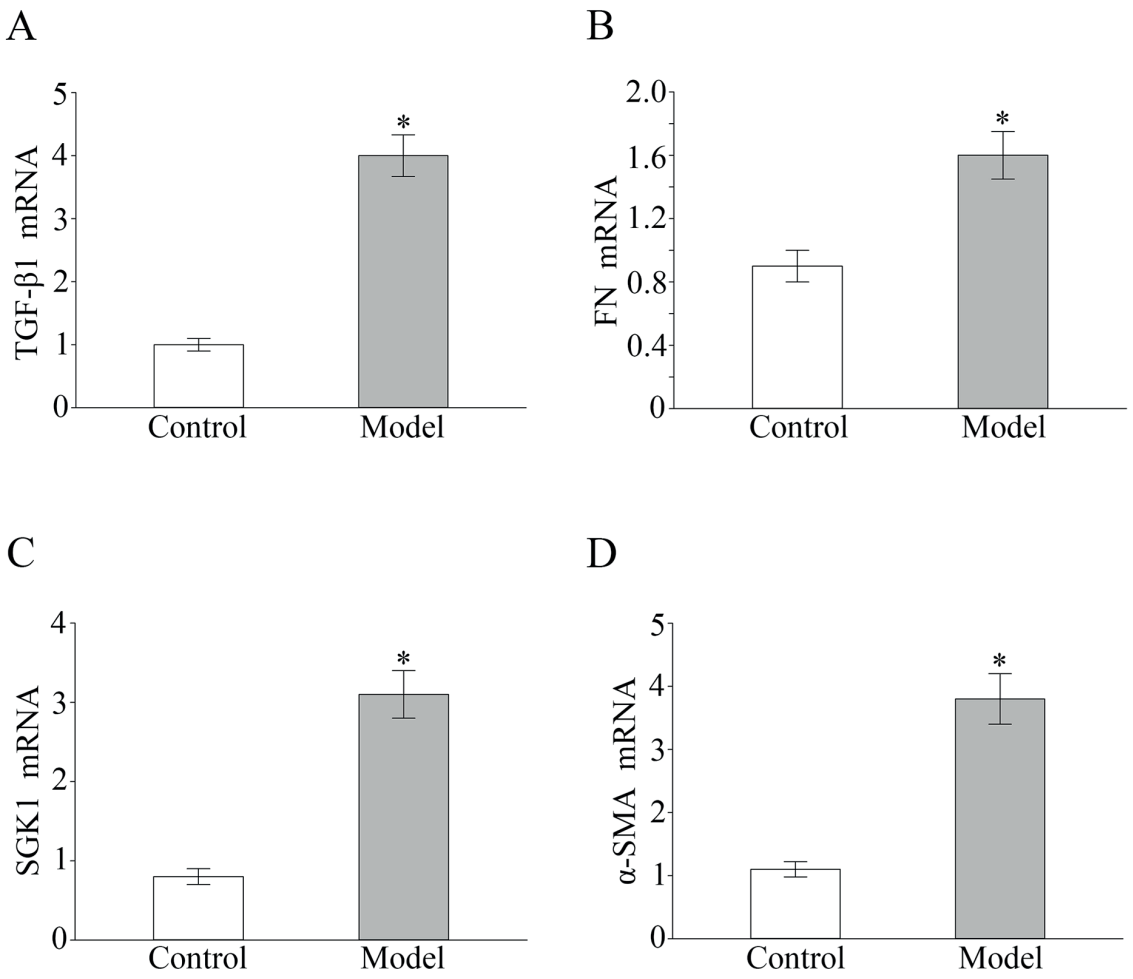

Figure 4. mRNA level changes of TGF-β1 (***A***), FN (***B***), SGK1 (***C***) and α-SMA (***D***) in control and model groups.

tients and animal models[25,26]. After blockage TGF-β1 by application of TGF-β1 neutralizing antibody, the expressions of FN and Col-IV in kidney tissues of diabetic mice are markedly down-regulated. Meanwhile, glomerular hypertrophy, sclerosis, and renal dysfunction are alleviated as well[26,27]. It has been demonstrated that multiple pathways are involved in TGF-β1-regulated DN progression, including Smads, P38MAPK, Notch, and Wnt pathways. In addition, TGF-β1 stimulation down-regulates TRPC6 expression and further aggravates glomerular sclerosis[28]. SGK1 is an early-stage immediate gene, which is sensitive to a variety of external stimuli. Meanwhile, it is regulated by many factors and participates in various physiological functions. SGK1 is related to fibrotic diseases, exerting a greater abundance in relevant fibrotic tissues than normal ones[29,30].

FN is mainly produced by mesangial cells in the glomerulus, presenting the function of binding and depositing various components of ECM. The abnormal up-regulation of FN indicates an excessive accumulation of ECM, which is one of the main manifestations of TIF in DN. As a major component of the basement membrane, FN interacts with type IV collagen to mediate cell attachment, migration, and organizational ordering[31,32]. α-SMA is a cytoskeletal protein unique to vascular smooth muscle. As a characteristic hallmark of myofibroblasts, α-SMA participates in TIF, eventually affecting the occurrence and progression of DN. A relative study has pointed out that the TGF-β1/Smads pathway aggravates renal function by up-regulating α-SMA and further leads to TIF[33]. In this paper, up-regulated TGF-β1 and SGK1 were observed in T2DM rats when compared with control rats. Besides, significantly up-regulated FN and α-SMA in kidney tissues of T2DM rats indicated the occurrence of EMT and tissue fibrosis. In the stimulation of high-sucrose, TGF-β1 is the key factor of fibrosis, while SGK1 is a downstream target of TGF-β1. Under the activation of the TGF-β1/SGK1 pathway, TIF is induced and developed.

To sum up, this study demonstrated the importance of the TGF-β1/SGK1 pathway in the occurrence and progression of TIF. In addition, our findings provided experimental references for the development of novel treatment strategies of DN.

Conclusions

We found that the TGF-β1/SGK1 pathway is closely related to tubulointerstitial fibrosis in T2DM rats.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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