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Original Article

Rapamycin blocks epithelial-mesenchymal transition of rat podocytes induced by high-glucose culturing

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Abstract: The aim of the current study was to investigate the effects of rapamycin on a murine epithelial-mesenchymal transition (EMT) model based on rat glomerular podocytes, providing the mechanisms of podocyte injuries in diabetic nephropathy. Primary rat glomerular podocytes were cultured *in vitro* and identified by cellular immunofluorescence. Differentiated podocytes were treated with glucose (30 mmol/L) after complete synchronization and differentiation, aiming to establish the EMT model. EMT cells were treated with rapamycin (200 ng/mL). Morphological alterations of podocytes were determined via inverted microscopy. Expression levels of α-SMA and E-cadherin were gauged by immunofluorescence and Western blotting. Present results suggest that high-glucose conditions could result in podocyte foot process effacement and cellular enlargement. Results of immunofluorescence and Western blotting demonstrated enhanced expression of α-SMA and decreased expression of E-cadherin. Compared with the high-glucose group, glucose challenges administered to podocytes in the rapamycin group partially inhibited expression of α-SMA induced by high glucose levels and antagonized expression of E-cadherin. Present results confirm that EMT was induced by high-glucose administration in podocyte injuries and was partially blocked by rapamycin.

Keywords: High sugar, podocyte, rapamycin, EMT

Introduction

Diabetic nephropathy (DN) is a common microvascular complication of diabetes, featured by progressive proteinuria and a gradual decline in the glomerular filtration rate, one of the leading causes for end-stage renal failure [1, 2]. Podocytes are an essential component of the glomerular filtration barrier, the severity of which could predict the occurrence and development of proteinuria. Thus, it may be a predicting factor for DN at its initial stage [3, 4]. In the process of DN, various pathogenic factors induce morphological and functional changes of podocytes, including foot process fusion, reduction in cell number and density, apoptosis, hypertrophy, and epithelial-mesenchymal transition (EMT) of podocytes [5]. As the primary manifestation of podocyte injuries, the process of EMT acts not only as an initiating factor for the induction of renal fibrosis but also as a target of various therapeutic approaches that aim to delay the progression of DN [6]. As the process of EMT occurs in the cells, markers of its epithelial phenotype (nephrin, podocin, synaptopodin) are lost, while markers of mesenchymal phenotype are expressed, including fibroblast-specific protein (FSP), integrins/integrin-linked kinase (ILK), fibronectin (FN), and matrix metalloproteinase (MMP)-9 [7]. In the last decade, numerous studies have demonstrated that EMT, which involves the subsequent migration from the phenotype of podocytes to tubular epithelial cells, is an essential cause of proteinuria and renal fibrosis [8, 9]. EMT in the disease is defined as the feature where interstitial myofibroblasts obtained by renal tubular epithelial cells gradually lose their characteristics of the original epithelium in the presence of various injury-related factors. It serves as one of the critical mechanisms underlying the development of renal interstitial fibrosis [10, 11]. The earliest changes that occur in EMT are the reduction or disappearance of

E-cadherin expression, destruction of tight junctions between cells, rearrangement of the cytoskeleton in the cytoplasm, and expression of new-coming cellular molecules, such as α-smooth muscle actin (α-SMA). These provide the morphological basis for occurrence of EMT [12].

Rapamycin (RAPA) is a specific inhibitor of mTORC1 (mammalian target of rapamycin complex 1). In animal experiments, rapamycin has been found to delay diabetes onset by protecting podocytes, further inhibiting renal interstitial inflammation and fibrosis. Kidney disease, membranous nephropathy, and other forms of chronic kidney disease, as well as a loss of kidney function, promote cell apoptosis in the cell-wall lining. These delay the formation and enlargement of cysts in polycystic kidneys [13, 14]. Rapamycin can partially inhibit occurrence of EMT in peritoneal mesothelial cells [15].

The present study established a glomerular podocyte-epithelial-stromal cell EMT model by treating differentiated primary podocytes with high levels of glucose. Via the blockade of mTORC1 with rapamycin, results revealed that rapamycin can inhibit α-SMA expression in high-glucose-induced rat podocytes and reduce inhibition of E-cadherin expression caused by high levels of glucose. Rapamycin appears to inhibit occurrence of EMT in podocytes. Therefore, the current study provides a new theoretical basis for the pathogenesis of diabetic nephropathy, offering new methods for treatment.

Materials and methods

Primary culture and identification of rat glomerular podocytes

According to methods described in detail in previous studies [16, 17] 3 Sprague-Dawley rats were sacrificed by cervical dislocation (Shanghai Slack Animal Center, Shanghai, China). The kidneys were extracted under sterile conditions. The renal cortex was dissected and shredded, then gently grounded. Completed phosphate-buffered saline (CPBS) was used to rinse each layer with screen, varied within 0, 150, and 200 mesh (Sangon Biotech Co., Shanghai, China). Next, these renal tissues were collected for centrifugation for 5 minutes at 1,500 rotations/minute. Afterward, 2 mL of complete medium (2 rats) was resuspended and subsequently inoculated into three separated culture flasks. Next, 10 mL of complete medium was added to each flask. The culture flasks were inverted so that the inoculum was placed in a humidified incubator at 37°C supplied with 5% CO_2, where the culture flasks were incubated for 4-5 hours. After 7-8 days of glomerular inoculation, the cells were harvested once the cells climbed up to the bottom of the bottle after passage for the first generation. Cellular morphology was visualized using an inverted-phase contrast microscope. The cells then underwent fixation via cold acetone and blocked via bovine serum albumin (BSA). A primary antibody against synaptopodin was added (Abcam plc, Cambridge, UK). Afterward, overnight at 4°C, a secondary antibody was added at 37°C in the dark for 30 minutes. Propidium iodide (PI) was used to counterstain the nucleus. The culture was observed and photographed under a fluorescence microscope.

Cell administration

Various agents, including complete medium (5% fetal bovine serum [FBS], 1% insulin-transferrin-selenium [ITS], and 1% streptomycin, were applied to the cells supplied with DMEM/F12 medium. They were then they were incubated for 7 days until the podocytes were fully differentiated and an intercellular junction was formed. Some of the cells were then cultured for 24 hours in serum-free medium to synchronize the cells. The cells were grouped based on different environmental conditions, named as the high glucose group (HG) and high glucose combined with rapamycin group (HG+RAPA). Cell apoptosis was induced using a high concentration of glucose at 30 mmol/L. The EMT cell model was treated with rapamycin (200 ng/mL). The other podocytes were treated with 5 mmol/L of glucose and 30 mmol/L of mannitol, serving as negative controls. Treated cells were replaced with different media for 48 hours.

Immunofluorescence staining

Immunofluorescence staining of rat podocyte sections was performed, as previously described. The sections were incubated with the primary antibody against E-cadherin (1:200; Abcam plc) and α-SMA (1:300; Abcam plc) at 4°C overnight. This was followed by incubation with the fluorescent-labeled secondary antibody. The sections were examined using a

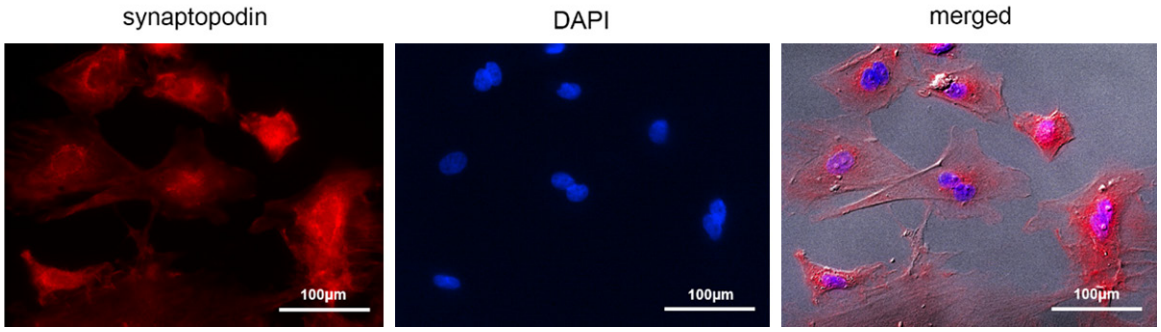

Figure 1. Identification of rat podocytes by immunofluorescence assay. The cell nucleus was stained by DAPI. Expression of synaptopodin was located in the cytoplasm and in the podocyte membrane. A merged staining image is also presented.

Leica confocal microscope (LEICA TCS SP5; Leica Microsystems, Wetzlar, Germany).

Western blot assay

Whole-cell proteins were extracted using mammalian cell total protein lysis buffer (Sangon Biotech Co., Shanghai, China) and quantified via BSA assay (Beyotime, Shanghai, China). The protein samples were then electrophoresed in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane (PVDF; EMD Millipore, Billerica, MA, USA). The membrane was probed with E-cadherin- and α-SMA-specific antibodies. Subsequently, enhanced chemiluminescence detection kits (EMD Millipore) were used for detection and visualization, according to manufacturer instructions. A Bio-Rad scanning system was used to detect immunoreactive protein bands (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

All experiments were performed in triplicate. Statistical analyses were carried out using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Analysis of variance (ANOVA) was performed for each group, as well as RT-PCR and Western blotting. Data are presented as the mean ± standard deviation (SD). *P*-values <0.05 indicate statistical significance.

Results

Primary culture and identification of glomerular podocytes

The glomerular inoculation culture was first digested and cultured for 7 days. It revealed that the podocytes were star-shaped and that the cytoplasm and nucleus could proliferate after passage. The maximum diameter reached up to 500 μm and the branches were prominent from the cell body. The cells were connected to each other. After 7 days of cell culturing, they were completely differentiated. Immunofluorescence results showed that synaptopodin was expressed in the podocyte cytoplasm and cell membrane (**Figure 1**).

Aiming to establish a more accurate EMT cell model, podocyte EMT was induced by administering high levels of glucose at different time points, observing changes in cell morphology. It was found that podocyte retraction occurred at 0 hours (**Figure 2A**), 12 hours (**Figure 2B**), 24 hours (**Figure 2C**), and 48 hours (**Figure 2D**) after podocyte hyperglycemia induction. Furthermore, some of the foot processes disappeared and lost their intercellular connections. This increased with the prolongation of culture time, being most obvious after 48 hours (**Figure 2D**).

Effects of rapamycin on expression of E-cadherin and α-SMA protein in EMT

To investigate the mechanisms by which rapamycin blocks EMT induced by high glucose in rat podocytes, immunofluorescence staining was performed for the rat podocyte sections. This was to determine expression levels of E-cadherin and α-SMA in the high-glucose-induced rat podocyte group (HG) and high glucose combined with rapamycin group (HG+RAPA). Results demonstrated that expression of E-cadherin was predominantly detected in the cytoplasm and membrane, while α-SMA

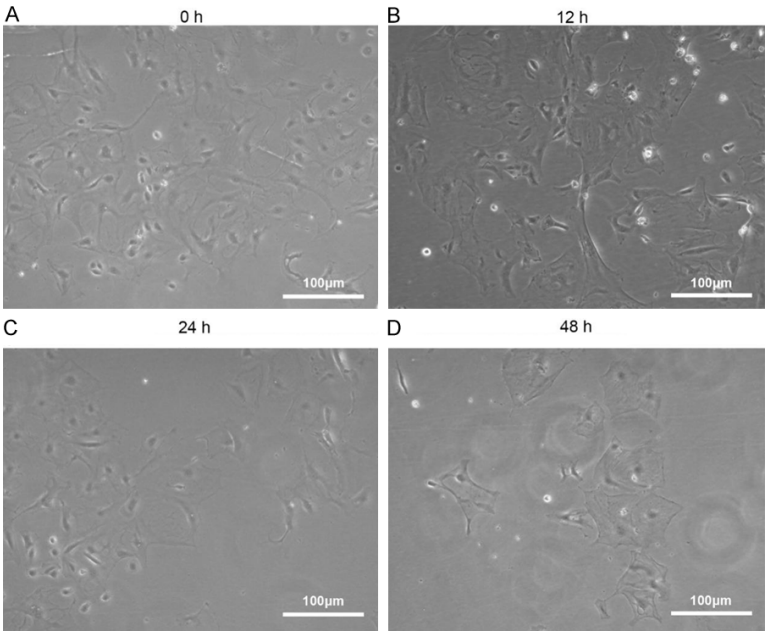

Figure 2. High levels of glucose were used to induce rat primary podocytes to establish an EMT cell model. Cellular morphological changes induced by high levels of glucose at 0 hours (A), 12 hours (B), 24 hours (C), and 48 hours (D).

was expressed in the nucleus (**Figure 3**). Expression of α-SMA increased and expression of E-cadherin decreased. Both processes were induced with the administration of high levels of glucose in the podocytes. Compared with the HG group, rapamycin inhibited α-SMA expression and attenuated inhibition of E-cadherin expression (**Figure 3**). Western blot results revealed that administration of high levels of glucose significantly upregulated protein expression levels of α-SMA and downregulated levels of E-cadherin (**Figure 4A**, **4B**). Additionally, protein expression levels of α-SMA notably decreased, while those of E-cadherin increased in the HG+RAPA group, compared to the HG group (**Figure 4A**, **4C**). Present results suggest that rapamycin can inhibit α-SMA expression in high-glucose-induced rat podocytes. Moreover, it can attenuate the inhibition of E-cadherin expression that occurs in response to high levels of glucose.

Discussion

DN is a common disease that can lead to end-stage renal failure. It is believed that long-term hyperglycemia, caused by insulin metabolism disorders, is the initiating factor leading to DN. The activation of numerous growth factors and cytokines is the basic mechanism underlying pathogenesis. These growth factors and cytokines include transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), connective tissue growth factor, and so forth [18]. Cytokines can activate the mTOR signaling pathway through PI3K/Akt and other pathways. Continuous activation of mTOR can cause hypertrophy in glomerular and tubular epithelial cells. Thus, the kidneys appear to be enlarged in the early stages of DN. The further activation of mTOR can lead to the dedifferentiation or shedding of podocytes, as well as the acceleration of glomerular matrix protein synthesis. These factors may lead to thickening of the glomerular basement membrane. Furthermore, they can induce excessive deposition of the mesangial matrix, resulting in the secretion of proinflammatory factors and fibrotic factors, leading to renal interstitial fibrosis [13]. Studies have shown that TGF-β1 induces podocyte apoptosis through Erk-mediated activation of the mTORC1/Nox4 axis [19]. In addition, in the presence of high glucose, Erk is activated in podocytes. This results in increased expression of VEGF. Previous studies have suggested that the Erk pathway may be involved in mTOR activation induced by high glucose in podocytes [20].

The glomerular filtration barrier is constituted by capillary endothelial cells, the glomerular basement membrane, and podocytes. Damage and loss of podocytes play important roles in the occurrence of diabetic nephropathy. The current study successfully established the primary culture of rat podocytes. This study used synaptopodin, which served as the cytoskeleton-related protein in podocytes, to identify whether these podocytes were differentiated and mature. Synaptopodin is a linear protein coupled with actin filaments, which has rich components of proline and a relative molecular mass of 73,700. It is expressed only in glomerular podocytes and in the posterior synapse in

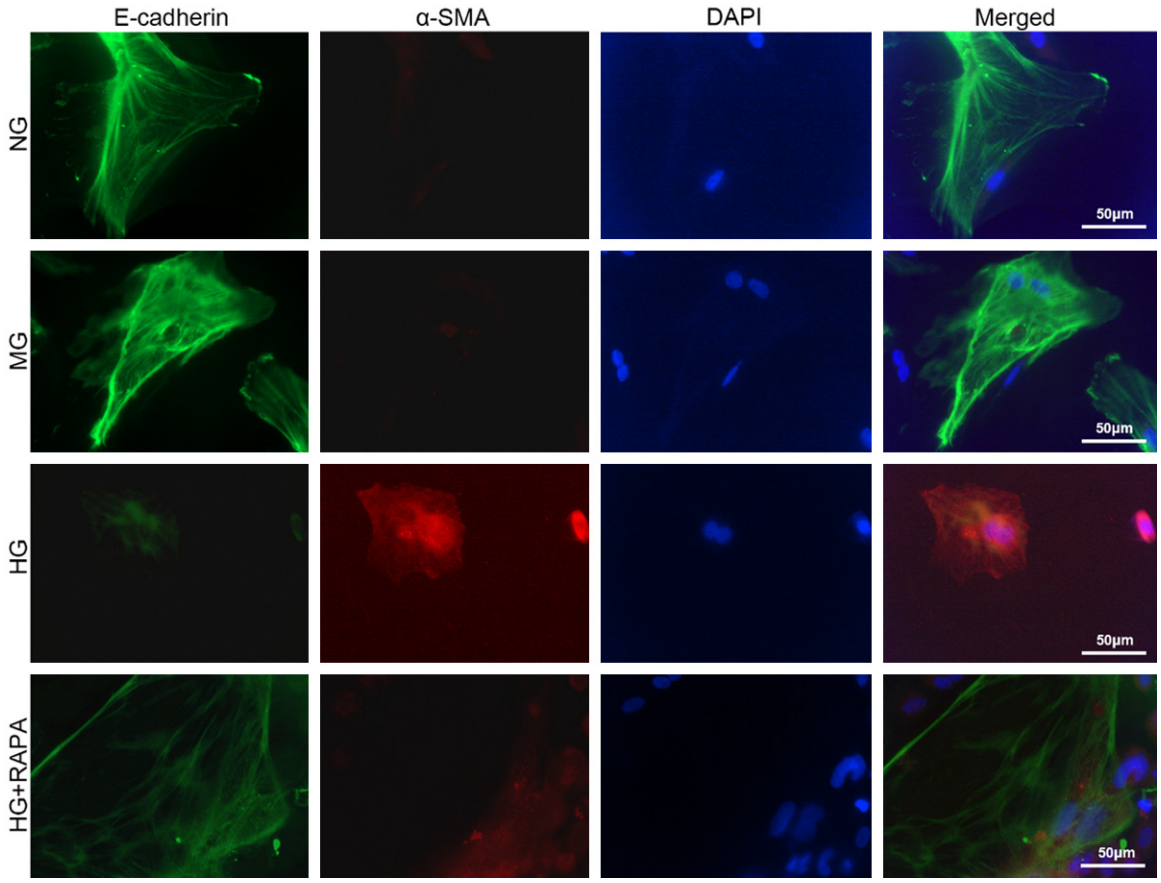

Figure 3. Immunofluorescence staining analysis of E-cadherin-positive cells/sections and α-SMA in the NG and HG+RAPA, respectively. Normal glucose-induced rat podocyte group (NG) and high mannitol-induced rat podocyte group (MG) served as the negative controls. The cell nucleus was stained with DAPI. A merged staining image is also shown.

the body [21]. Present results confirm that synaptopodin is expressed on the nuclear membrane and on the membrane of podocytes. It is also expressed in the cytoplasm, playing a role in stabilizing the structure of the podocyte cytoskeleton. Numerous studies have shown that podocyte damage occurs before podocyte loss. EMT can occur in podocyte injuries and is characterized by the gradual disappearance of epithelial properties, such as the tight junctional destruction between cells. It also results in the loss of cell polarity and the rearrangement of the intracellular skeleton and foot process. These factors are associated with the presence of various mesenchymal cell markers. Once EMT occurs, expression levels of substances such as actin and α-SMA are enhanced.

E-cadherin is an intercellular adhesion protein that maintains the structural integrity of epithelial cells. Decreased expression levels are a key marker of EMT, accompanied by increased expression levels of mesenchymal marker molecules, such as vimentin and N-cadherin [22]. Aiming to study the inhibitory effects of rapamycin on EMT in the pathogenesis of diabetic nephropathy, high levels of glucose were used to induce EMT in primary podocytes. Results showed that the podocytes appeared to exhibit a morphological change after being treated with high glucose for 12 hours. This change showed that the shortening of the foot process disappeared and that the cell volume was compensated first. Next, with the prolongation of high levels of sugar being administered over time, the number of podocytes decreased at 48 hours. This was accompanied by a decrease in the cell-body area decreased and disappearance of the foot process.

Fibroblasts activated by EMT are a significant source of renal fibroblasts. The latter play an

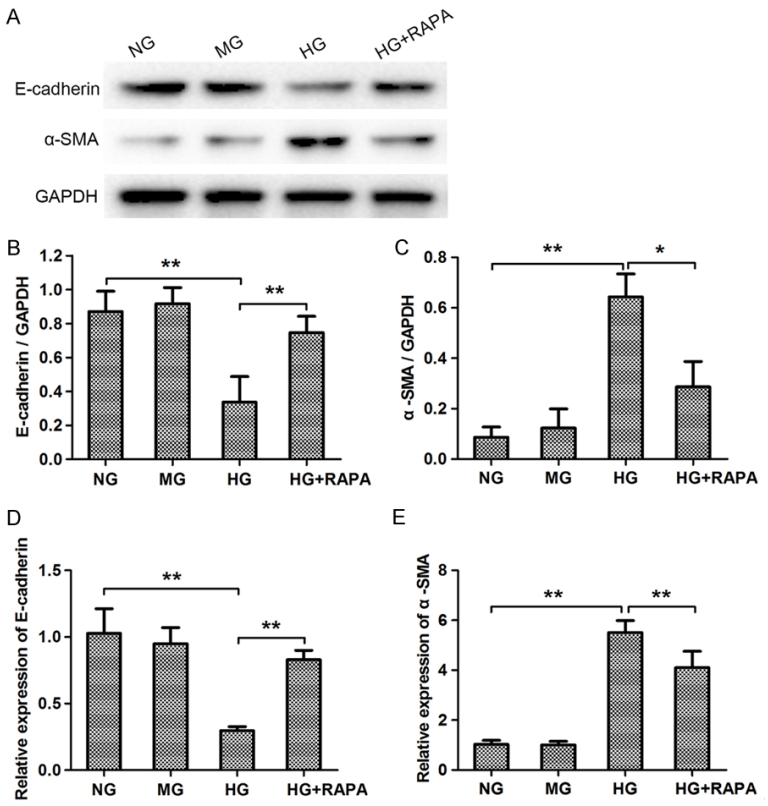

Figure 4. High levels of glucose induced expression of α-SMA and decreased expression of E-cadherin in podocytes. Rapamycin upregulated expression of E-cadherin and downregulated expression of α-SMA under high-glucose conditions in podocytes. (A) Western blot analyses were performed to evaluate expression of E-cadherin and α-SMA in the NG, MG, HG, and HG+RAPA groups, respectively; (B) Quantitative analysis of E-cadherin protein levels; (C) Quantitative analysis of α-SMA protein levels; mRNA levels of E-cadherin (D) and α-SMA (E) in the NG, MG, HG, and HG+RAPA groups detected by RT-PCR, respectively. **P*<0.05, ***P*<0.01.

important role in renal fibrosis development. Moreover, α-SMA is a well-recognized marker protein that suggests trans-differentiation of epithelial cells. Under physiological conditions, podocytes express only a small amount of α-SMA, which is in the nucleus. Expression of α-SMA in the podocyte cytoplasm and membrane can be a valuable indicator of EMT in podocytes. Previous studies have found that podocytes demonstrate decreases in nephrin levels in high-glucose environments. The present study further confirmed that high levels of glucose can induce expression of α-SMA in podocytes and decrease expression of E-cadherin, suggesting that high levels of glucose may pass through induced feet. In this way, cells undergo EMT, which subsequently affects the structure and function of podocytes. Thus, it may be involved in the development of DN proteinuria. Present results are consistent with the findings of Dai et al. They reported that phenotypic changes in podocytes were observed in high-glucose environments [23, 24]. The final outcome of these pathological changes is the development of glomerular sclerosis and renal interstitial fibrosis.

The reversal of podocyte EMT can delay or attenuate progression of DN. Thus, it is important to explore the mechanisms of podocyte EMT, further blocking this process. Rapamycin, also known as sirolimus, is a novel macrolide antibiotic isolated from soil bacteria. It does not inhibit any other protein kinases except for mTOR. Once rapamycin enters the cell, it binds to FKBP-12 to form a complex that inhibits mTOR activity. Moreover, mTOR mainly exerts its biological effects through the combination of the mTOR complex (mTORC)1 and mTORC2, with its corresponding downstream factors. Furthermore, mTORC1 includes Raptor, PRAS40, Deptor, and mLST8, while mTORC2 includes Rictor, Deptor, mSIN1, and mLST8. Of these, Raptor and Rictor are essential subunits of mTORC1 and mTORC2, respectively. Thus, mTORC1 mainly stimulates cell growth and proliferation, while mTORC2 mainly regulates cell polarity and cytoskeletal proteins and actin [25]. As a classic mTOR blocker, rapamycin has been studied in various diseases, such as membranous nephropathy, immunoglobulin (Ig) A nephropathy, focal segmental glomerulosclerosis, crescentic nephritis, and renal interstitial fibrosis. Several studies have shown that rapamycin has no effects on mTORC2 activity. Rather, rapamycin only inhibits mTORC1 specifically. Rapamycin exerts immunosuppressive, anti-inflammatory, anti-tumor, and anti-aging effects, thereby delaying the progression of kidney disease. In rat models of DN, rapamycin

reduces glomerular hypertrophy, inhibits extracellular matrix aggregation, delays DN progression, and downregulates kidney-associated inflammatory cytokines and chemokines by inhibiting lymphocyte proliferation and macrophage aggregation. These cytokines and chemokines include IL-8 and MCP1. They can alleviate the inflammatory response associated with DN. They can also reduce or delay the development of mitochondrial sclerosis, kidney disease, and DN by inhibiting the proliferation of myofibroblasts and promoting TGF-β fibrosis [26]. In addition, rapamycin can reduce oxidative damage and delay the aging of mesangial cells following high-glucose administration [27].

In the current study, the mTORC1 pathway was blocked using rapamycin. Under high-glucose conditions, expression of E-cadherin was up-regulated in podocytes, while α-SMA expression was down-regulated. This partially reduced EMT in podocytes. Present findings suggest that mTORC1 may be involved in the regulation of high glucose levels. Induction of podocyte EMT, therefore, offers a novel intervention for the future prevention and treatment of clinical DN.

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Disclosure of conflict of interest

None.

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