Overexpression of Myo1e promotes albumin endocytosis by mouse glomerular podocytes mediated by Dynamin

Huijun Shen Corresp., 1, Yu Bao 1, Chunyue Feng 1, Haidong Fu 1, Jianhua Mao 1

1 Department of Nephrology, The Children's Hospital of Zhejiang University School of Medicine, Hangzhou, Zhejiang, China

Corresponding Author: Huijun Shen
Email address: 6197005@zju.edu.cn

Background: As a fundamental process internalizing molecules from the plasma membrane, endocytosis plays a crucial role in podocyte biology. Our previous study has identified that overexpression of Myo1e may enhance podocyte endocytosis. However, its potential mechanism has been not well understand. Thus, we aimed to analyze whether albumin endocytosis by mouse glomerular podocytes is dependent on Myo1e expression. Also, we aimed to elucidate whether the underlying mechanism is mediated by Dynamin.

Methods: Firstly, mouse podocyte cells (MPC5) were treated with different concentrations of FITC-bovine serum albumin (BSA). The fluorescence intensity and cell viability were detected by flow cytometry and MTT assays, respectively. Afterwards, the optimal concentration of FITC-BSA was determined. Secondly, MPC5 cells were treated with Myo1e overexpression or knockdown. Cell morphology was observed under microscope. Immunofluorescence assay was used to determine the expression of F-actin. The protein expression of nephrin and podocin was detected by western blot. Flow cytometry was used to detect MPC5 cell apoptosis with annexin V. Finally, MPC5 cells were treated with Myo1e overexpression and/or Dynasore (a GTPase inhibitor of Dynamin). The fluorescence intensity was detected using flow cytometry assay.

Results: MPC5 endocytosis BSA was elevated with a concentration-dependent manner. MTT results showed that MPC5 cell viability was inhibited with a concentration-dependent manner. Myo1e overexpression promoted podocyte endocytic FITC-BSA, which was contrary to its knockdown. Under microscope, after inhibition of Myo1e, podocyte foot process fusion was observed. Myo1e overexpression promoted the expression of cytoskeleton F-actin and podocyte-specific molecules (nephrin and podocin) in podocyte endocytic FITC-BSA. Furthermore, we found that Myo1e promoted the apoptosis of podocytes. Dynasore attenuated the increase in endocytosis of FITC-BSA induced by Myo1e overexpression, suggesting that podocytes might mediate albumin endocytosis via Myo1e-Dynamin-Albumin.

Conclusion: Our findings revealed that overexpression of Myo1e promotes albumin endocytosis in mouse glomerular podocyte endocytic albumin mediated by Dynamin.
Overexpression of Myo1e promotes albumin endocytosis by mouse glomerular podocytes mediated by Dynamin

Authors:
Huijun Shen¹, *: 6197005@zju.edu.cn
Yu Bao¹: hybaoyu@163.com
Chunyue Feng¹: springmoonfeng@163.com
Haidong Fu¹: fhdhz@163.com
Jianhua Mao¹: maojh88@126.com

1. Department of Nephrology, The Children’s Hospital of Zhejiang University School of Medicine, No.57 Zhugan Lane, Xiacheng District, Hangzhou 310006, Zhejiang, P R China.
* Corresponding author: Huijun Shen
E-mail: 6197005@zju.edu.cn

Department of Nephrology, The Children’s Hospital of Zhejiang University School of Medicine, No.57 Zhugan Lane, Xiacheng District, Hangzhou 310006, Zhejiang, P R China.

Abstract:

Background: As a fundamental process internalizing molecules from the plasma membrane, endocytosis plays a crucial role in podocyte biology. Our previous study has identified that overexpression of Myo1e may enhance podocyte endocytosis. However, its potential mechanism has been not well understand. Thus, we aimed to analyze whether albumin endocytosis by mouse glomerular podocytes is dependent on Myo1e expression. Also, we aimed to elucidate whether the underlying mechanism is mediated by Dynamin.

Methods: Firstly, mouse podocyte cells (MPC5) were treated with different concentrations of FITC-bovine serum albumin (BSA). The fluorescence intensity and cell viability were detected by flow cytometry and MTT assays, respectively. Afterwards, the optimal concentration of FITC-BSA was determined. Secondly, MPC5 cells were treated with Myo1e overexpression or knockdown. Cell morphology was observed under microscope. Immunofluorescence assay was used to determine the expression of F-actin. The protein expression of nephrin and podocin was detected by western blot. Flow cytometry was used to detect MPC5 cell apoptosis with annexin V. Finally, MPC5 cells were treated with Myo1e overexpression and/or Dynasore (a GTPase inhibitor of Dynamin). The fluorescence intensity was detected using flow cytometry assay.

Results: MPC5 endocytosis BSA was elevated with a concentration-dependent manner. MTT results showed that MPC5 cell viability was inhibited with a concentration-dependent manner. Myo1e overexpression promoted podocyte endocytic FITC-BSA, which was contrary to its knockdown. Under microscope, after inhibition of Myo1e, podocyte foot process fusion was observed. Myo1e overexpression promoted the expression of cytoskeleton F-actin and podocyte-specific molecules (nephrin and podocin) in podocyte endocytic FITC-BSA. Furthermore, we
found that Myo1e promoted the apoptosis of podocytes. Dynasore attenuated the increase in endocytosis of FITC-BSA induced by Myo1e overexpression, suggesting that podocytes might mediate albumin endocytosis via Myo1e-Dynamin-Albumin.

**Conclusion:** Our findings revealed that overexpression of Myo1e promotes albumin endocytosis in mouse glomerular podocyte endocytic albumin mediated by Dynamin.

**Key words:** endocytosis; podocytes; MPC5; albumin; glomerular albuminuria

**Introduction:**

Albuminuria is a hallmark of nephropathy, usually caused by a deterioration in the integrity of the glomerular filtration barrier (Schiessl et al. 2016). The glomerular filtration barrier consists of a porous endothelium, basement membrane and podocytes. Podocytes are highly specialized and terminally differentiated visceral epithelial cells (Han et al. 2019). As an important component of the glomerular basement membrane, podocytes play an important role in maintaining the integrity of the glomerular filtration barrier (Brosius & Coward 2014). Hartleben et al. reported that endoplasmic reticulum stress, excessive ubiquitination, proteinuria and glomerular lesions were observed in aging mice after podocyte-specific knockdown of autophagy-related 5 (Atg5) (Hartleben et al. 2010). Furthermore, down-regulation of Cullin-5 expression in UPS as a cytoskeletal protein of E3 ligase in UPS can cause edema, proteinuria and glomerular structural abnormalities in zebrafish, and endoplasmic reticulum stress in podocytes (Mao et al. 2013). These findings reveal that it is necessary to explore the mechanisms of abnormally expressed genes in the podocyte cells in the development of albuminuria (Tryggvason et al. 2006).

Myosins constitute a large multigene family of actin-based molecular motors in eukaryotes (Guhathakurta et al. 2018; Heissler & Sellers 2016). According to the amino acid sequence of ATP hydrolysis region, myosins can be divided into 24 categories. Among them, Class I myosin consists of Myo1a~Myo1h (Dumont et al. 2002). Myo1e contains a proline-rich TH2 and a Src-homology 3 (SH3) domain in addition to the TH1 domain (Cheng et al. 2012). Recent studies have shown that Myo1e is involved in the development of proteinuria. In 2009, Krendel et al. reported that Myo1e is expressed in glomerular epithelial cells of mice (Krendel et al. 2009). In mice with Myo1e knockdown, disappearance of podocytes and thickening of the basement membrane are found, eventually leading to proteinuria (Chase et al. 2012). Moreover, mutated Myo1e is detected in patients with proteinuria appear (Mele et al. 2011; Sanna-Cherchi et al. 2011). These studies indicate that Myo1e in podocytes could be involved in the development of proteinuria, which requires further exploration.

Endocytosis is a process of transporting extracellular substances into cells through the deformation movement of the plasma membrane (Doherty & McMahon 2009). Studies have been found that albumin can directly induce podocyte and glomerular injury (Agrawal & Smoyer 2017). As an example, Notch signaling activation promotes the effect of Dynamin-dependent podocyte endocytosis of nephrin, leading to proteinuria (Waters et al. 2012). Both in vitro and in
vivo studies have found that podocytes possess the function of endocytic albumin with time- and concentration-dependent manners (He et al. 2011). After albumin aggregation in podocytes, knockdown of CD2AP and activation of TRPC6 channels could induce endoplasmic reticulum stress via mediating Ca\(^{2+}\) influx, thereby promoting podocyte apoptosis (He et al. 2011). These studies demonstrate that the endocytic BSA in podocytes is associated with the formation of proteinuria, but the specific mechanisms are poorly understood. Based on above studies, we hypothesized that overexpression of Myo1e could promote albumin endocytosis by mouse glomerular podocytes mediated by Dynamin.

**Materials and methods:**

**Cell culture**

Murine kidney podocyte cell line MPC5 (Mount Sinai School of Medicine, New York, NY) were cultured in RPMI-1640 medium (SH30809.01B, Hyclone, China) plus 10% fetal bovine serum (SH30084.03, Hyclone, China) and 10 U/ml mouse recombinant interferon-\(\gamma\) at 33°C with 5% CO\(_2\). To induce differentiation, MPC3 cells were cultured for 14 days at 37 °C without interferon-\(\gamma\).

**Endocytosis**

MPC5 cells were seeded in 6-well plates (2.5\(\times\)10\(^5\)/well) in three duplicates. After 24h, the cells were treated with different concentrations (0 ug/ml, 100 ug/ml, 250ug/ml, 500ug/ml, 1mg/ml) of FITC-BSA (SF063, Solarbio, China) for 4h in the dark. Flow cytometry was used to evaluate the endocytosis.

**MTT assay**

The MPC5 cells were seeded in 7\(\times\)10\(^3\)/well. 200ul of FITC-BSA (0 ug/ml, 100 ug/ml, 250ug/ml, 500ug/ml, 1mg/ml) and RPMI-1640 medium suspension was added to each well, and cultured at 37 °C for 4h. After medium change, the cells were cultured for another 24h. After that, the cells were treated with 20 ul MTT solution (5 mg/ml; M5655-1G, Sigma, USA) for 4h at 37°C. 150ul of DMSO was then added to each well for 10min. Absorbance was detected at 490nm.

**Plasmid transfection**

The Myole- or Dynamin- plasmid transfection was performed as our previous study (Jin et al. 2014). The shRNA targeting Myole (shMyole) and full length of Myole overexpression were synthesized in Invitrogen (Carlsbad, CA, USA). Furthermore, siRNAs targeting Dynamin (siDynamin) and full length of Dynamin overexpression were also synthesized. Then, the target sequences were cloned into pLenti6.3_MCS_IRES2-EGFP lentiviral vector. The lentiviral vectors with the target sequence of shMyole or siDynamin were transfected into HEK293FT cells. MPC5 cells were transfected with negative control shRNA (shNC), shMyole, empty pcDNA3.1 plasmid (empty vector), Myole-plasmid (Myole overexpression), negative control siRNA, siDynamin and Dynamin overexpression using Lipofectamine 2000 (Invitrogen), respectively. The cell morphology was observed under an optical microscope.

**Real-time quantitative PCR (RT-qPCR) analysis**
Total RNA was extracted from MPC5 cells using TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa, Beijing, China). The cDNA was synthesized with M-MLV (Vazyme, Jiang Su, China). RT-qPCR was carried out using SYBR Green I Master (Roche, Beijing, China). The primers were as follows: Mus Myo1e, 5'-ACAGTGCGCAACAACACACT-3' (forwards), 5'-TGATGCCAAGGCTTTGCTTC-3' (reverse); Mus Dynamin, 5'-TTTGCCAATGCTCAGCAGAG-3' (forwards), 5'-TGCTTGCTTGACATGAAGCC-3' (reverse); Mus GAPDH, 5'-TGATGCCAAGGCTTTGCTTC-3' (forwards), 5'-GATGGCAACAATCTCCACTTTG-3' (reverse). GAPDH was used as an internal control. The results were calculated using the $2^{-\Delta\Delta CT}$ method.

Immunofluorescence assay

The cells were seeded into a 24-well plate (1*10^5/well) at 37°C. After 24h of transfection with empty vector, Myo1e-plasmid, shNC and shMyo1e, the medium was removed from the 24-well plate. After washing twice in PBS, the expression of F-actin was stained by phalloidin and detected in MPC5 cells under a fluorescence microscopy (Olympus).

Western blot analysis

The MPC5 cells transfected with empty vector, Myo1e-plasmid, shNC and shMyo1e were lysed with RIPA buffer on ice for 10-20 min, and centrifuged at 12,000 rpm at 4°C for 3-5 min. Transfer the supernatant to a new 1.5 ml EP tube. The protein in the supernatant was collected for western blot analysis. After separating on SDS-PAGE, the protein was transferred onto PVDF membrane. After that, the membrane was blocked using 5% milk for 1h at room temperature. The primary antibody was diluted with 1% BSA/PBST, and the membrane was incubated overnight at 4 °C in a refrigerator. Afterwards, the PVDF membrane was incubated with a horseradish peroxidase-labeled secondary antibody diluted in 5% milk/PBST for 1h at room temperature. ECL was used to visualize the protein blots. The primary antibodies included goat anti-nephrin (1:100, Santa Cruz Biotechnology) and anti-podocin (1:100, Santa Cruz Biotechnology). The relative expression levels were corrected for GAPDH expression.

Flow cytometry for apoptosis

The MPC3 cells were plated in 6-well plates (2.5*10^5 cells/well) at 37°C and 5% CO₂. After 24h, Myo1e-plasmid and empty vector were transfected into MPC3 cells, respectively. After transfection for 24h, the cells were treated with Dynasore (160 uM) for 24h. After that, the cells were washed twice with PBS, and then added 500 ul of serum-free RPMI-1640 and different concentrations of 500 ug/ml FITC-BSA for 4h. Finally, the cells were collected for flow cytometry apoptosis assay. According to the manufactures’ protocols, apoptotic MPC5 cells were stained with Annexin V-FITC/Propidium Iodide (PI) (Beckman Coulter Trading Co., Ltd., China, Shanghai), and then analyzed on CytoFLEX S Flow Cytometer (BD Biosciences).

Statistical analysis

Graphpad Prism 7.0 (San Diego, CA, USA) was used for statistical analyses. All experiments were independently repeated at least three times. The data were presented as mean ± standard deviation (SD). Comparisons between two groups were analyzed with paired student’s t test. For pairwise multiple comparisons, one-way analysis of variance (ANOVA) was performed, followed by Tukey’s multiple comparison test. P-value<0.05 was considered statistically
Results:

The optimal concentration of BSA when treating MPC5 cells

We first determined the optimal concentration of endocytic BSA in podocytes. Flow cytometry was used to detect changes in fluorescence intensity after treatment with different concentrations of FITC-BSA for MPC-5 podocytes. The results showed that the higher the concentration of BSA, the higher the content of BSA phagocytized by MPC-5. When the concentration of BSA was 100ug/ml, the phagocytosis content of MPC5 was the lowest; when the concentration of BSA was 1mg/ml (p<0.001), the phagocytosis content of MPC5 was the highest (Figure 1A, B). Next, we examined the effect of BSA on the proliferation of MPC-5 podocytes. The proliferation of cells was detected by MTT assay after treatment with different concentrations of BSA for MPC-5. The results showed that BSA-FITC (250ug/ml, 500ug/ml and 1mg/ml) significantly inhibited MPC5 cell apoptosis (p<0.001; Figure 1C).

Immunofluorescence results showed that the content of BSA phagocytized by MPC-5 was increasing depending on the concentration of BSA, which was consistent with flow cytometry results (Figure 1D). Combined with above results, 500 ug/ml BSA was selected for subsequent experiments.

Figure 1. Identification of the optimal concentration of BSA when treating MPC5 cells. (A, B) Flow cytometry assay results showing the endocytosis effects of MPC5 cells treated with different concentrations (0 ug/ml, 100 ug/ml, 250ug/ml, 500ug/ml, 1mg/ml) of FITC-BSA. (C) The cell viability of MPC5 cells treated with different concentrations (0 ug/ml, 100 ug/ml, 250ug/ml, 500ug/ml, 1mg/ml) of FITC-BSA was evaluated using MTT assay. (D) Immunofluorescence showing the endocytosis effects of MPC5 cells treated with different concentrations of FITC-BSA. Scale bar, 50 μm. Compared to blank, ***p<0.001.

Overexpression of Myo1e enhances MPC5 glomerular podocyte endocytosis BSA

We further observed the effects of Myo1e on endocytosis BSA. RT-qPCR results showed that Myo1e was successfully overexpressed (Figure 2A; p<0.001) and inhibited (Figure 2B; p<0.01). Flow cytometry results showed that MPC5 endocytosis BSA increased significantly after overexpression of Myo1e compared to empty vector group (p<0.001), however, MPC5 endocytosis BSA was significantly decreased after Myo1e knockdown compared to shNC group (p<0.01; Figure 3A, B), which was consistent with immunofluorescence results (Figure 3C).

The above results indicated that Myo1e may promote MPC5 endocytic BSA. In addition, we observed the effect of abnormally expressed Myo1e on the morphology of MPC5 cells. As shown in Figure 4, compared with empty vector group, when Myo1e was overexpressed, we observed that the MPC5 cell morphology was irregular, the somas became larger, the foot processes were thinner and longer, and some of the podocytes also had a dendritic bifurcation. After the knockdown of Myo1e, the somas were shrunk, the edges were not neat, and the cells were fused.

Figure 2. The effects of Myo1e overexpression or knockdown. RT-qPCR results showed that
Myo1e was successfully overexpressed (A) or inhibited (B). **p<0.01; ***p<0.0001.

Figure 3. Overexpression of Myo1e enhances MPC5 glomerular podocyte endocytosis BSA. (A, B) Flow cytometry assay results showing the endocytosis effects of MPC5 cells. (C) Immunofluorescence results showing the endocytosis effects of MPC5 cells. Scale bar, 50 μm. **Myo1e overexpression vs. empty vector, p<0.001; ##shMyo1e vs. shNC, p<0.01.

Figure 4. Morphology changes of MPC5 cells when overexpression or knockdown of Myo1e (100×, 200×).

Myo1e may promote the expression of F-actin in MPC5 cells

We further analyzed the effect of Myo1e overexpression/knockout on F-actin expression in MPC5 cells. The immunofluorescence results showed that the fluorescence intensity of F-actin increased after overexpression of Myo1e gene compared with empty vector group (Figure 5). However, the fluorescence intensity of F-actin decreased when Myo1e expression was inhibited compared to shNC group. The experimental results suggested that Myo1e may promote the expression of F-actin in MPC5 cells.

Figure 5. Immunofluorescence detection of F-actin expression in MPC5 cells treated with Myo1e overexpression or knockdown. Scale bar, 50 μm.

Myo1e may promote the expression of nephrin and podocin in MPC5 cells

The western blot analysis results showed that the expression of nephrin and podocin increased after Myo1e overexpression compared with empty vector group (Figure 6A-C). However, the expression of nephrin and podocin decreased when knockdown Myo1e compared to shNC group (Figure 6A-C). Our results indicated that the Myo1e may promote the expression of nephrin and podocin in MPC5 cells.

Figure 6. Western blot analyses showing the expression levels of nephrin and podocin in MPC5 cells treated with Myo1e overexpression/knockdown. (A) Representative images of protein blots. (B) The expression levels of nephrin. (C) The expression levels of podocin. ** p<0.01; *** p<0.001.

Myo1e may promote MPC5 cell apoptosis

The flow cytometry results showed that compared with the empty vector group, after overexpression of Myo1e, the apoptosis of MPC5 cells significantly increased (p<0.05; Figure 7A, B). However, the apoptosis of MPC5 cells significantly decreased when Myo1e knockdown compared to shNC group (p<0.001; Figure 7A, B). The experimental results indicated that Myo1e may promote apoptosis of MPC5 cells.

Figure 7. Myo1e may promote MPC5 cell apoptosis. (A, B) Flow cytometry showing the apoptosis of MPC5 cells when treated with Myo1e overexpression/knockdown. * Myo1e overexpression vs. empty vector, p<0.05; ### shMyo1e vs. shNC, p<0.001.

Myo1e might promote MPC5 endocytosis BSA mediated by Dynamin

We further explored how Myo1e enhanced glomerular podocyte endocytosis of BSA. From the experimental results, it was found that MPC5 endocytosis BSA was significantly reduced after MPC5 cells treated with a GTPase inhibitor of Dynamin, Dynasore (Figure 8A, B). In addition, compared with the MPC5 + Myo1e overexpression group, MPC5 endocytosis BSA was significantly inhibited after MPC5 cells treated with Dynasore (Figure 8A, B). The effect of
Dynosore on both Myo1e and Dynamin expression was examined using RT-qPCR. The results showed that Dynosore significantly inhibited the expression of Dynamin (Figure 8C), however, it did not affect the expression of Myo1e (Figure 8D). Above results indicated that Dynasore can inhibit MPC5 endocytosis BSA.

As shown in Figure 9A, RT-qPCR results showed that Dynamin was successfully overexpressed (p<0.001). Furthermore, three siRNAs targeting Dynamin was synthesized. RT-qPCR results showed that MPC5 cells treated with Dynamin-siRNA2 had the lowest expression levels of Dynamin (p<0.001; Figure 9B). Thus, Dynamin-siRNA2 was used for further experiments. Immunofluorescence results showed that Dynamin knockdown inhibited MPC5 endocytosis BSA, while its overexpression promoted MPC5 endocytosis BSA (Figure 10).

Furthermore, Dynamin knockdown decreased the effect of MPC5 endocytosis BSA induced by Myo1e overexpression (Figure 10). We also found that Dynamin overexpression ameliorated the inhibitory effect of MPC5 endocytosis BSA induced by Myo1e knockdown (Figure 10). These results indicated that podocytes mediate albumin endocytosis through Myo1e-Dynamin-albumin.

**Figure 8.** Myo1e might promote MPC5 endocytosis BSA mediated by Dynamin. (A, B) Flow cytometry showing the endocytosis effects of MPC5 cells. (C, D) RT-qPCR showing the effect of Dynosore on both Myo1e and Dynamin expression. * p<0.05; ** p<0.01; *** p<0.001.

**Figure 9.** The effects of Dynamin overexpression or knockdown. RT-qPCR results showed that Dynamin was successfully overexpressed (A) or silenced (B). ***p<0.0001.

**Figure 10.** Immunofluorescence showing Myo1e might promote MPC5 endocytosis BSA mediated by Dynamin. Scale bar, 50 μm.

**Discussion:**

Proteinuria, mainly characterized by albuminuria, is not only a marker but also a known risk factor for progressive glomerular disease. Tubulointerstitial damage has been a field of widespread interest in this animal model. In addition, in vitro studies have revealed the role of serum albumin and its binding factors as mediators of proximal tubule cell damage, however, its molecular role in podocytes is not well understood. The response of podocytes to serum albumin includes albumin endocytosis and apoptosis. Myo1e plays an important role in renal function. Previous research has reported that the podocyte-specific knockout myo1e was performed using Cre-mediated recombination controlled by the podocin promoter (Chase et al. 2012). Loss of Myo1e in podocytes results in proteinuria, disappearance of the podocyte process and disintegration of the glomerular basement membrane. Podocytes can endocytose proteins, including albumin, immunoglobulins and transferrin, in a receptor-mediated manner. In our previous studies, we analyzed endocytic FITC-transferrin by podocyte analysis by quantitative analysis and fluorescence microscopy. After co-culture of podocytes with FITC-transferrin, the number of cells with FITC-positive vesicles in somatic cells treated with Myo1e knockdown was significantly decreased. However, FITC-transferrin was observed in abundant large vesicles in podocytes, especially in podocytes overexpressing Myo1e. Our previous study indicated that inhibition of Myo1e expression may reduce the efficiency of endocytic FITC-transferrin in...
podocytes. Our previous study has identified that Myo1e was expressed in the mouse podocytes of glomeruli, furthermore, overexpression of Myo1e may promote cellular endocytosis, migration, and adhesion (Jin et al. 2014). However, the specific mechanisms remain unclear. BSA is a main protein component of proteinuria, therefore, in our current study, we observed the podocyte endocytosis of FITC-BSA by fluorescence microscopy in a concentration-dependent manner. The MTT assay showed that FITC-BSA inhibited podocyte proliferation in a concentration-dependent manner.

In this study, we found that overexpression/knockdown of Myo1e can cause changes in the function and morphology of endocytic albumin in podocytes. Our results showed that overexpression of Myo1e promoted the ability of podocytes to endocytosis and while knockdown of Myo1e inhibited the ability of podocytes to endocytosis. Renal biopsy in patients with proteinuria usually manifests as the disappearance of podocyte foot processes. We found that MPC5 cells treated with knockdown of Myo1e appeared foot process fusion, which was contrary to MPC5 cells treated with overexpression of Myo1e. Myo1e may ameliorate podocyte foot process fusion of patients with proteinuria (Perysinaki et al. 2011).

F-actin cytoskeletal disruption is a typical characteristic of podocyte injury. F-actin cytoskeleton has been shown to be critical for maintaining the specific morphology of podocyte foot processes (Allison 2015; Hu et al. 2017; Schiffer et al. 2015). Destruction of the F-actin cytoskeleton in podocytes results in the disappearance of the foot process and is associated with the pathogenesis of proteinuria (Ni et al. 2018). In our study, immunofluorescence results showed that Myo1e overexpression promoted the expression of F-actin in MPC5 cells, which was contrary to its knockdown. Thus, Myo1e may play an important role in podocyte endocytosis by regulating the actin cytoskeleton F-actin.

Slit membrane proteins are essential molecular components of the glomerular filtration barrier and are also involved in actin polymerization (Kim et al. 2016). As podocyte-specific proteins, nephrin and podocin play important roles in the function of the glomerular filtration barrier (Ni et al. 2017). The disappearance of the foot process may be due to diaphragm rupture and podocyte damage. Nephrin is a structural component of a slit membrane formed by adjacent podocytes (Ruotsalainen et al. 1999). The absence of nephrin contributes to proteinuria and foot process effacement (Teng et al. 2016). Podocin is a key factor in maintaining the steady state of the slit diaphragm. Our results showed that overexpression of Myo1e promoted the expression of nephrin and podocin in MPC5 cells. However, the expression of nephrin and podocin decreased when knockdown of Myo1e in MPC5 cells. These results indicated that Myo1e may be involved in maintaining slit membrane by nephrin and podocin. Increasing evidence suggests that the expression of nephrin and podocin could be regulated by many factors. For example, microRNA-29a may promote nephrin acetylation to improve hyperglycemia-induced podocyte dysfunction (Lin et al. 2014). Another research found that vitamin D3 ameliorates podocyte injury via targeting nephrin (Trohatou et al. 2017).

Podocyte injury has been shown to contribute to the development of proteinuria (Li et al. 2019). Our findings showed that overexpression of Myo1e promoted apoptosis of MPC5 cells that were co-cultured with BSA, indicating that overexpression of Myo1e may induce podocyte
injury. There are several pathways of albumin endocytosis, such as Dynamin-dependent podocyte endocytosis, FcRn-mediated albumin transcytosis (Kinugasa et al. 2011), clathrin-mediated endocytosis (Soda et al. 2012), caveolin-mediated endocytosis (Dobrinskikh et al. 2014), fluid phase-endocytosis (Palm et al. 2015) and dynein-microtubule related vesicle transport (Tojo et al. 2017). Dynamin plays a crucial role in maintaining the structure and function of the glomerular filtration barrier. Dynamin regulates the actin cytoskeleton and the turnover of nephrin in podocytes, furthermore, knockdown of dynamin leads to proteinuria (Khalil et al. 2019). Dynamin-dependent podocyte endocytosis is one of the pathways of albumin endocytosis. In our study, we found that Dynasore attenuated the increase in endocytosis of albumin induced by Myo1e overexpression. As a GTPase inhibitor of Dynamin, these results indicated that podocytes might mediate albumin endocytosis by Myo1e-Dynamin-Albumin.

**Conclusion:**

In our study, our results showed that Myo1e promoted podocyte endocytic albumin, however, after inhibition of Myo1e, podocyte foot process fusion was observed. Furthermore, we found that Myo1e promoted apoptosis of podocytes. Myo1e elevated the expression of podocyte-specific molecules (nephrin and podocin) and cytoskeleton F-actin in podocyte endocytic albumin. Dynasore attenuated the increase in endocytosis of albumin induced by Myo1e overexpression, suggesting that podocytes might mediate albumin endocytosis via Myo1e-Dynamin-Albumin.

**Abbreviations:**

BSA: bovine serum albumin.

**Declarations:**

1. Acknowledgements
Not applicable.

2. Funding
This work was funded by The Zhejiang Provincial Natural Science Foundation of China (LH14H050002).

3. Ethics approval and consent to participate
Not applicable.

4. Consent for publication
Not applicable.

5. Conflicts of Interest
The authors declare no conflicts of interest.

**References:**
Agrawal S, and Smoyer WE. 2017. Role of albumin and its modifications in glomerular injury. *Pflugers Arch* 469:975-982. 10.1007/s00424-017-2029-4

Allison SJ. 2015. Chronic kidney disease: Actin cytoskeleton alterations in podocytes: a therapeutic target for chronic kidney disease. *Nat Rev Nephrol* 11:385. 10.1038/nrneph.2015.79

Brosius FC, and Coward RJ. 2014. Podocytes, signaling pathways, and vascular factors in diabetic kidney disease. *Adv Chronic Kidney Dis* 21:304-310. 10.1053/j.ackd.2014.03.011

Chase SE, Encina CV, Stolzenburg LR, Tatum AH, Holzman LB, and Krendel M. 2012. Podocyte-specific knockout of myosin 1e disrupts glomerular filtration. *Am J Physiol Renal Physiol* 303:F1099-1106. 10.1152/ajprenal.00251.2012

Cheng J, Grassart A, and Drubin DG. 2012. Myosin 1E coordinates actin assembly and cargo trafficking during clathrin-mediated endocytosis. *Mol Biol Cell* 23:2891-2904. 10.1091/mbc.E11-04-0383

Dobrinskikh E, Okamura K, Kopp JB, Doctor RB, and Blaine J. 2014. Human podocytes perform polarized, caveolae-dependent albumin endocytosis. *Am J Physiol Renal Physiol* 306:F941-951. 10.1152/ajprenal.00532.2013

Doherty GJ, and McMahon HT. 2009. Mechanisms of endocytosis. *Annu Rev Biochem* 78:857-902. 10.1146/annurev.biochem.78.081307.110540

Dumont RA, Zhao YD, Holt JR, Bahlner M, and Gillespie PG. 2002. Myosin-I isozymes in neonatal rodent auditory and vestibular epithelia. *J Assoc Res Otolaryngol* 3:375-389. 10.1007/s101620020049

Guhathakurta P, Prochniewicz E, and Thomas DD. 2018. Actin-Myosin Interaction: Structure, Function and Drug Discovery. *Int J Mol Sci* 19. 10.3390/ijms19092628

Han X, Li Q, Wang C, and Li Y. 2019. MicroRNA-204-3p Attenuates High Glucose-Induced MPC5 Podocytes Apoptosis by Targeting Braykinin B2 Receptor. *Exp Clin Endocrinol Diabetes* 127:387-395. 10.1055/a-0630-0173

Hartleben B, Godel M, Meyer-Schwesinger C, Liu S, Ulrich T, Kobler S, Wiech T, Grahammer F, Arnold SJ, Lindenmeyer MT, Cohen CD, Pavenstadt H, Kerjaschki D, Mizushima N, Shaw AS, Walz G, and Huber TB. 2010. Autophagy influences glomerular disease susceptibility and maintains podocyte homeostasis in aging mice. *J Clin Invest* 120:1084-1096. 10.1172/jci39492

He F, Chen S, Wang H, Shao N, Tian X, Jiang H, Liu J, Zhu Z, Meng X, and Zhang C. 2011. Regulation of CD2-associated protein influences podocyte endoplasmic reticulum stress-mediated apoptosis induced by albumin overload. *Gene* 484:18-25. 10.1016/j.gene.2011.05.025

Heissler SM, and Sellers JR. 2016. Various Themes of Myosin Regulation. *J Mol Biol* 428:1927-1946. 10.1016/j.jmb.2016.01.022

Hu M, Fan M, Zhen J, Lin J, Wang Q, Lv Z, and Wang R. 2017. FAK contributes to proteinuria in hypercholesterolaemic rats and modulates podocyte F-actin re-organization via activating p38 in response to ox-LDL. *J Cell Mol Med* 21:552-567. 10.1111/jcmm.13001
Jin X, Wang W, Mao J, Shen H, Fu H, Wang X, Gu W, Liu A, Yu H, Shu Q, and Du L. 2014. Overexpression of Myo1e in mouse podocytes enhances cellular endocytosis, migration, and adhesion. *J Cell Biochem* 115:410-419. 10.1002/jcb.24676

Khalil R, Koop K, Kreutz R, Spaink HP, Hogendoorn PC, Bruijn JA, and Baelde HJ. 2019. Increased dynamin expression precedes proteinuria in glomerular disease. *J Pathol* 247:177-185. 10.1002/path.5181

Kim SH, Kim HJ, and Kim CW. 2016. GLCCI1 is a novel component associated with the PI3K signaling pathway in podocyte foot processes. *Exp Mol Med* 48:e233. 10.1038/emm.2016.28

Kinugasa S, Tojo A, Sakai T, Tsumura H, Takahashi M, Hirata Y, and Fujita T. 2011. Selective albuminuria via podocyte albumin transport in puromycin nephrotic rats is attenuated by an inhibitor of NADPH oxidase. *Kidney Int* 80:1328-1338. 10.1038/ki.2011.282

Krendel M, Kim SV, Willinger T, Wang T, Kashgarian M, Flavell RA, and Mooseker MS. 2009. Disruption of Myosin 1e promotes podocyte injury. *J Am Soc Nephrol* 20:86-94. 10.1681/asn.2007111172

Li X, Ma A, and Liu K. 2019. Geniposide alleviates lipopolysaccharide-caused apoptosis of murine kidney podocytes by activating Ras/Raf/MEK/ERK-mediated cell autophagy. *Artif Cells Nanomed Biotechnol* 47:1524-1532. 10.1080/21691401.2019.1601630

Lin CL, Lee PH, Hsu YC, Lei CC, Ko JY, Chuang PC, Huang YT, Wang SY, Wu SL, Chen YS, Chiang WC, Reiser J, and Wang FS. 2014. MicroRNA-29a promotion of nephrin acetylation ameliorates hyperglycemia-induced podocyte dysfunction. *J Am Soc Nephrol* 25:1698-1709. 10.1681/asn.2013050527

Mao J, Wang D, Mataleena P, He B, Niu D, Katayama K, Xu X, Ojala JR, Wang W, Shu Q, Du L, Liu A, Pikkarainen T, Patrakka J, and Tryggvason K. 2013. Myo1e impairment results in actin reorganization, podocyte dysfunction, and proteinuria in zebrafish and cultured podocytes. *PLoS One* 8:e72750. 10.1371/journal.pone.0072750

Mele C, Iatropoulos P, Donadelli R, Calabria A, Maranta R, Cassis P, Buelli S, Tomasoni S, Piras R, Krendel M, Bettoni S, Morigi M, Delledonne M, Pecoraro C, Abbate I, Capobianchi MR, Hildebrandt F, Otto E, Schaef er F, Macciardi F, Ozaltin F, Emre S, Ibsrligliu T, Benigni A, Remuzzi G, and Noris M. 2011. MYO1E mutations and childhood familial focal segmental glomerulosclerosis. *N Engl J Med* 365:295-306. 10.1056/NEJMoa1101273

Ni Y, Wang X, Yin X, Li Y, Liu X, Wang H, Liu X, Zhang J, Gao H, Shi B, and Zhao S. 2018. Plectin protects podocytes from adriamycin-induced apoptosis and F-actin cytoskeletal disruption through the integrin alpha6beta4/FAK/p38 MAPK pathway. *J Cell Mol Med* 22:5450-5467. 10.1111/jcm.13816

Ni Z, Tao L, Xiaohui X, Zelin Z, Jiangang L, Zhao S, Weikang H, Hongchao X, Qiujing W, and Xin L. 2017. Polydatin impairs mitochondria fitness and ameliorates podocyte injury by suppressing Drp1 expression. *J Cell Physiol* 232:2776-2787. 10.1002/jcp.25943

Palm W, Park Y, Wright K, Pavlova NN, Tuveson DA, and Thompson CB. 2015. The Utilization of Extracellular Proteins as Nutrients Is Suppressed by mTORC1. *Cell*
Perysinaki GS, Moysiadiis DK, Bertsias G, Giannopoulou I, Kyriacou K, Nakopoulou L, Boumpas DT, and Daphnis E. 2011. Podocyte main slit diaphragm proteins, nephrin and podocin, are affected at early stages of lupus nephritis and correlate with disease histology. *Lupus* 20:781-791. 10.1177/0961203310397412

Ruotsalainen V, Ljungberg P, Wartiovaara J, Lenkkeri U, Kestila M, Jalanko H, Holmberg C, and Tryggvason K. 1999. Nephrin is specifically located at the slit diaphragm of glomerular podocytes. *Proc Natl Acad Sci U S A* 96:7962-7967. 10.1073/pnas.96.14.7962

Sanna-Cherchi S, Burgess KE, Nees SN, Caridi G, Weng PL, Dagnino M, Bodria M, Carrea A, Allegretta MA, Kim HR, Perry BJ, Gigante M, Clark LN, Kisselev S, Cusi D, Gesualdo L, Allegri L, Scolari F, D'Agati V, Shapiro LS, Pecoraro C, Palomer T, Ghiggeri GM, and Gharavi AG. 2011. Exome sequencing identified MYO1E and NEIL1 as candidate genes for human autosomal recessive steroid-resistant nephrotic syndrome. *Kidney Int* 80:389-396. 10.1038/ki.2011.148

Schiessl IM, Hammer A, Kattler V, Gess B, Theilig F, Witzgall R, and Castrop H. 2016. Intravital Imaging Reveals Angiotensin II-Induced Transcytosis of Albumin by Podocytes. *J Am Soc Nephrol* 27:731-744. 10.1681/asn.2014111125

Schiffer M, Teng B, Gu C, Shechedrina VA, Kasaikina M, Pham VA, Hanke N, Rong S, Gueler F, Schroder P, Tossidou I, Park JK, Staggs L, Haller H, Erschow S, Hilfiker-Kleiner D, Wei C, Chen C, Tardi N, Hakroush S, Selig MK, Vasilyev A, Merscher S, Reiser J, and Sever S. 2015. Pharmacological targeting of actin-dependent dynamin oligomerization ameliorates chronic kidney disease in diverse animal models. *Nat Med* 21:601-609. 10.1038/nm.3843

Soda K, Balkin DM, Ferguson SM, Paradise S, Milosevic I, Giovedi S, Volpicelli-Daley L, Tian X, Wu Y, Ma H, Son SH, Zheng R, Moeckel G, Cremona O, Holzman LB, De Camilli P, and Ishibe S. 2012. Role of dynamin, synaptojanin, and endophilin in podocyte foot processes. *J Clin Invest* 122:4401-4411. 10.1172/jci65289

Teng B, Schroder P, Muller-Deile J, Schenk H, Staggs L, Tossidou I, Dikic I, Haller H, and Schiffer M. 2016. CIN85 Deficiency Prevents Nephrin Endocytosis and Proteinuria in Diabetes. *Diabetes* 65:3667-3679. 10.2337/db16-0081

Togita A, Hatakeyama S, Kinugasa S, Fukuda S, and Sakai T. 2017. Enhanced podocyte vesicle transport in the nephrotic rat. *Med Mol Morphol* 50:86-93. 10.1007/s00795-016-0151-6

Trohatou O, Tsilibray EF, Charonis A, Iatrou C, and Drossopoulou G. 2017. Vitamin D3 ameliorates podocyte injury through the nephrin signalling pathway. *J Cell Mol Med* 21:2599-2609. 10.1111/jcmm.13180

Tryggvason K, Patrakka J, and Wartiovaara J. 2006. Hereditary proteinuria syndromes and mechanisms of proteinuria. *N Engl J Med* 354:1387-1401. 10.1056/NEJMra052131

Waters AM, Wu MY, Huang YW, Liu GY, Holmyard D, Onay T, Jones N, Egan SE, Robinson LA, and Piscione TD. 2012. Notch promotes dynamin-dependent endocytosis of nephrin. *J Am Soc Nephrol* 23:27-35. 10.1681/asn.2011010027
Figure legends:

Figure 1. Identification of the optimal concentration of BSA when treating MPC5 cells. (A, B) Flow cytometry assay results showing the endocytosis effects of MPC5 cells treated with different concentrations (0 ug/ml, 100 ug/ml, 250ug/ml, 500ug/ml, 1mg/ml) of FITC-BSA. (C) The cell viability of MPC5 cells treated with different concentrations (0 ug/ml, 100 ug/ml, 250ug/ml, 500ug/ml, 1mg/ml) of FITC-BSA was evaluated using MTT assay. (D) Immunofluorescence showing the endocytosis effects of MPC5 cells treated with different concentrations of FITC-BSA. Scale bar, 50 μm. Compared to blank, ***p<0.001.

Figure 2. The effects of Myo1e overexpression or knockdown. RT-qPCR results showed that Myo1e was successfully overexpressed (A) or inhibited (B). **p<0.01; ***p<0.001.

Figure 3. Overexpression of Myo1e enhances MPC5 glomerular podocyte endocytosis BSA. (A, B) Flow cytometry assay results showing the endocytosis effects of MPC5 cells. (C) Immunofluorescence results showing the endocytosis effects of MPC5 cells. Scale bar, 50 μm. ***Myo1e overexpression vs. empty vector, p<0.001; ##shMyo1e vs. shNC, p<0.01.

Figure 4. Morphology changes of MPC5 cells when overexpression or knockdown of Myo1e (100×, 200×).

Figure 5. Immunofluorescence detection of F-actin expression in MPC5 cells treated with Myo1e overexpression or knockdown. Scale bar, 50 μm.

Figure 6. Western blot analyses showing the expression levels of nephrin and podocin in MPC5 cells treated with Myo1e overexpression/knockdown. (A) Representative images of protein blots. (B) The expression levels of nephrin. (C) The expression levels of podocin. ** p<0.01; *** p<0.001.

Figure 7. Myo1e may promote MPC5 cell apoptosis. (A, B) Flow cytometry showing the apoptosis of MPC5 cells when treated with Myo1e overexpression/knockdown. * Myo1e overexpression vs. empty vector, p<0.05; ### shMyo1e vs. shNC, p<0.001.

Figure 8. Myo1e might promote MPC5 endocytosis BSA mediated by Dynamin. (A, B) Flow cytometry showing the endocytosis effects of MPC5 cells. (C, D) RT-qPCR showing the effect of Dynosore on both Myo1e and Dynamin expression. * p<0.05; ** p<0.01; *** p<0.001.

Figure 9. The effects of Dynamin overexpression or knockdown. RT-qPCR results showed that Dynamin was successfully overexpressed (A) or silenced (B). ***p<0.0001.

Figure 10. Immunofluorescence showing Myo1e might promote MPC5 endocytosis BSA mediated by Dynamin. Scale bar, 50 μm.
Figure 1

Identification of the optimal concentration of BSA when treating MPC5 cells.

(A, B) Flow cytometry assay results showing the endocytosis effects of MPC5 cells treated with different concentrations (0 ug/ml, 100 ug/ml, 250ug/ml, 500ug/ml, 1mg/ml) of FITC-BSA. (C) The cell viability of MPC5 cells treated with different concentrations (0 ug/ml, 100 ug/ml, 250ug/ml, 500ug/ml, 1mg/ml) of FITC-BSA was evaluated using MTT assay. (D) Immunofluorescence showing the endocytosis effects of MPC5 cells treated with different concentrations of FITC-BSA. Scale bar, 50 μm. Compared to blank, ***p<0.001.
Figure 2

The effects of Myo1e overexpression or knockdown.

RT-qPCR results showed that Myo1e was successfully overexpressed (A) or inhibited (B).

**p<0.01; ***p<0.0001.
Figure 3

Overexpression of Myo1e enhances MPC5 glomerular podocyte endocytosis BSA.

(A, B) Flow cytometry assay results showing the endocytosis effects of MPC5 cells. (C) Immunofluorescence results showing the endocytosis effects of MPC5 cells. Scale bar, 50 μm.

***Myo1e overexpression vs. empty vector, p<0.001; ##shMyo1e vs. shNC, p<0.01.
Figure 4

Morphology changes of MPC5 cells when overexpression or knockdown of Myo1e (100×, 200×).
| Condition       | 100× Image | 200× Image |
|-----------------|------------|------------|
| Blank           | ![Blank 100×](#) | ![Blank 200×](#) |
| Empty vector    | ![Empty vector 100×](#) | ![Empty vector 200×](#) |
| Myolet overexpression | ![Myolet overexpression 100×](#) | ![Myolet overexpression 200×](#) |
| shNC            | ![shNC 100×](#) | ![shNC 200×](#) |
| shMyolet        | ![shMyolet 100×](#) | ![shMyolet 200×](#) |
Figure 5

Immunofluorescence detection of F-actin expression in MPC5 cells treated with Myo1e overexpression or knockdown. Scale bar, 50 μm.
**F-actin** | **DAPI** | **Merge**
--- | --- | ---
**Blank** | | |
**Empty vector** | | |
**Myocyte overexpression** | | |
**shNC** | | |
**shMyocyte** | | |
Figure 6

Western blot analyses showing the expression levels of nephrin and podocin in MPC5 cells treated with Myo1e overexpression/knockdown.

(A) Representative images of protein blots. (B) The expression levels of nephrin. (C) The expression levels of podocin. ** p<0.01; *** p<0.001.
Figure 7

Myo1e may promote MPC5 cell apoptosis.

(A, B) Flow cytometry showing the apoptosis of MPC5 cells when treated with Myo1e overexpression/knockdown. * Myo1e overexpression vs. empty vector, p<0.05; ### shMyo1e vs. shNC, p<0.001.
Myo1e might promote MPC5 endocytosis BSA mediated by Dynamin.

(A, B) Flow cytometry showing the endocytosis effects of MPC5 cells. (C, D) RT-qPCR showing the effect of Dynosore on both Myo1e and Dynamin expression. * p<0.05; ** p<0.01; *** p<0.001.
Figure 9

The effects of Dynamin overexpression or knockdown.

RT-qPCR results showed that Dynamin was successfully overexpressed (A) or silenced (B). ***p<0.0001.
Figure 10

Immunofluorescence showing Myo1e might promote MPC5 endocytosis BSA mediated by Dynamin. Scale bar, 50 μm.