

p66Shc regulates podocyte autophagy in high glucose environment through the Notch-PTEN-PI3K/Akt/mTOR pathway

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Summary. Background and aims. Autophagy has been found to be involved in podocyte injury, which is a key factor in the progression of diabetic kidney disease (DKD). p66Shc is an important protein adaptor that regulates production of reactive oxygen species (ROS) and induction of apoptosis, and is a novel biomarker for oxidative damage of renal tubules. Our preliminary studies showed that p66Shc expression in podocytes of DKD patients is increased, while autophagic flux and podocyte number is decreased in DKD patients. The mechanism by which p66Shc may regulate podocyte autophagy and injury remains unknown. The present study aimed to investigate the molecular function of p66Shc under high glucose condition and its possible therapeutic utility in DKD.

Methods. We histologically evaluated kidney injury in a streptozocin (STZ)-induced mouse model of diabetes using HE, PAS, PASM, and Masson staining and assessed glomerular structure by transmission electron microscopy. The apoptosis rate of high glucose-treated podocytes was assessed by TUNEL and Annexin V/PI staining. Markers of podocyte autophagy were measured by immunofluorescence and western blotting. DHE/ET fluorescence quantification was used for ROS detection and quantification.

Results. Urine creatinine, serum creatinine, urinary microalbumin, and p66Shc expression were significantly increased in STZ-induced diabetic mice. Cultured MPC5

podocytes subjected to high glucose showed reduced viability, and p66Shc overexpression further accelerated apoptosis. p66Shc knockdown enhanced HG-induced autophagy, while p66Shc overexpression reduced the expression of PTEN and increased the expression of mTOR and phospho-mTOR. LC3 protein expression was higher in cells with p66Shc knockdown, indicating that activation of p66Shc inhibits podocyte autophagy. DAPT, an inhibitor of the Notch pathway, downregulated the expression of p66Shc.

Conclusion. These findings indicate that p66Shc inhibits podocyte autophagy and induces apoptosis through the Notch -PTEN-PI3K/Akt/ mTOR signaling pathway in high glucose environment, providing novel evidence for its potential role in DKD treatment.

Key words: Notch-PTEN-PI3K/AKT/mTOR pathway, p66Shc, Apoptosis, Podocyte, Autophagy

Introduction

Diabetic kidney disease (DKD) is a serious microvascular complication of diabetes mellitus and a major contributor to the prevalence of end stage renal disease (ESRD) in Western countries. Approximately 40% of patients with type 1 diabetes and 25% of patients with type 2 diabetes will progress to DKD within ten years of diagnosis (Adler et al., 2003). In addition, approximately 45% of patients undergoing dialysis have diabetes. According to the WHO, although diabetics in China account for one-third of the global patient population, the incidence of diabetes continues to

increase in China. It is therefore urgent to better understand the mechanisms of DKD, which has broad implications for global human health.

Podocytes are highly differentiated glomerular epithelial cells that comprise the outer aspect of the glomerular basement membrane (GBM) and are located on the outside of glomerular capillaries to form the kidney filtration barrier. Loss or injury of podocytes can lead to proteinuria, and can cause progression to chronic kidney failure. Since podocyte injury occurs in many different diseases, including hyperglycemia, hypertension-induced kidney disease, and in the puromycin aminonucleoside (PAN) nephropathy model (Kang et al., 2014; Becherucci and Romagnani, 2015; Kakimoto et al., 2015; Kato et al., 2015), understanding mechanisms of podocyte injury can provide further insights into the pathogenesis of proteinuria and glomerular diseases.

Autophagy is an evolutionarily conserved cellular pathway that maintains podocyte homeostasis by degrading damaged organelles and aggregated proteins via the lysosome, and regulates many critical aspects of kidney disease (Takabatake et al., 2014). Autophagy dysfunction is an important factor underlying podocyte injury, which may lead to progression of DKD (Mizushima et al., 2008; Li et al., 2015). In recent years, the mechanism by which podocyte autophagy interferes with development of diabetic nephropathy has been reported. Accordingly, beta-arrestins enhance podocyte injury by inhibiting autophagy, leading to development of DKD (Liu et al., 2016), while rapamycin activates podocyte autophagy and protects podocytes from injury in diabetic mice (Xiao et al., 2014). Autophagy protects against podocyte injury by regulating the production of VEGF (Wei et al., 2016), and ginsenoside Rg1 can alleviate angiotensin II-induced autophagy in podocytes through AMPK/mTOR/PI3K signaling (Mao et al., 2016). HDAC4 inhibits autophagy and stimulates podocyte injury in diabetic nephropathy (Wei and Dong, 2014), and podocyte apoptosis provoked by inhibition of autophagy may be related to activation of endoplasmic reticulum stress (Fang et al., 2014). Our previous study found that the number of podocytes in DKD patients is significantly reduced along with a reduction in the number of autophagosomes, suggesting that autophagy plays an important role in mediating DKD podocyte injury.

p66Shc is regarded as a new biomarker of renal tubular oxidative injury, and its expression is clearly increased in peripheral blood monocytes from DKD patients (Xu et al., 2016). Moreover, p66Shc mediates cell apoptosis, cell survival, and the production of reactive oxygen species (ROS) (Giorgio et al., 2005). In renal proximal tubular epithelial cells, nicotine-mediated oxidative stress via p66Shc-dependent induction of ROS and inactivation of FOXO caused cell damage, to a similar degree as knockdown of MnSOD (Arany et al., 2016). In STZ-induced p66Shc knockout mice, the production of ROS, glomerular injury, and podocyte

apoptosis was reduced, indicating that deletion of p66Shc protects against oxidative stress induced by high glucose (Menini et al., 2006, 2007). Although p66Shc appears to mediate glomerular damage induced by hyperglycemia, whether it participates in the pathogenesis of DKD by impairing podocyte autophagy remains unknown.

Notch signaling is an evolutionarily conserved pathway that regulates cell fate and differentiation during development (Artavanis-Tsakona et al., 1999). Previous studies have shown an interplay between p66Shc and Notch3 in regulating self-renewal and cell survival of breast stem cells under hypoxic conditions through induction of the Notch ligand Jagged-1 (Sansone et al., 2006). It has been proved that blocking Notch signaling pathway by γ -secretase inhibitors provokes amelioration of diabetic glomerulopathy by preventing podocyte dedifferentiation and loss and tubulointerstitial fibrosis (Bonegio and Susztak, 2012). In mesenchymal stem cells, the Notch inhibitor DAPT inactivates PTEN-PI3K/AKT/mTOR to induce early autophagy and promotes bone marrow mesenchymal stem cell differentiation, while the autophagy inhibitors CQ and 3-MA block this effect (Song et al., 2015).

We propose that p66Shc regulates podocyte injury by sensing high levels of ROS induced by elevated glucose, and activating the Notch-PTEN-PI3K/AKT/mTOR signaling pathway to inhibit podocyte autophagy. This hypothesis predicts that regulating p66Shc expression can improve podocyte autophagy flux and protect against podocyte injury in high glucose environment.

Materials and methods

Cell culture and transfections

MPC5 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in 5% CO₂. pEGFP-p66Shc, p66Shc siRNA, and a negative control were transfected into cells using Lipofectamine 2000 reagent according to the manufacturer's instructions.

Detection of Reactive Oxygen Species

DCFH-DA probe kit (Beyotime, China) diluted in serum-free media was used to detect ROS production. Cells were treated with high glucose and differing concentrations of vitamin C, then washed with phosphate-buffered saline (PBS) and loaded with DCFH-DA (1:1000) for 25 min at 37°C. Cells were then collected by centrifugation and cultured in serum-free media. Fluorescence intensity was quantified by flow cytometry.

MDC staining assay

Cells were fixed with 4% paraformaldehyde for 15

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min, then washed with PBS and incubated with monodansylcadaverine (MDC) staining solution (40 μ M) at 4°C for 30 min in the dark. Cells were then washed with PBS and immediately examined under an inverted microscope (Olympus).

Annexin V/PI staining assay

The number of apoptotic cells in each treatment group was determined using an Annexin V/PI apoptosis detection kit (MultiSciences Biotech, Hangzhou, PRC). Briefly, the cell pellet was resuspended in 500 μ l 1 \times binding buffer followed by incubation with 5 ml of Annexin V (conjugated with FITC) and 10 ml of PI for 5 min, protected from light. Cell fluorescence was then analyzed using a flow cytometer (BD AccuriC6, Becton, Dickinson and Company).

Streptozotocin-induced diabetic kidney disease model and treatment with vitamin C and DAPT

15 male C57BL/6 mice, weighing about 23–25 g, were housed in a ventilated animal room at 20–25°C with a 12 h light/dark cycle and maintained on rodent diet and water ad libitum. After a 12-hour overnight fast, mice were subjected to a single intraperitoneal injection of 150 mg/kg streptozotocin (Sigma Chemical). Three days after injection, blood glucose was measured weekly from tails. Mice with blood glucose >16.7 mmol/L eight weeks later were defined as demonstrating successful induction of diabetes. A total of nine mice with diabetes were randomly divided into three groups. Mice in each group received intraperitoneal injections with 100 ml/kg/day normal saline (n=3), vitamin C (n=3), or DAPT (n=3) every other day for eight weeks, respectively. Nine mice injected with normal saline rather than streptozotocin were used as controls. Urine samples were collected from all mice for measurement of protein and creatinine concentrations using commercially available assay kits. After eight weeks, blood was collected from each group of mice, then all mice were killed and kidney tissues were harvested.

Histology and immunohistochemistry

Kidneys were fixed in 4% PBS-buffered formaldehyde, embedded in paraffin, and then sliced longitudinally into 5-mm thick sections. Kidney sections were stained using HE, PAS, PASM, and Masson staining, then observed and photographed using an optical microscope (BX43, Olympus, Japan).

Immunofluorescence staining

After antigen retrieval and blocking, kidney sections were incubated at room temperature with anti-p66Shc (1:150, Abcam) or anti-synaptopodin (1:100, Acris),

followed by incubation with secondary antibodies conjugated with Alexa Fluor 488 or 555 (Life Technologies). Sections were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) and then examined by fluorescence microscopy.

Assessment of glomerular structure by transmission electron microscopy

Kidney tissues were minced into 1 mm³ pieces and processed for electron microscopy

Western blotting analysis

Kidney or cell protein lysates were electrophoresed and transferred to membranes, and the membranes were incubated overnight at 4°C with anti-p66Shc (Abcam), anti-PTEN (CST), anti-mTOR (CST), anti-LC3 (CST), and anti-phospho-mTOR (CST) antibodies, as well as mouse anti- β -actin (Santa Cruz). The membranes were washed extensively, and then incubated with HRP-conjugated secondary antibodies for 1 h. Western ECL substrate (Beyotime, China) was used to visualize the proteins.

Statistical analysis

All results are expressed as the mean \pm standard deviation (S.D.). Differences between groups were determined using Wilcoxon test. All statistical analyses were performed using GraphPad Prism 5. Results were considered significant if P values were <0.05.

Results

High glucose causes kidney injury both in vivo and in vitro

As shown in Fig. 1A, the body weight of the DKD group (DKD) sharply decreased compared with the control group (Control), while the blood glucose of the DKD group showed a significant increase. Renal indexes such as urine creatinine, serum creatinine, and urinary microalbumin increased in the DKD group compared to the Control, of which urine creatinine showed the largest change in magnitude. Further, PAS, PASM, and Masson staining revealed substantial histological changes between the DKD group and Control. There was obvious mesangial expansion associated with a variable increase in glomerular interstitial fibrosis in the DKD group compared to Control (Fig. 1B). The expression of synaptopodin, a podocyte marker, was significantly decreased in the DKD group compared to Control (Fig. 1C). We performed transmission electron microscopy to visualize the thickness of the GBM and podocyte structure, and observed thickening of the GBM and fusion of podocyte foot processes in the DKD group (Fig. 1D). Altogether, these data indicate that high

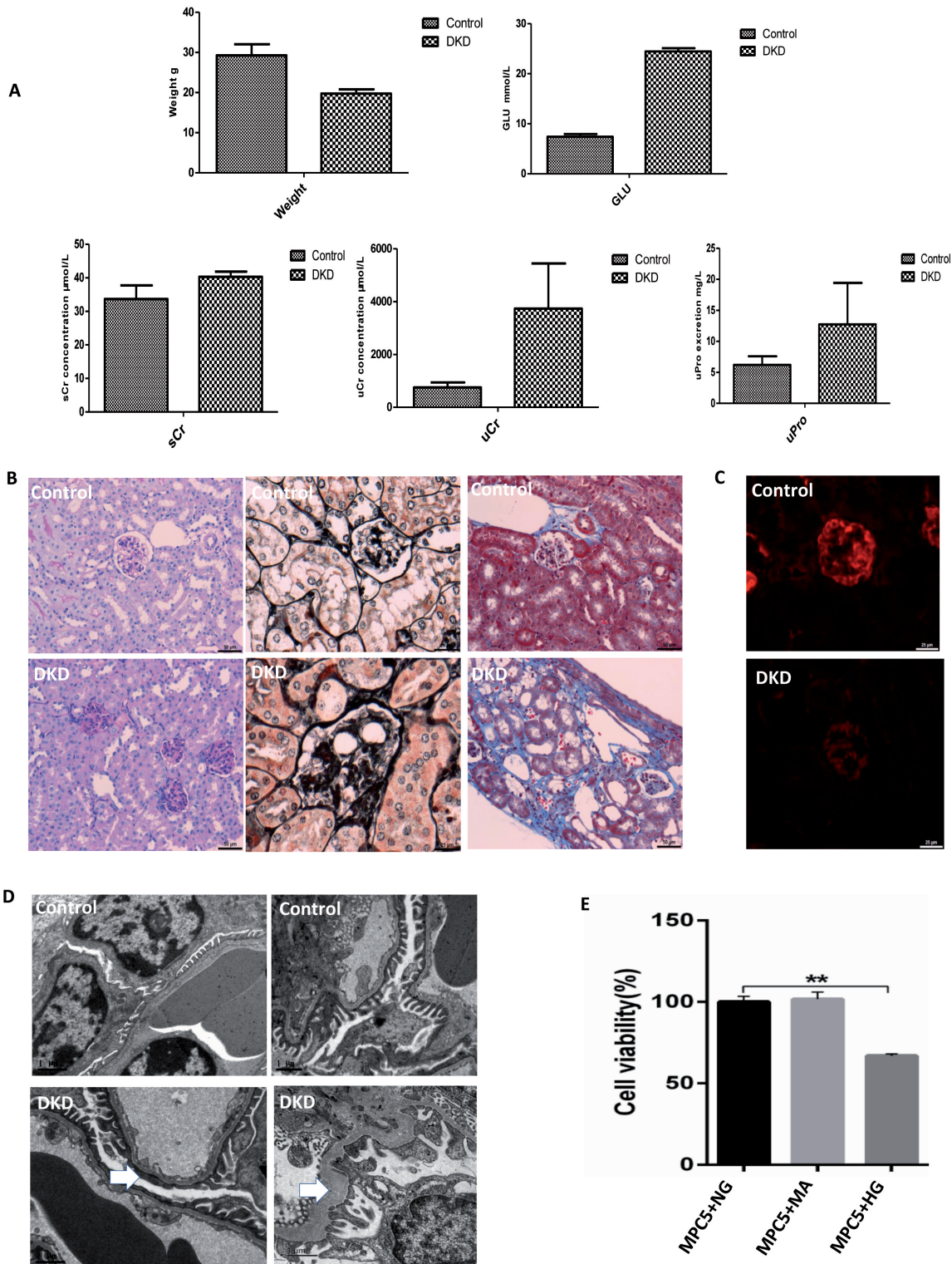
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Fig. 1. High glucose induces kidney damage both in streptozotocin (STZ)-diabetic mice and MPC5 cells. **A.** Body weight, blood glucose, urine creatinine, serum creatinine, and urinary microalbumin from the Control group and STZ-treated (DKD) group were measured. Values are means \pm SD ($n=9$). * $P<0.05$ vs. control. **B.** Pathology of kidney biopsies from DKD ($n=9$) and control group ($n=9$) was assessed by PAS, PASM, and Masson staining. **C.** Synaptopodin expression in podocytes was visualized by immunofluorescence staining. **D.** Glomerular structure from the Control and DKD groups by transmission electron microscopy (TEM). **E.** MPC5 cell viability among normal glucose (NG), mannitol (MA), and high glucose (HG) groups. Control: normal mice; CKD: STZ-induced diabetic mice; MPC5+NG: MPC5 cells treated with normal glucose (5.6 mM); MPC5+MA: MPC5 cells treated with mannitol (30 mM); MPC5+HG: MPC5 cells treated with high glucose (30 mM).

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glucose can destroy kidney structure, leading to renal dysfunction.

We also evaluated viability of MPC5 cells treated *in vitro* with normal glucose (5.6 mM) (NG group), high glucose (30 mM) (HG group), and mannitol (30 mM) (MA group), and observed a significant decrease in viability in the HG group (Fig. 1E). Taken together, these data demonstrate that high glucose causes podocyte injury *in vivo* and *in vitro*.

HG reduces kidney cell autophagy in vitro

We evaluated the effect of three different glucose concentrations on MPC5 cells: normal glucose (5.6 mM) (NG group), high glucose (30 mM) (HG group), and normal glucose (5.6 mM) with mannitol (24.4 mM) (MA group), which served as an osmotic control for HG. As shown in Fig. 2, the expression of mTOR, phospho-mTOR, PTEN, and p66Shc protein in the HG and MA

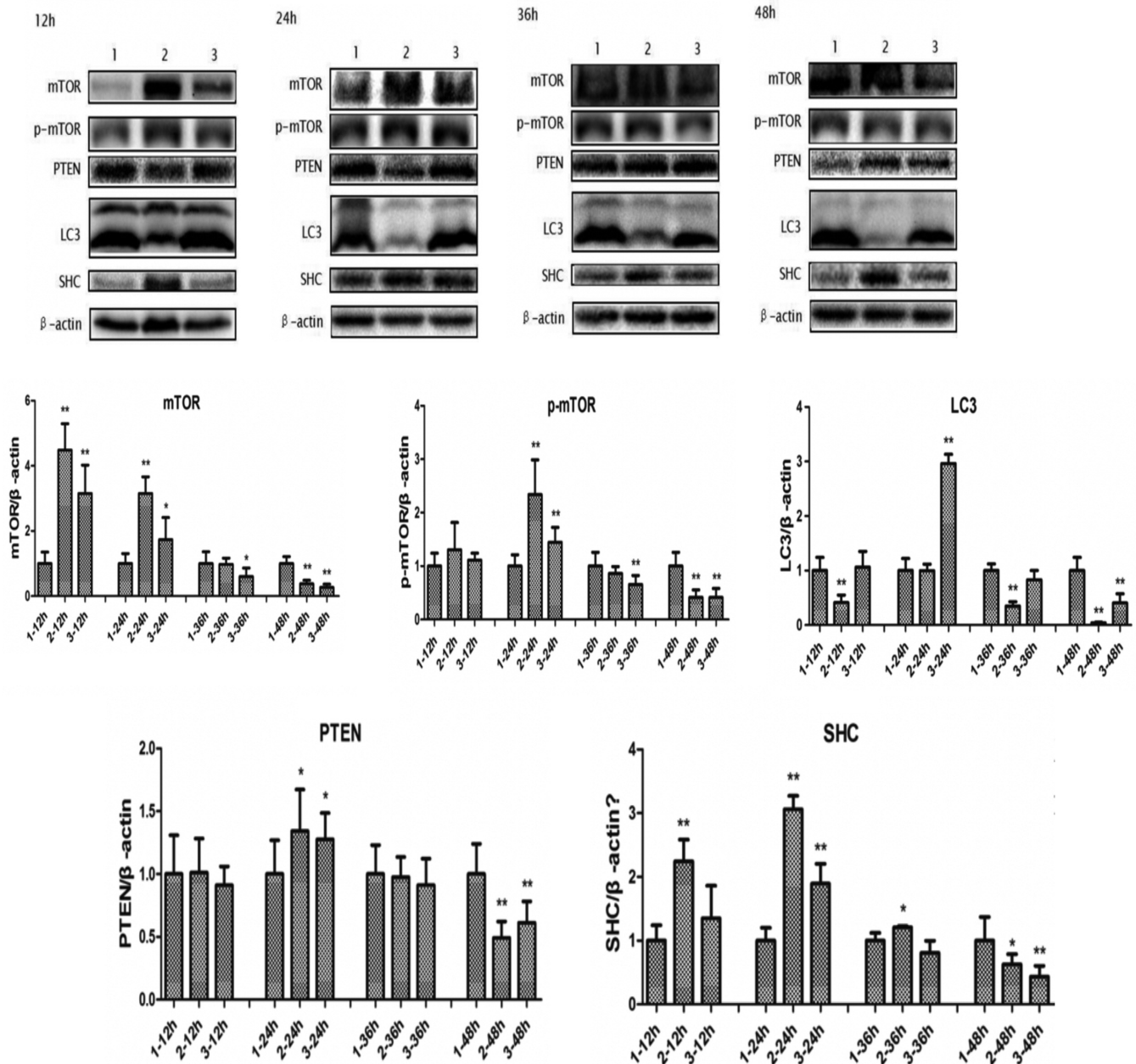


Fig. 2. Effects of high glucose on podocyte autophagy. Measurement of protein levels of mTOR, phospho-mTOR, PTEN, and p66Shc from the NG, HG, and MA groups by Western blotting. (*P<0.05; **P<0.01). 1: MPC5 cells treated with normal glucose (5.6 mM); 2: MPC5 cells treated with high glucose (30 mM); 3: MPC5 cells treated with normal glucose (5.6 mM) and mannitol (24.4 mM).

groups was notably increased compared to the NG group within the first 24 hours, and then decreased in a time-dependent manner during the next 24 hours. In contrast, the expression of LC3 protein, a marker of autophagy, was consistently significantly decreased in the HG group compared to the NG group. These data suggest that high glucose can inhibit autophagy in MPC5 cells.

HG-induced p66Shc expression influences ROS production

To elucidate whether p66Shc regulates the production of ROS in MPC5 cells, p66Shc expression was reduced by gene knockdown and increased by overexpression. As shown in Fig. 3A, ROS level in the HG group was consistently increased compared to the NG group, especially during the first 24 hours, which was consistent with the change in p66Shc expression. The relative fluorescence intensity of ROS in the p66Shc overexpression group was consistently greater than that in the CK (Control kidney) group, while production of ROS in the p66Shc knockdown group was decreased at most timepoints except during the first 12 hours (Fig. 3B). Taken together, these data show that p66Shc is a positive regulator of ROS independent of glucose status, an observation that corresponds with results from several other studies (Trinei et al., 2002; Giorgio et al., 2005).

p66Shc knockdown ameliorates HG-induced autophagy

To confirm whether p66Shc is an essential regulator of autophagy in HG-treated MPC5 cells, p66Shc expression was knocked down using a pEGFP small

interfering RNA (siRNA). The protein level of LC3 increased in both the p66Shc overexpression group and the p66Shc siRNA group, but showed a more substantial increase in expression in the p66Shc siRNA group (Fig. 4A). Additionally, the expression of mTOR and phospho-mTOR protein decreased in the p66Shc knockdown group, but increased slightly in the overexpression group. The expression of PTEN protein was consistently decreased. Vitamin C is used as a suppressor of oxidative stress response, and can also be regarded as an inhibitor of ROS production. As shown in Fig. 4B, the relative ROS fluorescence intensity decreased in a vitamin C concentration-dependent manner. Varying concentrations of vitamin C downregulated mTOR and phospho-mTOR levels (Fig. 4C). However, the expression of PTEN and LC3 increased slightly in the presence of 20 mM and 60 mM vitamin C. These data demonstrate significant negative correlations between p66Shc and autophagy.

HG induces p66Shc overexpression via the Notch signaling pathway

To evaluate the relationship between HG-induced overexpression of p66Shc and the Notch signaling pathway, we treated podocyte cells with DAPT, an inhibitor of Notch signaling. As shown in Fig. 5A, podocytes overexpressing p66Shc exhibited a notably enhanced rate of HG-induced apoptosis in a time-dependent manner, as determined by TUNEL assay and flow cytometry. The apoptosis rate also increased significantly in the presence of DAPT-induced Notch signaling pathway blockade, especially at 24 hours and

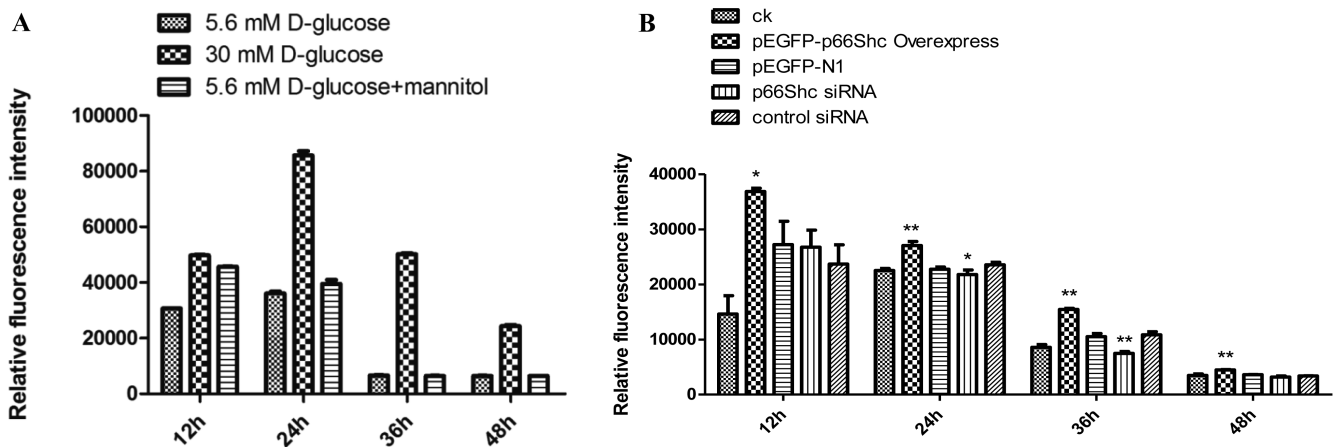


Fig. 3. Relationship between the expression of p66Shc and the production of ROS in podocytes induced by high glucose. **A.** Differences in the production of ROS among the NG, HG, and MA groups were measured by relative fluorescence intensity. **B.** Differences in the production of ROS among the CK, pEGFP-p66Shc overexpression, pEGFP-N1, p66Shc siRNA, and control groups were measured by relative fluorescence intensity. NG: MPC5 cells treated with normal glucose (5.6 mM); HG: MPC5 cells treated with high glucose (30 mM); MA: MPC5 cells treated with normal glucose (5.6 mM) and mannitol (30 mM); CK: control kidney; pEGFP-p66Shc overexpression group: MPC5 cells transfected with p66Shc overexpression sector; pEGFP-N1 group: MPC5 cells transfected with control sector; p66Shc siRNA group: MPC5 cells verified with the p66Shc small interference RNA; control siRNA: MPC5 cells verified with control small interference RNA.

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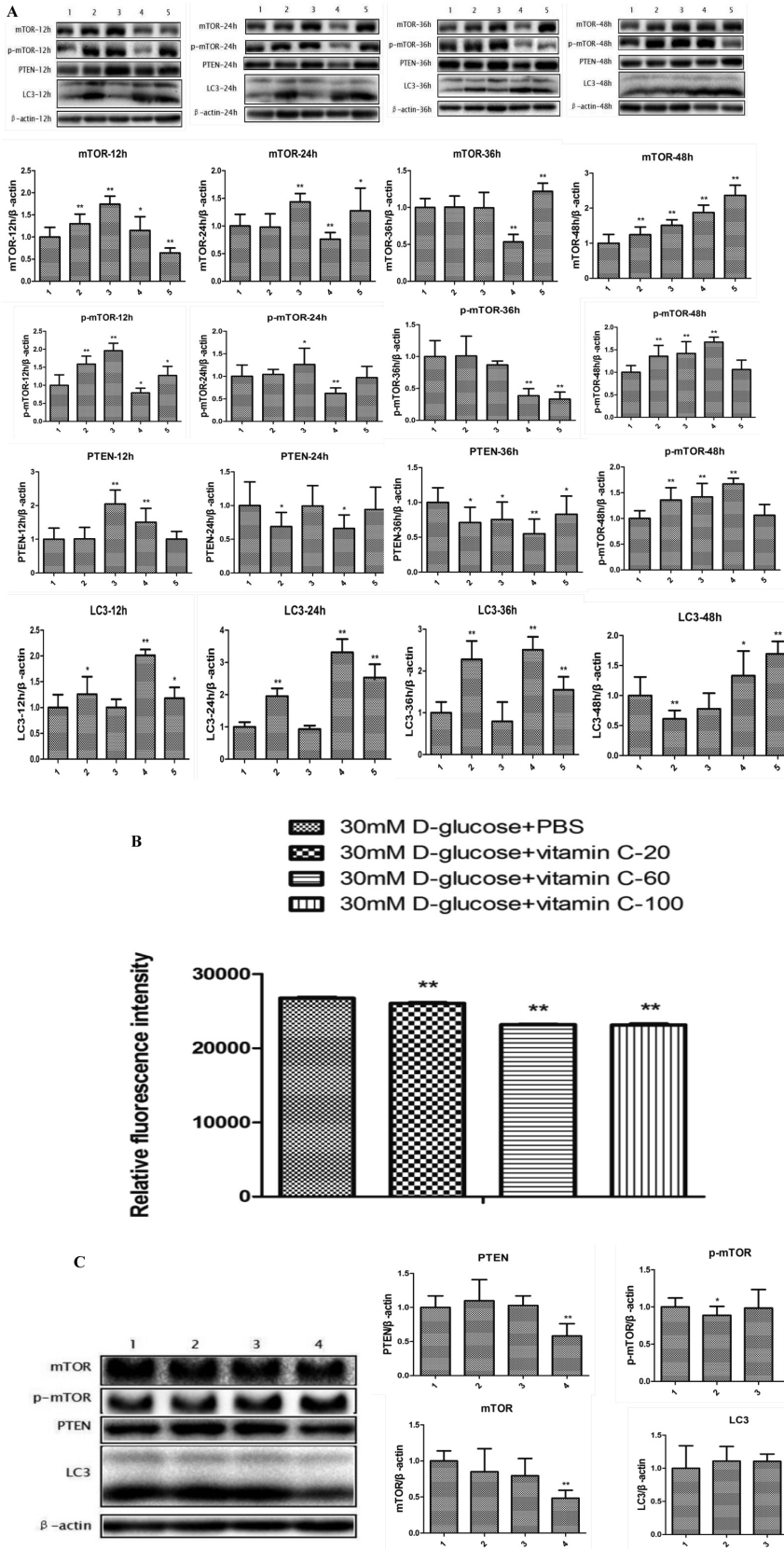


Fig. 4. HG-induced autophagy can be restored by inhibiting the expression of p66Shc. **A.** Measurement of protein levels of mTOR, phospho-mTOR, PTEN, and LC3 from the CK, pEGFP-p66Shc overexpression, pEGFP-N1, p66Shc siRNA, and control groups by Western blotting. (* $P < 0.05$; ** $P < 0.01$). **B.** MPC5 cells were cultured in four media with different vitamin C concentrations (0 mmol/L, 20 mmol/L, 60 mmol/L, 100 mmol/L, respectively). Differences in the production of ROS among these groups were measured by relative fluorescence intensity. **C.** The expression of mTOR, phospho-mTOR, PTEN, and LC3 in four different media was measured by Western blotting. (* $P < 0.05$; ** $P < 0.01$). Fig. 4A-1: MPC5 cells; Fig. 4A-2: MPC5 + pEGFP-p66Shc Overexpression; Fig. 4A-3: MPC5 + pEGFP-N1; Fig. 4A-4: MPC5 + p66Shc siRNA; Fig. 4A-5: MPC5 + control siRNA; Fig. 4C-1: MPC5 cells +30 mM D-glucose + PBS; Fig. 4C-2: MPC5 cells + 30 mM D-glucose + vitamin C (20 mmol/L); Fig. 4C-3: MPC5 cells + 30mM D-glucose + vitamin C (60 mmol/L); Fig. 4C-4: MPC5 cells + 30 mM D-glucose + vitamin C (100 mmol/L).

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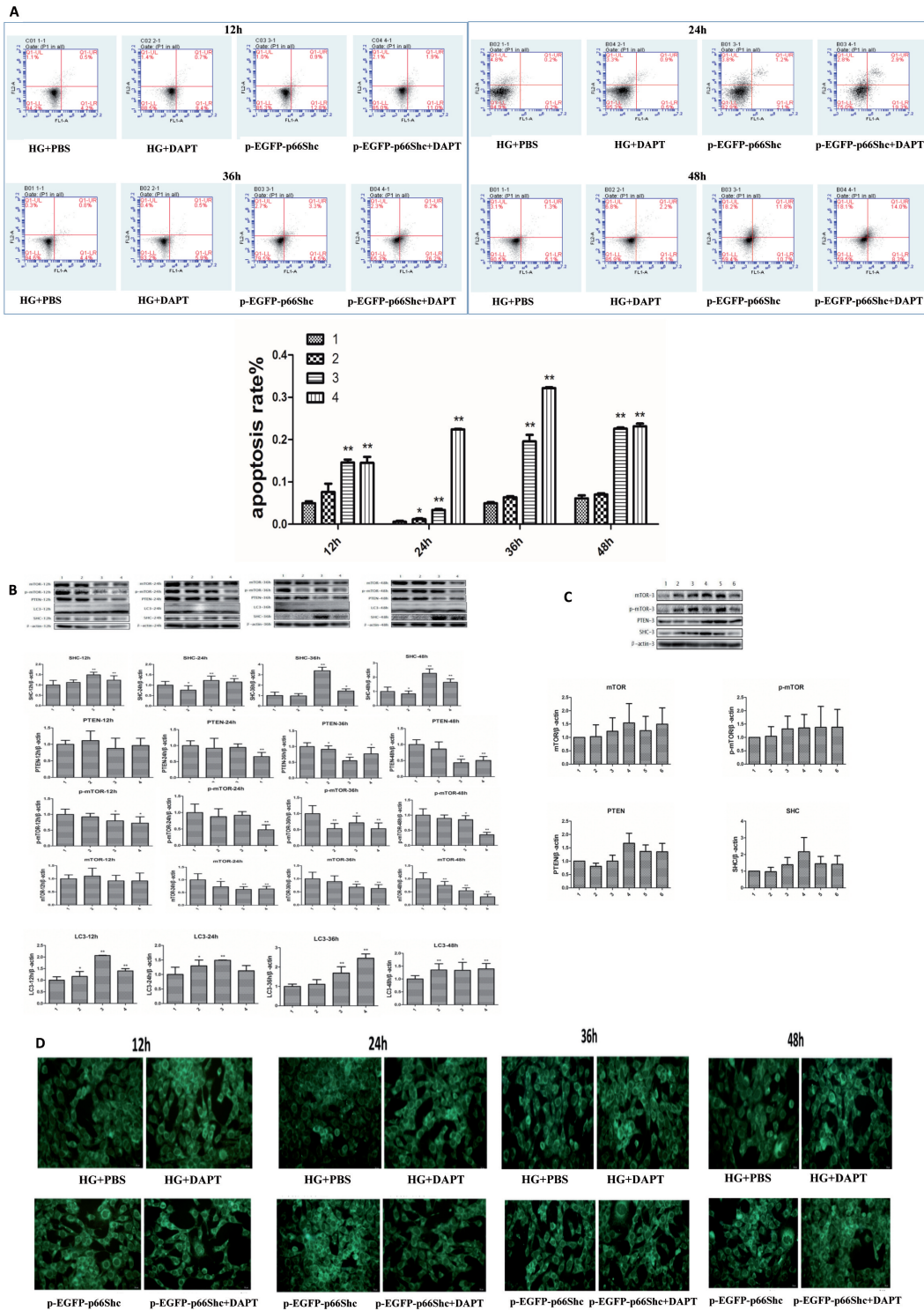


Fig. 5. Notch signaling pathway may be responsible for the HG-induced overexpression of p66Shc in MPC5 cells. **A.** Apoptotic MPC5 cells were determined by flow cytometry. **B.** Measurement of protein levels of mTOR, phospho-mTOR, PTEN, p66Shc, and LC3 from the HG + PBS, HG + DAPT, HG + pEGFP-p66Shc overexpression, and HG + pEGFP-p66Shc overexpression + DAPT groups by Western blotting. (* $P < 0.05$; ** $P < 0.01$). **C.** Measurement of protein levels of mTOR, phospho-mTOR, PTEN, and p66Shc from DKD mice injected with physiological saline, DAPT, and vitamin C, respectively, as well as control mice, as measured by Western blotting. (* $P < 0.05$; ** $P < 0.01$). **D.** Changes in number of podocyte autophagosomes were observed by fluorescence microscope. Fig. 5B-1: MPC5+30 mM D-glucose + PBS; Fig. 5B-2: MPC5+30 mM D-glucose + DAPT (10 μ M); Fig. 5B-3: MPC5+pEGFP-p66Shc overexpression; Fig. 5B-4: MPC5+pEGFP-p66Shc overexpression+ DAPT (10 μ M); Fig. 5C-1: DKD mice + 0.9% NaCl; Fig. 5C-2: DKD mice + DAPT (100 mg/kg i.p); Fig. 5C-3: DKD mice + vitamin C (100 mg/kg.i.p); Fig. 5C-4: normal mice + 0.9% NaCl; Fig. 5C-5: normal mice + DAPT (100 mg/kg. i.p); Fig. 5C-6: normal + vitamin C (100 mg/kg.i.p).

36 hours, indicating that blocking the Notch signaling pathway can enhance podocyte apoptosis induced by p66Shc. Expression of p66Shc decreased with DAPT treatment, and p66Shc expression in the DAPT + pEGFP-overexpression group was also slightly reduced compared to the untreated overexpression group. The expression of PTEN, mTOR and phospho-mTOR decreased in the p66Shc overexpression group, and DAPT treatment was associated with a further decrease in mTOR and phospho-mTOR levels. In contrast, the protein level of LC3 increased in the p66Shc overexpression group, and DAPT augmented this increase (Fig. 5B). In *in vivo* test, STZ-induced diabetic mice and normal mice were treated with saline, DAPT, or vitamin C, respectively. mTOR and phospho-mTOR in diabetic mice group treated with DAPT or vitamin C were both increased compared with saline treatment, additionally, vitamin C treatment group increased more. In normal mice group, the expression of mTOR and phospho-mTOR were also increased, but when treated with DAPT, the amplification was slightly decreased. The expression of PTEN in normal mice was lower than the diabetic mice group. As for p66Shc, compared with diabetic mice+ saline group, the expression in diabetic mice+ DAPT group was slightly decreased, while vitamin C group increased. In normal mice + saline group, the level of p66Shc was higher, when the normal mice + DAPT group was decreased, as well as normal mice + vitamin C group. Furthermore, immunofluorescence analysis revealed that p66Shc modestly promoted the formation of autophagosomes, but DAPT treatment did not appear to further influence autophagosome formation (Fig. 5D).

Discussion

There are three types of cell death, including type I (apoptosis), type II (autophagic cell death), and type III (necrosis). Autophagy plays a key role in keeping cellular homeostasis physiologically under various stress conditions, persisting autophagy is able to induce the type II cell death (Liu and Levine, 2015). It means that autophagy served as a double-edged sword in regulation cell survival and death, including podocytes, which can relieve the injury of podocytes, as well as lead to cell death. Podocyte apoptosis results in podocyte loss, which regard as a pathogenic factor of DKD (Guhr et al., 2013). In general, autophagy can protect cells from apoptosis to keep most cells alive, but when excessive, autophagy and apoptosis will lead to cell death together (De Rechter et al., 2016).

Podocytes comprise the last component of the glomerular filtration barrier (GFB), which when injured plays a pivotal role in the development of diabetic kidney disease (DKD) (Lemley, 2003; Hayden et al., 2005; Wolf et al., 2005). Hyperglycemia-induced oxidative stress can result in podocyte injury (Ha et al., 2008; Giacco and Brownlee, 2010), which can be

mitigated in part through podocyte autophagy mechanisms that can provide energy and materials to repair damaged cells. p66Shc is an important regulator of oxidative stress, which participates in the development of DKD (Sun et al., 2010; Bock et al., 2013). Our preliminary work showed that autophagosomes were decreased and the expression of p66Shc was increased in DKD patients, suggesting a relationship between podocyte autophagy and p66Shc and a potential new target for DKD treatment.

In DKD, many pathways regulate podocyte autophagy. The mTOR pathway is a classical nutrient-sensing pathway that downregulates autophagy activities. Activation of the mTOR pathway can enhance the development of DKD (Ding and Choi, 2015). The PI3K/Akt pathway, a classical signaling pathway regulating cell growth, proliferation, differentiation, motility, and survival (McGonnell et al., 2012), is the upstream signaling pathway of mTOR and suppresses mTOR expression (Bian et al., 2013; Bahrami et al., 2017). PTEN is a negative regulator of PI3K/Akt/mTOR signaling, and is considered a positive regulator of autophagy (Arico et al., 2001). Numerous sources of evidence suggest that the Notch pathway plays a role in the pathogenesis of glomerular disease, including DKD. The Notch signaling pathway can inhibit autophagy by downregulating the expression of PTEN (Liu et al., 2018). ROS induced by high glucose activate podocyte autophagy during early stages of DKD (Ma et al., 2013). p66Shc serves as an inducer of ROS (Yang et al., 2014), which can amplify the podocyte injury induced by hyperglycemia.

In the present study, high glucose caused histologically evident kidney damage in STZ-induced diabetic mice *in vivo* and decreased MPC5 cell viability *in vitro*. LC3 protein, an indicator of autophagy, showed reduced expression in MPC5 cells treated with high glucose, suggesting that hyperglycemia inhibits podocyte autophagy. LC3 protein expression in cells with p66Shc siRNA knockdown was higher than in cells overexpressing p66Shc. Apoptosis induced by hyperglycemia could be enhanced by p66Shc, even more serious while blocking the Notch pathway by DAPT. Additionally, inhibiting the Notch pathway downregulated the expression of p66Shc. These data suggest that p66Shc downregulation of HG-induced podocyte autophagy and upregulation of podocyte apoptosis, and Notch signaling blockade amplified this change. Overall, our data demonstrate that in settings of hyperglycemia, Notch pathway activation leads to upregulated expression of p66Shc, which induces production of ROS, and regulates autophagy via the PTEN-PI3K/Akt/mTOR pathway.

In conclusion, our study has demonstrated the role of p66Shc in regulating podocyte autophagy in high glucose-induced MPC5 cells and diabetic mice, providing novel evidence for its potential role in DKD treatment.

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Availability of data and materials. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate. The present study was approved by Zhejiang University Animal Care and Use Committee.

Patient consent for publication. Not applicable.

Competing interests. The authors declare that they have no competing interests.

Author Contributions. Conceptualization, Qiang He and Juan Jin; Data curation, Juan Jin; Formal analysis, Juan Jin and Danna Zheng; Funding acquisition, Qiang He; Investigation, Qiang He and Juan Jin; Methodology, Danna Zheng, Mei Tao, Xudong Liang and Yiwen Li; Project administration, Qiang He; Visualization, Juan Jin and Danna Zheng; Writing - original draft, Juan Jin and Danna Zheng; Writing - review & editing, Qiang He

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