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From Cell Biology to Tissue Engineering

Tripterygium glycoside protects diabetic kidney disease mouse serum-induced podocyte injury by upregulating autophagy and downregulating β-arrestin-1

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Summary. Background. Diabetic kidney disease (DKD), one of the most common causes of end-stage renal disease (ESRD), remains prevalent in many populations. Podocyte loss and apoptosis play a crucial role in the progression of DKD. Tripterygium glycoside (TG), a widely used Chinese herb, exerted comprehensive protective effects on preventing DKD progression. This study was performed to assess the podocyte protective effect of tripterygium glycoside on DKD by the potential role of activation of autophagy and downregulating β-arrestin-1.

Methods. Tripterygium glycoside and small interfering RNA (siRNA) of β-arrestin-1 were added to 10% db/db mice high-glucose serum induced podocytes *in vitro*. Autophagic activity was evaluated by transmission electronic microscopy, immunofluorescence staining and western blot analysis. Apoptotic activity was evaluated by Annexin V-FITC/PI flow cytometric analysis. The levels of nephrin and podocin, a marker protein of podocytes, were examined using western blot analysis.

Results. Significantly ameliorated podocyte apoptosis, increased nephrin and podocin levels and inhibited expression of β-arrestin-1 were observed after pretreatment of tripterygium glycoside in DKD mouse serum treated podocytes. Significantly higher levels of autophagic activity were also observed. Silencing β-arrestin-1 upregulated autophagic activity and ameliorated podocyte apoptosis. Silencing β-arrestin-1 in combination with tripterygium glycoside enhanced the levels of LC3-II and LC3-II/LC3-I ratios and reduced the expression of p62. Finally, we observed a notable reduction in podocyte apoptotic rate in DKD serum + siRNA-β-arrestin-1 + TG group compared to DKD serum + siRNA-β-arrestin-1 group, and upregulated protein levels of nephrin and podocin compared to treatment with siRNA-β-arrestin-1 only.

Conclusions. This study demonstrated that tripterygium glycoside provided protection against podocyte injury induced by high-glucose serum, and that this effect was mediated by the concomitant activation of autophagy and downregulation of β-arrestin-1.

Key words: Tripterygium glycoside, Autophagy, Apoptosis, β-arrestin-1, Podocyte

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Introduction

Diabetic kidney disease (DKD) is one of the most common causes of chronic kidney failure requiring dialysis and leading to severe complications of diabetes (Nanditha et al., 2016; Ma, 2018). Podocytes play a crucial role in maintaining the normal function of the glomerular filtration barrier and are a central target for the prevention of the development and progression of diabetic albuminuria. Podocyte injury in diabetic kidney

disease can lead to massive proteinuria and cause a variety of glomerulopathies, starting with glomerulosclerosis and chronic progression and eventually leading to end-stage renal disease (Li et al., 2007; Wolf and Ziyadeh, 2007; Ziyadeh and Wolf, 2008). Protection of podocyte injury in DKD is a key therapeutic strategy to prevent or ameliorate the progression to end-stage renal failure.

Tripterygium wilfordii Hook F (TwHF), a widely used Chinese herb, is a member of the Celastraceae family of perennial vine-like plants. Tripterygium glycoside (TG), extracted and purified from the root xylem of TwHF is the active component of TwHF and is widely used in glomerulopathy. We have previously reported that TG provided protection against podocyte injury via apoptosis induced by puromycin aminonucleoside, and this effect was mediated by the concomitant activation of autophagy (Gong et al., 2018). It has been reported that a TwHF/irbesartan combination could synergistically reduce the urinary excretion of proteins and podocytes in DKD patients (Ma et al., 2015). However, the mechanisms of TG ameliorating the proteinuria of diabetic kidney disease remain unclear.

In this study, podocytes were incubated in 10% db/db mice high-glucose serum and treated with TG and small interfering RNAs (siRNA) of β-arrestin-1, and apoptotic activity was evaluated by Annexin V-FITC/PI flow cytometric analysis. Autophagic activity was evaluated by transmission electronic microscopy, immunofluorescence staining and western blot analysis. The aim of this study was to investigate whether TG could alleviate podocyte injury in extracellular high-glucose conditions and explore its underlying mechanisms.

Materials and methods

Reagents and antibodies

Tripterygium glycoside was purchased from Zhejiang Deende Pharmaceutical Co., Ltd (Deende, Zhejiang, China). Annexin V-FITC Apoptosis Detection Kits APC were purchased from Ebioscience (Ebioscience, San Diego, USA). The antibodies for LC3 and p62 were obtained from Proteintech Group (Proteintech Group, Rosemont, USA). The antibodies against nephrin and podocin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody for β-arrestin-1 was obtained from Abcam (Burlingame, CA, USA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG antibody was obtained from Beyotime Institute of Biotechnology (Beyotime, Shanghai, China).

Preparation of serum of diabetic kidney disease mice

This study was approved by the Animal Ethics Committee at Zhejiang province Hospital, Hangzhou, China. db/db mice, a spontaneous model of diabetic nephropathy, was chosen as the animal model for serum preparation. Male db/db mice (n=12) weighing 16 to 20 g at 8 weeks old and age and weight-matched SPF male C57BL/6 control mice (n=6) were obtained from Shanghai Sippr-BK Laboratory Animal Co. Ltd. The mice were maintained on a 12-hour light/dark cycle with free access to food and water for 5 weeks. The mice were sacrificed and the serum was isolated. Serum glucose in db/db and control mice was measured. The glucose concentration in db/db mice was 26.2±2.83 mmol/l.

Cell culture and drug treatment

Conditionally immortalized differentiated mouse podocyte cells (MPC5) were provided by the American Type Culture Collection (ATCC-Y2859). Podocytes were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin G, and 100 mg/ml streptomycin. Podocytes were maintained and expanded at 33°C with 100 U/ml interferon-γ in medium. For podocytes to acquire a differentiated phenotype, cells were grown under restrictive conditions at 37°C. The MPC5 were divided into three groups: the control serum group (MPC5 + control serum), the DKD serum group (MPC5 + 10% db/db mouse serum) and the DKD serum + TG group (MPC5 + 10% db/db mouse serum + 1.25 μg/mL tripterygium glycoside). Tripterygium glycoside (1.25 μg/mL) was added into the DKD serum + TG group 1 hour before treatment with 10% db/db mouse serum, and podocytes were cultured for 24 hours continually and collected for experiments.

Transfection of small interfering RNAs

Small interfering RNA (siRNA) duplex sequences targeting β-arrestin-1 were synthesized and purified by GenePharma Co., Ltd (GenePharma, Shanghai, China.). The sequences of the oligonucleotides were as follows: sense 5' GGGACUUUGUGGA CCACAUUU 3' and anti-sense 5' AUGUGGUCCACA AAGUCCCUU 3'. The internal control using the following of oligonucleotides: sense 5' UCUCCGAACGUGUCA-CGUUU 3' and anti-sense 5' ACGUGACACGUU CGGAGAAUU 3'. Cells were transfected by Lipofectamine RNAi MAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Transmission electronic microscopy

The cell cultures were centrifuged for 10 min at 800 r/min and the supernatant was discarded. The cells were then fixed in 2.5% glutaraldehyde, dehydrated with graded ethanol and embedded in Epon 812 using standard laboratory procedures. Ultrathin sections were subsequently cut and mounted on nickel grids and

stained with lead citrate for transmission electron microscopy (JEOL, JSM-IT300LV, Japan).

Immunofluorescence analysis

The cells were seeded onto glass cover slips, washed twice with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 30 min, and incubated at 4°C overnight with the primary antibody (anti-LC3 1:500, anti-p62 1:500). After three PBS washes, cells were incubated with the FITC-conjugated anti-rabbit IgG antibody for 1 hour at room temperature. Coverslips were then mounted onto glass microscope slides with DAPI (1:500, Beyotime, Shanghai, China). The cells were observed and photographed using a laser scanning confocal fluorescence microscope (Perkin Elmer UltraVIEW VoX, Perkin Elmer, USA).

Western blot analysis

The cells were washed three times with ice-cold PBS and lysed with cell lysis buffer (RIPA, Solarbio, R0010), and total protein was extracted for western blot analysis. Proteins were electrophoretically separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked at room temperature with 5% milk in tris-buffered saline Tween-20 (TBS-T) for 1 hour and then probed with the following primary antibodies overnight at 4°C: anti-LC3 (1:1000), anti-nephrin (1:500), anti-podocin (1:400), and anti-β-actin (1:1000). Membranes were then incubated with secondary antibody (anti-rabbit IgG, 1:1000) for 1 h at room temperature. Membranes were washed three times with TBST and developed using an enhanced chemiluminescence reagent, followed by exposure to Luminescence imaging analysis system (Tanon-5200, Shanghai, China). Finally, images were collected and analyzed using ImageJ software. Protein levels were expressed as the protein/β-actin ratio to minimize differences in sample loading.

Analysis of apoptosis by flow cytometry

The FITC Annexin V Apoptosis Detection kit (BD Biosciences, San Diego, CA, USA) was used to analyze podocyte apoptosis. The cells were washed twice with cold PBS and then suspended in 1X binding buffer at a concentration of $1x10^6$ cells/ml. The solution was then transferred to a 5 ml culture tube and 5 μl of Annexin V-FITC and 5 μl propidium iodide (PI) were added to the

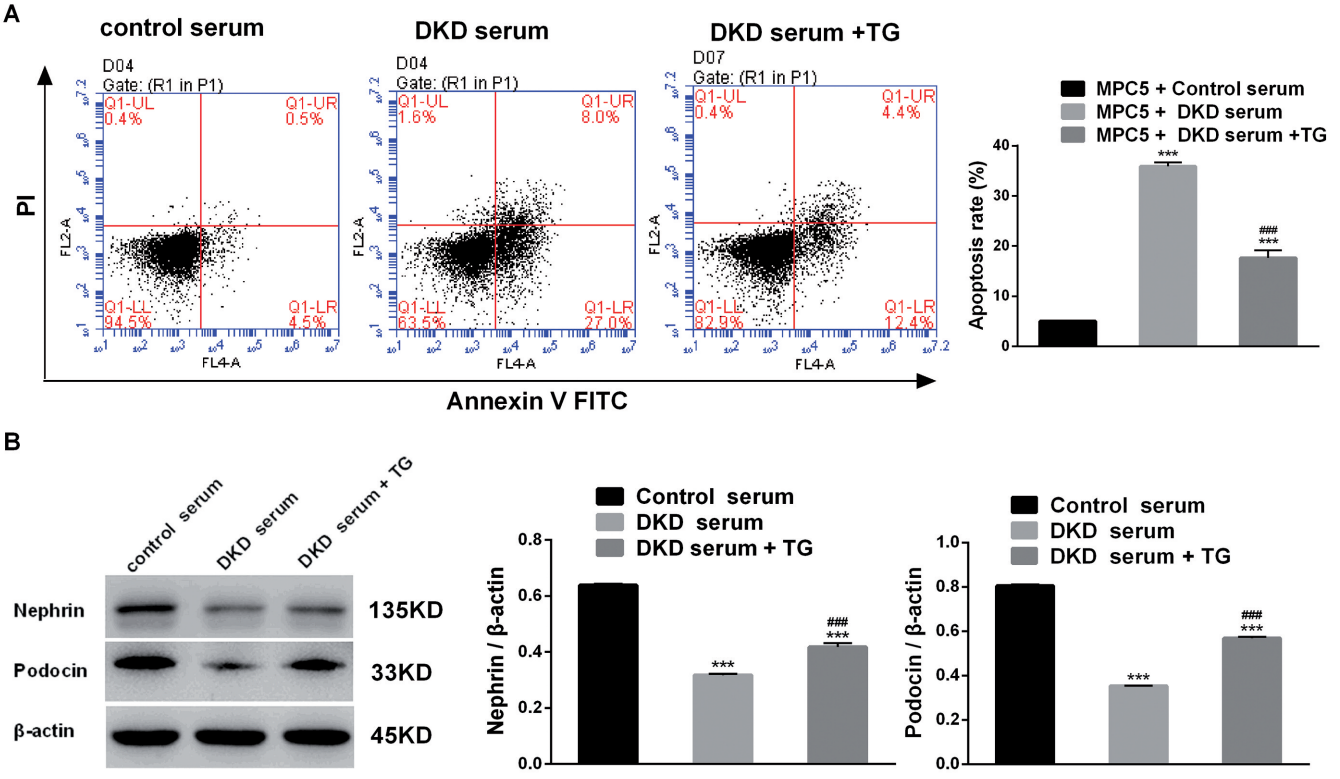


Fig. 1. Tripterygium glycoside ameliorates podocyte apoptosis induced by DKD mouse serum. **A.** Apoptosis was detected by flow cytometry and the representative images and quantitative analysis of apoptotic cells are shown. **B.** Representative western blotting and quantitative evaluation of the expression of nephrin and podocin. Data are reported as the mean ± SD (n=3/group). *p<0.05, **p<0.01, ***p<0.001., vs. control serum group. #p<0.05, ##p<0.01, ###p<0.001, vs. DKD serum group.

solution. The mixture was gently vortexed and incubated for 15 min at room temperature (25°C) in the dark. Cell apoptosis was determined using the BD FACS Calibur flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA) and data were analyzed by CellQuest software (BD Biosciences 2.5).

Statistical analysis

All statistical analyses were performed using SPSS 19.0 software (Stanford University, Stanford, CA, USA). Results are expressed as mean ± standard deviation. Group means were compared using a one-way ANOVA

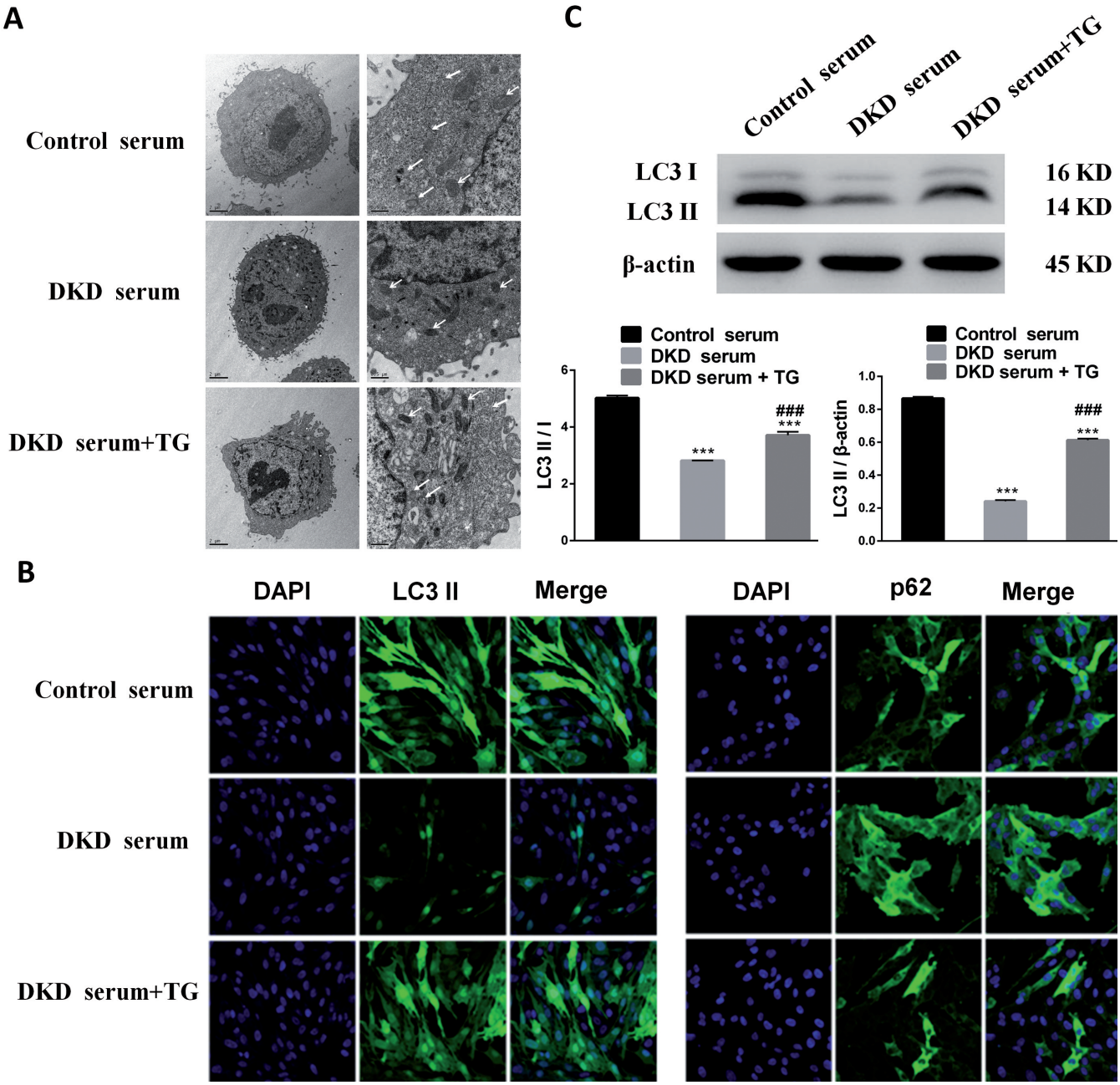


Fig. 2. Tripterygium glycoside promotes autophagic activity in DKD mouse serum-treated podocytes. **A.** Autophagosomes (arrows) detected by transmission electron microscopy in the podocytes of individual groups. **B.** Immunofluorescence stain of LC3 II and p62 showed that autophagic activity was heightened in the DKD serum + TG group versus the DKD serum group. **C.** Representative bands of LC3 in the individual groups. Bar graphs showing the relative expression of LC3II/I and LC3II protein. The expression of LC3II/I and LC3II dramatically increased in the DKD serum + TG group versus the DKD serum group. Data are presented as the mean ± SD (n=3/group). *p<0.05, **p<0.01, ***p<0.001 vs. control serum group. #p<0.05, ##p<0.01, ###p<0.001 vs. DKD serum group. A left, x 10,000; A right, x 20,000; B, x 400.

followed by a least significant difference (LST) test for independent data. All p-values were two-tailed and $p<0.05$ was considered to indicate statistical significance.

Results

The protective effect of tripterygium glycoside on diabetic kidney disease mouse serum-induced podocyte injury

To observe the effect of tripterygium glycoside on DKD mouse serum-induced podocyte apoptosis, we examined podocyte apoptosis by flow cytometry after treatment with DKD mouse serum and tripterygium glycoside. We found that the number of apoptotic cells was significantly increased in the DKD serum group compared with the cells incubated with C57BL/6 control mouse serum (35.90±0.82 vs 5.03±0.06, $p<0.001$). In the DKD serum + TG group, podocyte apoptosis level was inhibited by tripterygium glycoside compared to the DKD serum group (17.67±1.50 vs 35.90±0.82, $p<0.001$) (Fig. 1A). Nephrin and podocin were also detected to evaluate podocyte injury. As shown in Fig. 1B, DKD serum significantly reduced the expression levels of nephrin and podocin. However, in tripterygium glycoside-treated podocytes, there was a significant increase in the protein expression of nephrin and podocin ($p<0.01$).

Tripterygium glycoside promotes basal autophagy in podocytes with diabetic kidney disease mouse serum treatment

Transmission electronic microscopy (TEM), immunofluorescence and western blot were used to observe the effect of mouse serum and tripterygium glycoside on podocyte autophagy. As shown in Fig. 2A, TEM showed that the number of typical autophagosomes with double membranes was significantly increased in podocytes treated with tripterygium glycoside compared with the DKD serum group. Moreover, immunofluorescence analysis showed that the fluorescent spot of LC3 II was significantly reduced and p62 was significantly increased in the DKD serum group, indicating that autophagic flux was inhibited. In tripterygium glycoside-treated podocytes, the fluorescent spot of LC3 II was increased remarkably and the fluorescent spot of p62 was significantly decreased (Fig. 2B). These results were further confirmed by western blot. We found that tripterygium glycoside increased LC3-II/LC3-I conversion and the amount of LC3-II was much higher in DKD serum + TG group than the DKD serum group (Fig. 2C).

Tripterygium glycoside inhibits expression of β-arrestin-1 in podocytes with diabetic kidney disease mouse serum treatment

We studied the regulation of β-arrestin-1 protein in podocytes with diabetic kidney disease mouse serum treatment and with tripterygium glycoside treatment via western blot. As shown in Fig. 3A,B, the expression of β-arrestin-1 was significantly increased in the podocytes treated with diabetic kidney disease mouse serum versus the control group. Nonetheless, β-arrestin-1 protein expression was significantly reduced in the DKD serum + TG group compared with the DKD serum group. Taken together, these data suggest that β-arrestin-1 might serve as one of the triggers in DKD serum-induced cell apoptosis, and tripterygium glycoside can

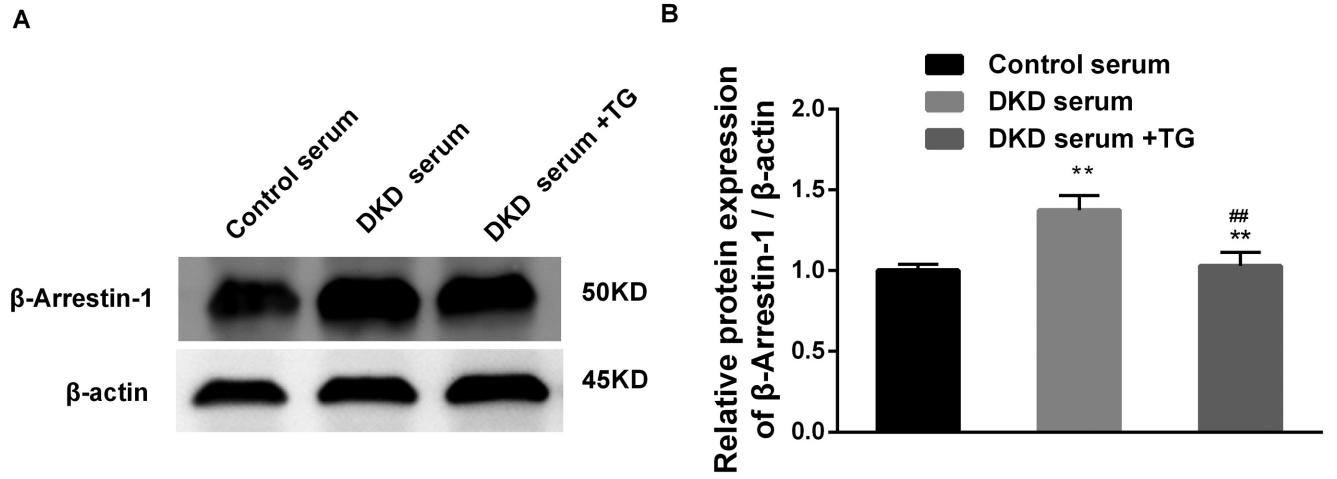


Fig. 3. Tripterygium glycoside inhibits expression of β-arrestin-1 in DKD mouse serum- treated podocytes. **A.** Representative bands of β-arrestin-1 in the individual groups. **B.** Bar graphs showing the relative expression of β-arrestin-1 protein. The expression of β-arrestin-1 significantly decreased in DKD serum + TG group versus DKD serum group. Data are presented as the mean ± SD, n=3. *p<0.05, **p<0.01, ***p<0.001 versus control group. #p<0.05, ##p<0.01, ###p<0.001 versus DKD serum group.

counteract this detrimental effect.

Silencing β-arrestin-1 in combination with tripterygium glycoside promotes autophagic activity in podocytes treated with diabetic kidney disease mouse serum

To determine the role of β-arrestin-1 and tripterygium glycoside in podocyte autophagy, small interfering RNA (siRNA-β-arrestin-1) was obtained to silence β-arrestin-1, and immunofluorescence and western blot was performed to detect the biomarker of autophagic activity. As shown in Fig. 4, siRNA-β-arrestin-1 application significantly increased the expression of LC3 II and relative LC3 II/I level in DKD mouse serum-treated podocytes. More interestingly, when podocytes were treated with tripterygium glycoside for 1 h before siRNA-β-arrestin-1 transfection, the expression of LC3 II and relative LC3 II/I levels were further increased. Similar differences were observed by immunofluorescence. We found that the fluorescent spot of LC3 II was significantly increased and p62 was significantly decreased in the DKD serum + siRNA-β-arrestin-1 group (Fig. 5). In addition, the fluorescent spot of LC3 II was significantly increased and the fluorescent spot of p62 was significantly decreased in the DKD serum + siRNA-β-arrestin-1 + TG

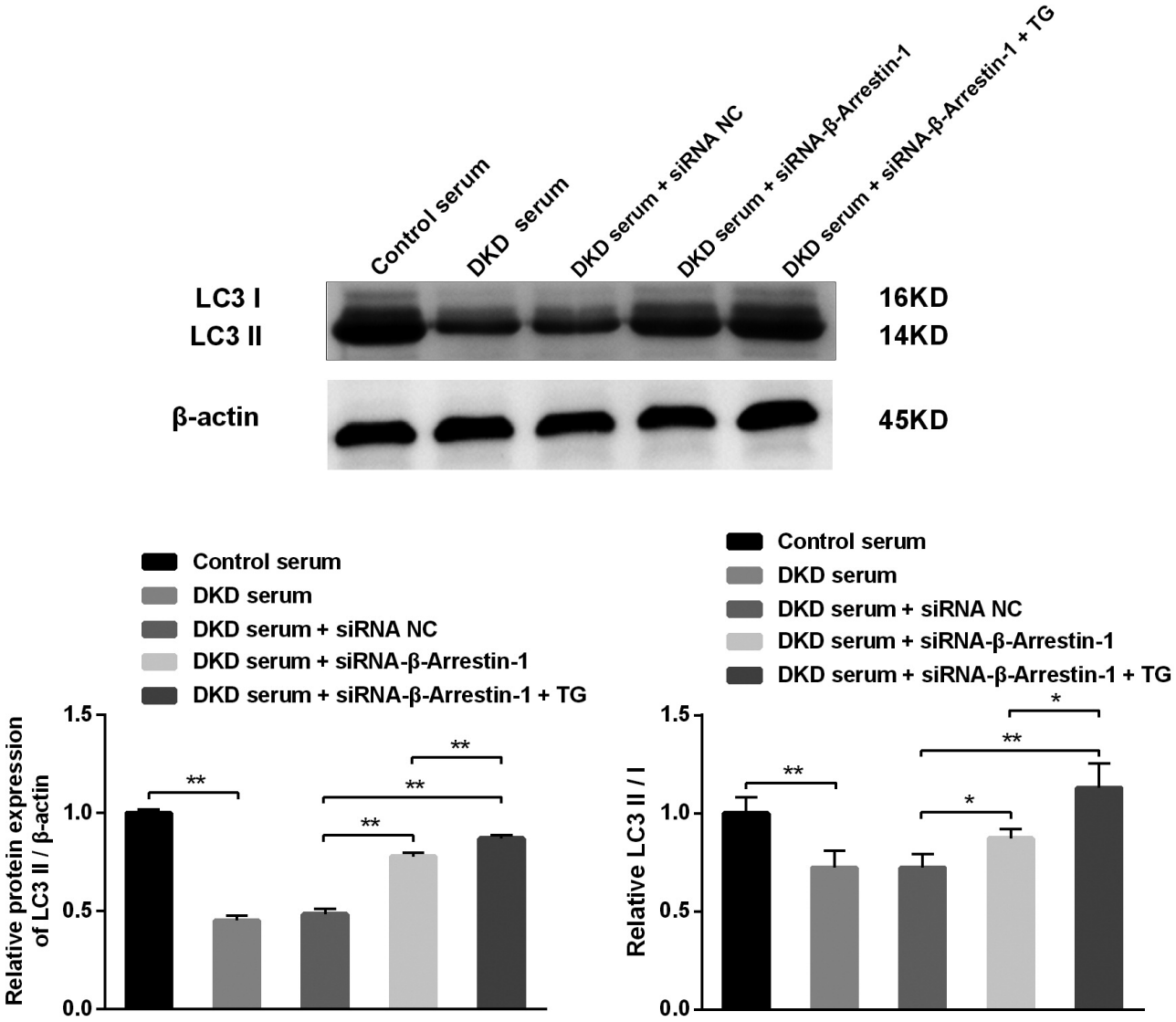


Fig. 4. Silencing β-arrestin-1 in combination with tripterygium glycoside promotes autophagic activity of DKD mouse serum-treated podocytes. Representative bands and bar graphs of LC3 in individual groups by western blot. Silencing β-arrestin-1 in combination with TG treatment increased the expression of LC3 II and relative LC3 II/I level in DKD serum treated podocytes. Data are presented as the mean ± SD, n=3. *p<0.05, **p<0.01, ***p<0.001.

group compared to the DKD serum + siRNA-β-arrestin-1 group (Fig. 5).

Silencing β-arrestin-1 in combination with tripterygium glycoside ameliorates podocyte injury induced by diabetic kidney disease mouse serum

To determine whether β-arrestin-1 downregulation is a critical factor in the protective effect of tripterygium glycoside in podocytes, we used siRNA-β-arrestin-1 to silence β-arrestin-1; we then examined DKD mouse serum-induced apoptosis in podocytes via flow cytometry and the expression of nephrin and podocin by western blot. We found that siRNA-β-arrestin-1 application significantly ameliorated DKD mouse serum-induced podocyte apoptosis in line with an increase of nephrin and podocin (Fig. 6). Furthermore, when podocytes were treated with tripterygium glycoside for 1 hour before siRNA-β-arrestin-1 transfection, the protective effect in podocytes was much more evident as the apoptosis ratio was reduced to 11.9%. Similarly, silencing β-arrestin-1 in combination with tripterygium glycoside significantly promoted the upregulation of nephrin and podocin.

Discussion

Diabetic kidney disease (DKD), a major cause of end-stage renal disease (ESRD), is one of the most severe complications of diabetes mellitus and poses a

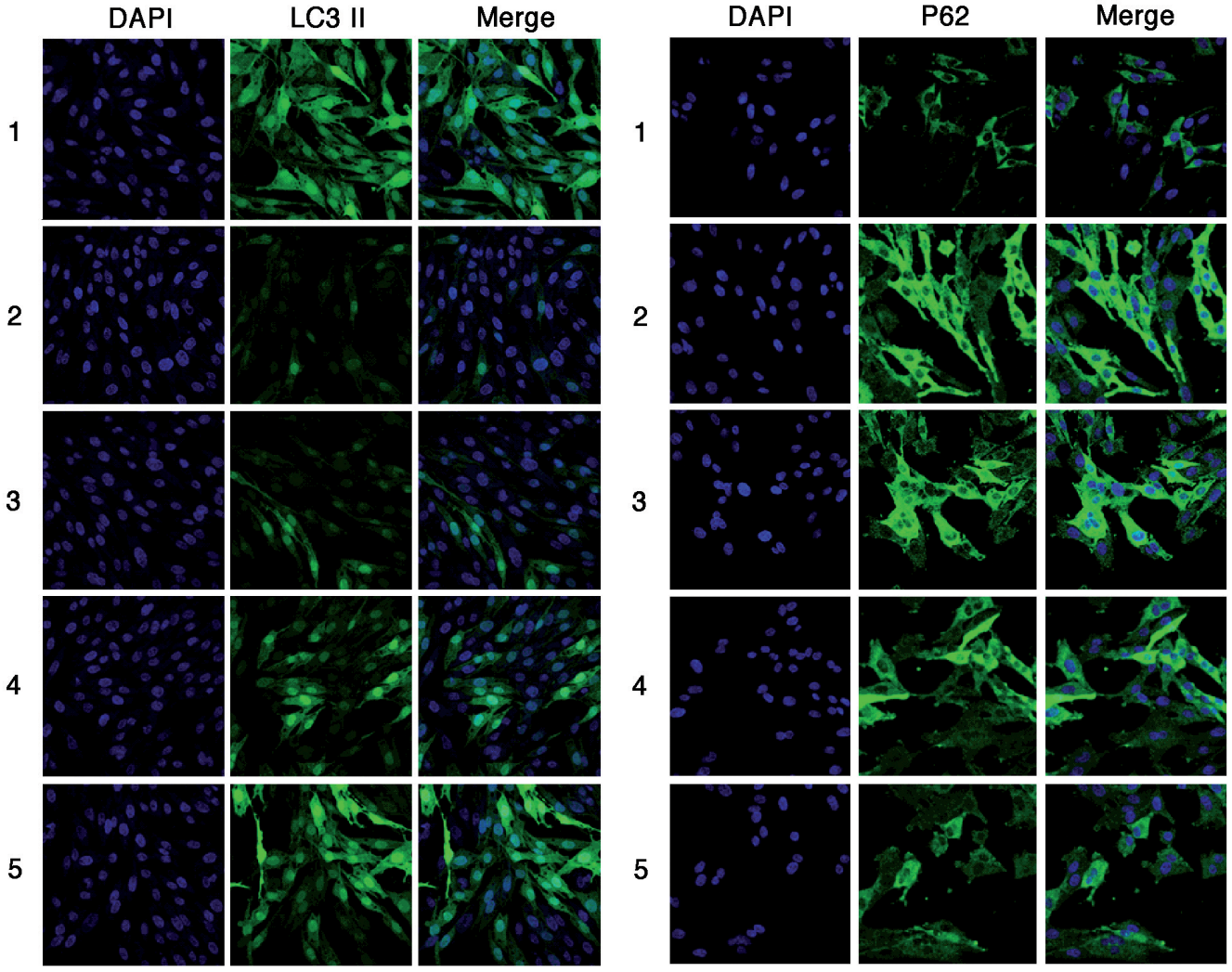
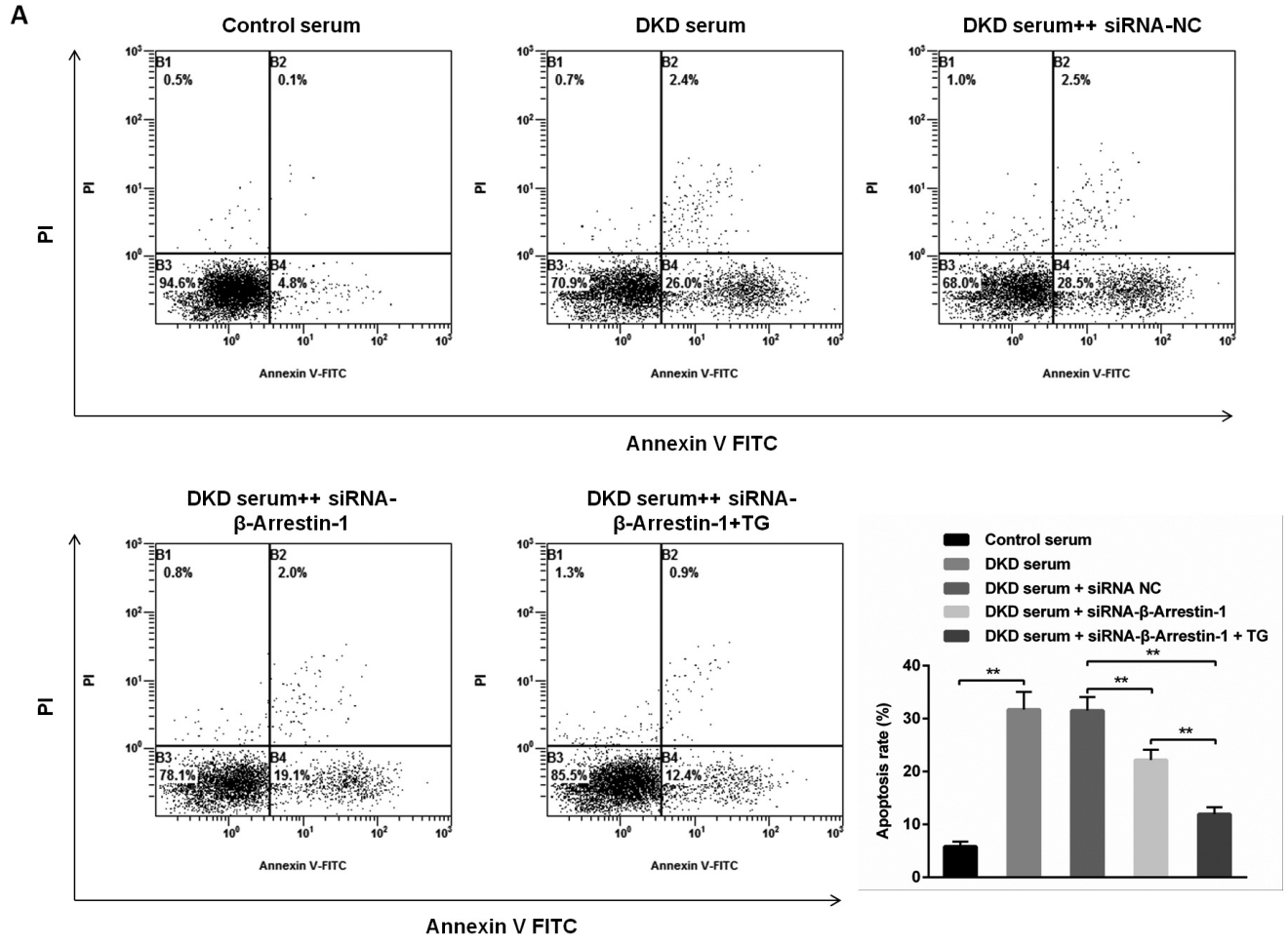


Fig. 5. Silencing β-arrestin-1 in combination with tripterygium glycoside promotes autophagic activity of DKD mouse serum-treated podocytes. Immunofluorescence stain of LC3 II and p62 showed that autophagic activity was promoted in DKD serum + siRNA-β-arrestin-1 + TG group versus DKD serum group. 1: control group; 2: DKD serum group; 3: DKD serum + siRNA NC group; 4: DKD serum + siRNA-β-arrestin-1 group; 5: DKD serum + siRNA-β-arrestin-1 + TG group. x 400.



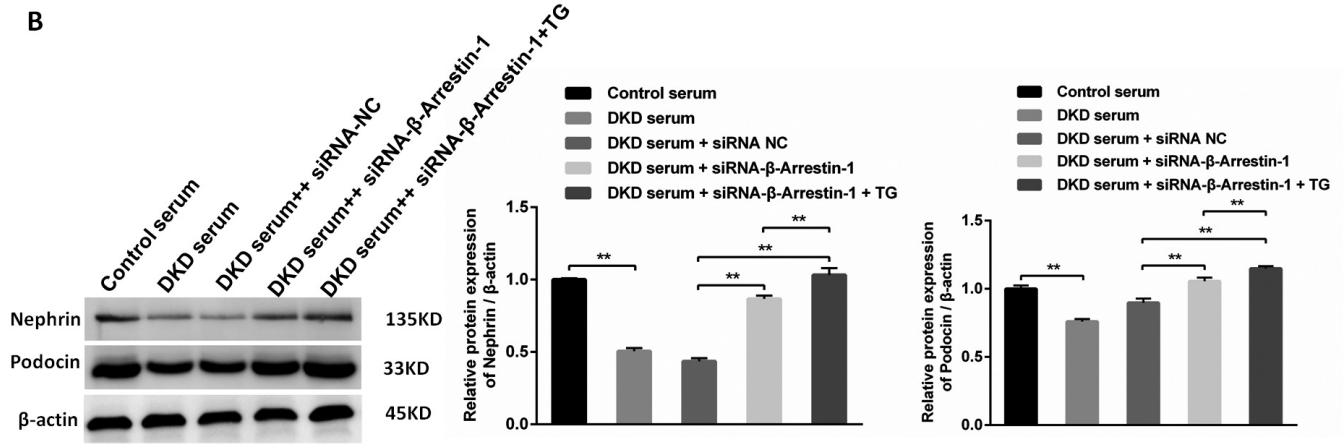


Fig. 6. Silencing β-arrestin-1 in combination with tripterygium glycoside ameliorates podocyte injury induced by DKD mouse serum. **A.** Apoptosis was detected by flow cytometry and the representative images and quantitative analysis of apoptotic cells were presented in individual groups. The podocyte apoptotic rate was obviously decreased in the DKD serum + siRNA-β-arrestin-1 group and the DKD serum + siRNA-β-arrestin-1 + TG group versus the DKD serum + siRNA-β-arrestin-1 NC group, respectively. **B.** Representative western blotting and quantitative evaluation of the expression of nephrin and podocin. The expression of nephrin and podocin were significantly increased in the DKD serum + siRNA-β-arrestin-1 + TG group versus DKD serum + siRNA-β-arrestin-1 NC group and the DKD serum + siRNA-β-arrestin-1 + group respectively. Data are reported as the mean ± SD, n=3/group. *p<0.05, **p<0.01, ***p<0.001.

direct threat to public health. It is imperative to develop novel strategies to prevent or ameliorate the progression of DKD. In recent years, studies have demonstrated that podocyte apoptosis and detachment play an essential role in the pathogenesis of deteriorated renal function in DKD (Kim et al., 2014; Ilatovskaya et al., 2015). Studies have shown that *Tripterygium wilfordii* Hook F (TwHF) extract can clearly reduce the urine protein level of diabetic nephropathy (DN) patients (Ge et al., 2013; Gong et al., 2018). In this study, we found that tripterygium glycoside significantly attenuated DKD mouse serum-induced podocyte apoptosis *in vitro*. Our studies suggest that treatment of tripterygium glycoside results in a reduction of proteinuria and improvement in renal function in DKD by inhibiting podocyte injury. Increased expression of nephrin and podocin by western blot provides additional proof of our conclusions.

The molecular mechanisms of podocyte injuries are not clearly understood. Mechanical stress, oxidative stress, immunological stress are implicated in podocyte injuries in glomerulonephritis (Goldman et al., 2010; Inagi et al., 2014; Locatelli et al., 2014). Autophagy, a major intracellular lysosomal degradation system, performs homeostatic functions linked to metabolism and organelle turnover, and it has been known to be involved in the pathogenesis of DKD. Generally, when kidney cells are exposed to stressful conditions, including hypoxia, genotoxic damage, oxidative stress, and ER stress, autophagy is activated and plays a critical role in cell survival (Kitada et al., 2017; Yang et al., 2018). Although the role of autophagy in DKD remains to be elucidated, inhibition of autophagy in podocytes has been demonstrated in other DKD models (Vallon et al., 2013; Lim et al., 2018). To clarify the role of autophagy in podocyte injury of DKD, we characterized DKD mouse serum-induced podocyte autophagic activity. Our data showed that autophagy was inhibited, which is in line with previous reports (Vallon et al., 2013; Lim et al., 2018) (Fig. 2). Interestingly, we found that autophagic activity was heightened, while there was a decline in apoptotic rate when the podocytes were preincubated with tripterygium glycoside. This was confirmed by the increased autophagosomes and LC3 II and decreased p62. Our data suggest that tripterygium glycoside possibly alleviates podocyte injury by upregulating autophagic activity. Further research is required to explore which signal path is involved in autophagy activation.

It has been reported that β-arrestins are upregulated in the kidney in STZ-induced diabetic mice and human diabetic renal tissues (Liu et al., 2016). The study also demonstrates that β-arrestins are critical components of signal transduction pathways that link renal injury to inhibit autophagy in DKD (Liu et al., 2016). In our study, we found that β-arrestin-1 was upregulated in podocytes treated with db/db mouse serum, which is in line with *in vivo* study. Furthermore, we found that tripterygium glycoside treatment significantly decreased the expression of β-arrestin-1 (Fig. 3). β-arrestins are adaptor proteins and signal transduction proteins that play an important role in molecular regulation. In recent years, β-arrestins have been shown to activate signaling cascades of G-protein activation, scaffold many intracellular signaling networks, play important roles in cell growth and apoptosis, affect the deposition of ECM and are involved in the pathological process of fibrotic diseases (Lovgren et al., 2011). Renal fibrosis is central to the progression of DKD. It has been confirmed that β-arrestin-1 expression increases in the glomerular tuft and in the area of hyperplastic lesions in patients with extracapillary glomerulonephritis. Further studies have found that the podocyte differentiation marker synaptopodin was reduced when they were exposed to endothelin-1 (ET-1) and ET-1 promoted podocyte migration via endothelin-A receptor activation and increased β-arrestin-1 expression (Buelli et al., 2014). Another study showed that β-arrestin-1/2 overexpression inhibited the expression of nephrin and podocin proteins and promoted podocyte apoptosis of DKD (Wang et al., 2018). In our study, we found that β-arrestin-1 knockdown promoted autophagic activity and ameliorated podocyte apoptosis of DKD mouse serum-treated podocytes. We demonstrated that the protective effect was enhanced and autophagy was further activated when silencing β-arrestin-1 in combination with tripterygium glycoside treatment. Nephrin and podocin are components of the glomerular slit diaphragm and single transmembrane spanning receptor, which play an important role in maintaining normal glomerular filtration function. Our study indicated that silencing β-arrestin-1 in combination with tripterygium glycoside treatment remarkably increased nephrin and podocin expression. Taken together, these data suggest that tripterygium glycoside can downregulate β-arrestin-1, promote autophagic activity and ameliorate podocyte injury induced by high-glucose serum.

To our knowledge, this is the first study that demonstrates that tripterygium glycoside provides protection against podocyte injury induced by high-glucose serum, and that this effect is mediated by the concomitant activation of autophagy and downregulation of β-arrestin-1. In our study, we used DKD mouse serum instead of high-glucose solution to construct a podocyte injury model. The use of mouse serum simulates the *in vivo* environment more closely and reflects the genuine disease state. Our findings provide new insights into understanding the effects of tripterygium glycoside, and may be useful for identifying an innovative therapeutic strategy for treating patients with diabetic kidney disease.

However, there are still some limitations in the present study. First, this is just an *in vitro* study and lacks data from *in vivo* experiments. Another limitation of this study is that we have not designed experiments to observe whether the protective effect of TG on DKD mice serum-induced podocyte injury still exists after inhibiting autophagy. Furthermore, it is still an open question as to how TG regulates autophagy and β-

Arrestin-1 to protect podocyte injury.

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

Authors' contributions. Jianguang Gong designed the study. Qiang He proofread and checked the article, Juan Jin and Li Zhao performed the laboratory assays. Shikai Liang performed the statistical analyses. Huifang Zhan wrote the manuscript. The final version of the manuscript was approved by all authors. Jianguang Gong and Qiang He contributed equally to this manuscript.

Ethics approval and consent to participate. This study was approved by the local ethics committee of Zhejiang Provincial People's Hospital. The study was performed according to the ethical standards laid down in the 1964 Declaration of Helsinki.

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