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Overexpression of Myo1e in Mouse Podocytes Enhances Cellular Endocytosis, Migration, and Adhesion

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ABSTRACT

Podocytes are a terminally differentiated and highly specialized cell type in the glomerulus that forms a crucial component of the glomerular filtration barrier. Recently, Myo1e was identified in the podocytes of glomeruli. Myo1e podocyte-specific knockout mice exhibit proteinuria, podocyte foot process effacement, glomerular basement membrane disorganization, signs of chronic renal injury, and kidney inflammation. After overexpression of Myo1e in a conditionally immortalized mouse podocyte cell line (MPC5), podocyte migration was evaluated via transwell assay, endocytosis was evaluated using FITC-transferrin, and adhesion was evaluated using a detachment assay after puromycin aminonucleoside treatment. Myo1e overexpression significantly increased the adherence of podocytes. ANOVA analysis indicated significant differences for cell adhesion between the overexpression and control groups (overexpression vs. control, $t = 11.3199$, $P = 0.005$; overexpression vs. negative control, $t = 12.0570$, $P = 0.0006$). Overexpression of Myo1e inhibited puromycin aminonucleoside-induced podocyte detachment, and the number of cells remaining on the bottom of the culture plate increased. Cell migration was enhanced in Myo1e-overexpressing podocytes in the transwell migration assay. Internalization of FITC-transferrin also increased in Myo1e-overexpressing podocytes relative to control cells. Overexpression of Myo1e can enhance podocyte migration ability, endocytosis, and attachment to the glomerular basement membrane. Restoration of Myo1e expression in podocytes may therefore strengthen their functional integrity against environmental and mechanical injury. J. Cell. Biochem. 115: 410–419, 2014.

KEY WORDS: MYO1E; OVEREXPRESSION; PODOCYTE; MIGRATION; ENDOCYTOSIS; ADHESION

Podocytes are a terminally differentiated and highly specialized cell type in the glomerulus that form a crucial component of the glomerular filtration barrier [Greka and Mundel, 2011]. Podocyte damage leads to proteinuria, and podocyte injury occurs in many glomerular diseases and can progress to chronic renal failure. Elucidation of the regulation of the physiological properties of podocytes and the mechanisms of their cellular [Mathieson, 2011] response to injury may therefore advance the understanding of the pathogenesis of proteinuria and glomerular diseases.

Myo1e is one of two Src homology 3 domain-containing "long-tailed" type I myosins in vertebrates. Recently, Myo1e was identified in the podocytes of glomeruli [Krendel et al., 2009]. Myo1e knockout mice and Myo1e podocyte-specific knockout mice exhibit proteinuria, podocyte foot process effacement, glomerular basement membrane disorganization, signs of chronic renal injury, and kidney inflammation [Krendel et al., 2009; Chase et al., 2012]. Following specific knockdown of Myo1e in zebrafish, the injected larvae exhibited pericardial edema and pronephric cysts, consistent with the

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appearance of protein in the condensed incubation supernatant (our previous study). Furthermore, Mele et al. [2011] and Sanna-Cherchi et al. [2011] reported that two mutations (A159P and Y695X) in Myo1e segregated with focal segmental glomerulosclerosis in two independent pedigrees, indicating that Myo1e mutations are associated with childhood-onset glucocorticoid-resistant focal segmental glomerulosclerosis and that Myo1e plays a key role in podocyte function and the consequent integrity of the glomerular filtration barrier.

In the present study, we overexpressed Myo1e in mouse podocytes by transient transfection with a Myo1e cDNA plasmid and observed alterations in endocytosis, migration, and adhesion to the glomerular basement membrane (GBM).

MATERIALS AND METHODS

IMMUNOFLUORESCENCE

The methods and primary antibodies used have been previously described [Patrakka et al., 2007]. We raised antisera directed against novel glomerular proteins by purifying recombinant proteins with affinity tags and by immunizing New Zealand white (NZW) rabbits with these antigens using standard protocols (SVA, Uppsala, Sweden; KTH, Stockholm, Sweden). Briefly, for the production of Myo1e antigen, we generated mouse recombinant proteins. Myo1e residues 50 to 262 were cloned into the pET-28a(+) expression vector (Novagen, Madison, WI). The his-tagged Myo1e recombinant proteins were solubilized from inclusion bodies in 8 M urea. Then, Myo1e antigen was purified using sequential S-Sepharose ion exchange and Sephadex S-200 gel filtration columns (Amersham Biosciences).

For double-labeling experiments, we used anti-mouse synaptopodin (Progen, Heidelberg, Germany) and anti-human nephrin 50A9 antibodies [Ruotsalainen et al., 2000; Patrakka et al., 2007]. Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Microscopy was performed using a Leica standard fluorescent microscope or a standard Leica confocal laser-scanning microscope. For immunofluorescence staining, kidney samples were collected from adult human cadaver kidneys that were unsuitable for transplantation (obtained from the IV Department of Surgery of Helsinki, Finland under the body donation scheme in which the donor or the donor's relatives signed a consent form before the use of the body for research purposes) or patients with congenital nephrotic syndrome of the Finnish type (from the IV Department of Surgery of Helsinki, Finland). Informed written consent was obtained from all patients [Patrakka et al., 2007].

Expression of F-actin in glomerular tissues and cultured podocytes was detected by phalloidin staining.

VECTOR AND REAGENTS

The whole coding region of mouse Myo1e was cloned into the pcDNA3.1 plasmid (GenePharma Co., Ltd, Shanghai, China). The recombinant plasmid pcDNA3.1-Myo1e (Myo1e-plasmid) was extracted using a kit (EndoFree Plasmid Max kit, Qiagen, Hilden, Germany). The mouse Myo1e coding sequence was obtained from the web site www.ensembl.org (Gene ID: ENSMUSG00000032220).

PODOCYTE CULTURE

The conditionally immortalized mouse podocyte cell line (MPC5) used in this study was kindly provided by Dr. Peter Mundel (Mount Sinai School of Medicine, New York, NY). To propagate podocytes, the cells were cultured at 33°C in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 10 U/ml mouse recombinant interferon-γ (R&D Systems, Minneapolis, MN) to increase the expression of a thermosensitive T antigen. To induce differentiation, podocytes were grown under nonpermissive conditions at 37°C in the absence of interferon-γ for 14 days.

PLASMID TRANSFECTION

When the podocytes grew to approximately 70–90% confluence, they were transfected with the Myo1e-plasmid using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Briefly, 4 μg of Myo1e-plasmid was diluted in 250 μl of Opti-MEM I Reduced Serum Medium without serum and mixed gently. Then, 10 μL of Lipofectamine 2000 was diluted in 250 μl of Opti-MEM I Reduced Serum Medium, mixed gently, and incubated for 5 min at room temperature. After the 5-min incubation, the diluted Myo1e-plasmid was combined with the diluted Lipofectamine 2000 (total volume of 500 μl). The solution was mixed gently and incubated for 20 min at room temperature to allow DNA-Lipofectamine 2000 complexes to form. Then, 500 μl of Myo1e-plasmid-Lipofectamine 2000 complexes and 1.5 ml of Opti-MEM I Reduced Serum Medium were added to each well containing cells and medium. The cells were mixed gently by rocking the plate back and forth and were incubated at 37°C in a CO_2 incubator for 7–8 h. Then, the cells were cultured at 33°C in RPMI 1640 medium supplemented with 10% FBS and 10 U/ml mouse recombinant interferon-γ for a further 12–18 h until they were ready to be assayed for transgene expression. Podocytes transfected with an empty pcDNA3.1 plasmid (without Myo1e cDNA inside) were defined as the negative controls, and podocytes not subjected to any transfection were defined as the control.

REAL-TIME PCR FOR MYO1E EXPRESSION IN CULTURED PODOCYTES

Expression of Myo1e in podocytes in the negative control group transfected with an empty pcDNA3.1 plasmid, the control group without a plasmid, and the group transfected with Myo1e-plasmid was studied by real-time PCR following standard procedures (SYBR® Premix EX Taq). The primer sequences and sizes of the expected PCR products for Myo1e were as follows: upstream, ACCCATTCAAGCAAATGCCATAT; downstream, TGTCTTTCCAGCACCACTTTCAC; and size, 179 bp. The primer sequences and sizes of the expected PCR products for β-actin were as follows: upstream, CTGAGAGGGAAATCGTGCGTGAC; downstream, GAACCGCTCGTTGCCAATAGTGA; and size, 142 bp. The company that supplied the reagents did not supply the sequences of the probes for Myo1e and β-actin because the sequences are trade secrets.

WESTERN BLOT

Using Western blotting, we compared Myo1e expression among the negative control group transfected with the empty pcDNA3.1 plasmid, the control group without a plasmid, and the group transfected with the Myo1e-plasmid. Western blotting was performed

following standard procedures using polyvinyl difluoride membranes and a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences). As a positive loading control, we used a polyclonal anti–β-actin antibody (Protech, West Chester, PA).

ELISA MEASUREMENT OF MYO1E EXPRESSION IN CULTURED PODOCYTES AT DIFFERENT TIME POINTS

Expression of Myo1e in podocyte lysates was studied in the negative control group transfected with an empty pcDNA3.1 plasmid, the control group without a plasmid, and the group transfected with Myo1e-plasmid. The variation of Myo1e expression in cultured podocytes at different time points (12, 24, 36, and 48 h) was measured by ELISA (AD Company) following manufacturer's instructions.

Briefly, set blank wells, standard wells, and sample wells, respectively. Blank wells: don't add samples and horseradish peroxidase (HRP), Standard wells: Add 50 μL standard solution to standard wells. Sample wells: Add 50 μL of diluted sample. Add 100 μL horseradish peroxidase to each well, except blank well. Then

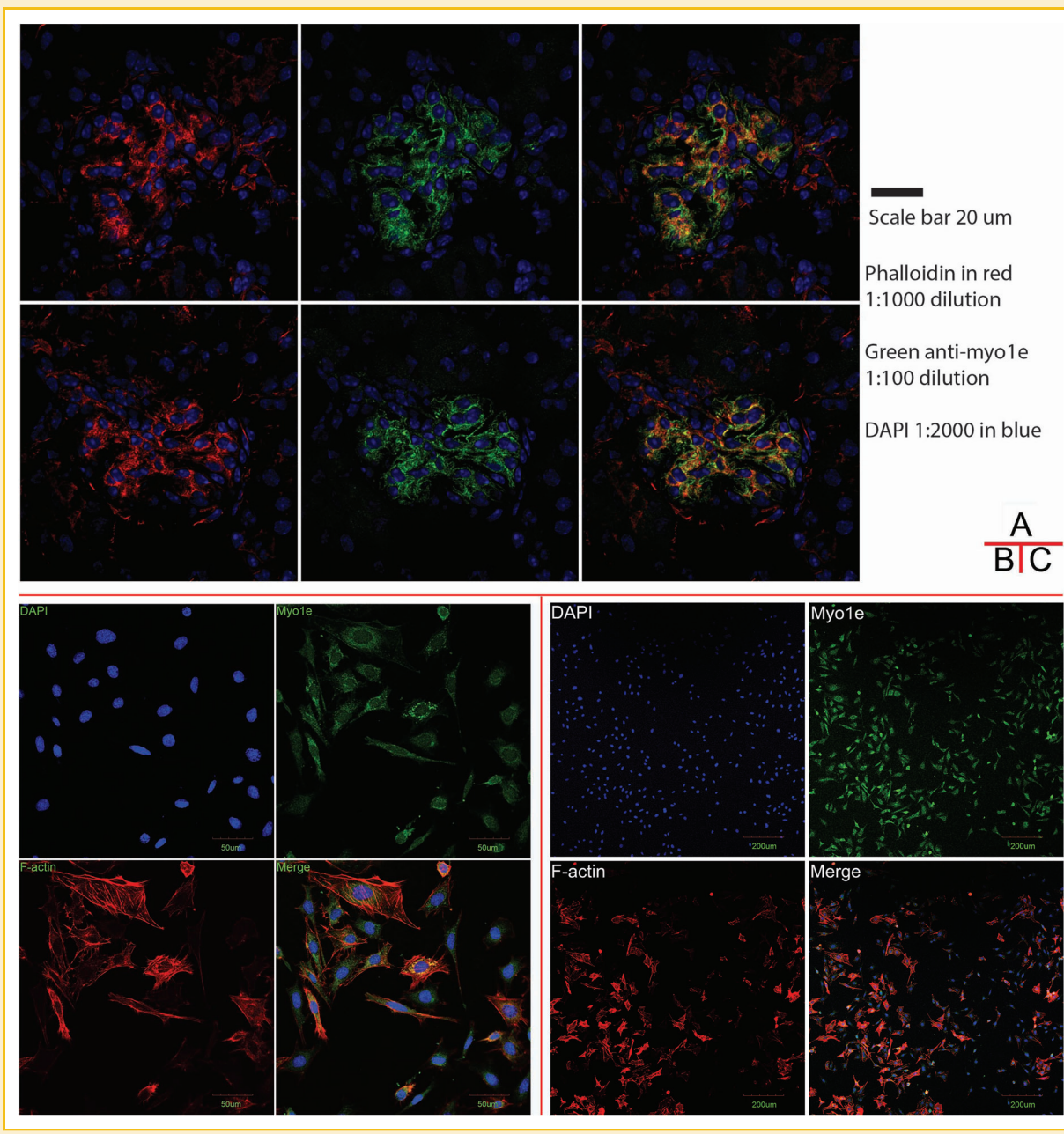

Fig. 1. Expression of Myo1e in the normal human kidney and cultured mouse podocytes. A: Co-expression of Myo1e and F-actin in normal human kidney cells as observed by immunofluorescence and confocal microscopy. Myo1e partially co-localized with F-actin in the podocyte foot processes. B and C: Co-expression of Myo1e and F-actin in cultured mouse podocytes observed by immunofluorescence and confocal microscopy. Myo1e was predominantly expressed in the peri-nuclear cytoplasm as a spot-like pattern. Myo1e expression was lower in the lamellipodia and cellular ridge structure than in the peri-nuclear cytoplasm.

seal with sealing membrane and incubate 60 min at 37°C with gentle shaking. Discard liquid, drying, fill each well with diluted washing liquid, oscillation for 30 s, discard the washing liquid with absorbent paper. Repeat five times, and pat dry. Add 50 μL chromogen solution A, then 50 μL chromogen solution B to each well. Gently mix, incubate for 10 min at 37°C away from light. Add 50 μL stop solution into each well to stop the reaction. For measurement, take blank well as zero, measure the optical density (OD) under 450 nm wave-length which should be carried out within 15 min after adding the stop solution. According to standards′ concentration and the corresponding OD values, calculate the standard curve linear regression equation, and then apply the OD values of the sample on the regression equation to calculate the corresponding sample′s concentration.

ADHESION ASSAYS

Six-well plates were coated with collagen I (10 μg/ml), with 1% bovine serum albumin as the control. Podocytes were harvested using trypsin/EDTA, resuspended in serum-free medium, and then allowed to attach at 37°C for 1 h. The unbound cells were removed by washing twice with PBS. The attached cells were fixed in 4% paraformaldehyde and counted. Two investigators recorded the cell attachment in these experiments. The cell counts were obtained by averaging the cell numbers from five wells.

DETACHMENT ASSAY

Wild-type and Myo1e overexpressing podocytes were evaluated after puromycin aminonucleoside (PAN)-induced detachment. All cells were cultured on six-well cell culture plates under nonpermissive conditions for 14 days before analysis. The fields of cells were marked, and the cells in each field were counted to establish a baseline. The cells were treated with 50 μg/ml PAN, and the cells in each field were counted at 24 h. The average cell counts of four fields in three independent sets of experiments were analyzed by Student′s *t*-test.

TRANSWELL MIGRATION ASSAY

For this assay, 24-well tissue culture plates with inserts containing a polycarbonate membrane with a pore size of 8 μm (Falcon) were used. Podocytes at the first passage were quiesced for 72 h, trypsinized, and resuspended in RPMI 1640 medium supplemented with 0.25% bovine serum albumin (migration medium). The podocytes and culture medium were subsequently loaded into the upper wells (2×10^4 cells in 100 μl) and incubated for 7 h at 37°C. Serum-containing medium as a

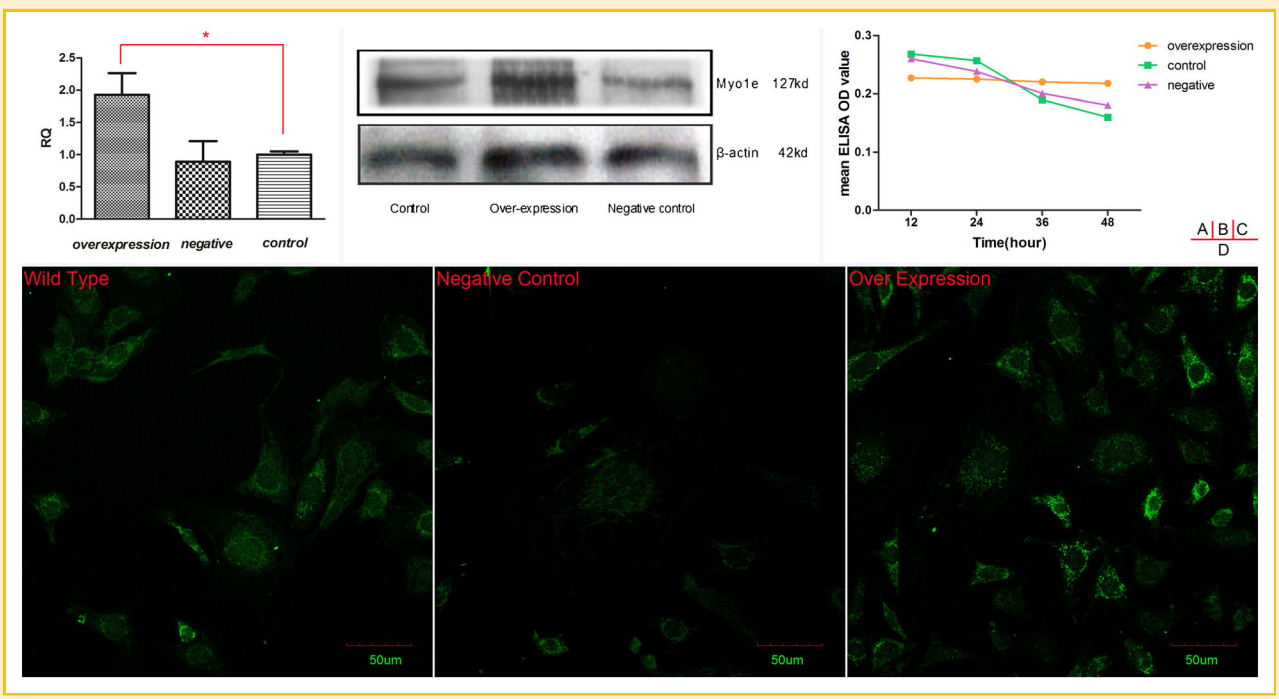

Fig. 2. Confirmation of Myo1e overexpression in cultured podocytes. A: mRNA expression of Myo1e in cultured podocytes determined by real-time PCR in three different groups. *Compared with the control groups, Myo1e expression was upregulated in podocytes from the overexpression group (overexpression vs. control, $t = 5.2567$, $P = 0.033$). β-actin was used as an internal control in RT-PCR. Also see Table I. B: Protein expression of Myo1e (127 kDa) in cultured podocytes measured by Western blot in three different groups. β-actin was used as a control (47 kDa) for the Western blots. Also see Table II. C: Myo1e expression at different time points (12–48 h) measured by ELISA. Expression of Myo1e was sustained for a longer time in the overexpression group than in the other two groups. Compared with the control groups, the expression of Myo1e was more stable in the Myo1e overexpression group after 48 h of podocyte culture. At the 48 h time point, Myo1e expression was more abundant in the overexpression group than in the control ($P < 0.001$) and negative control ($P = 0.005$) groups. Also see Table III. D: Myo1e expression in podocytes measured by immunofluorescence in different groups. In cultured mouse podocytes, Myo1e was predominately localized on the bottom surface of the plasma membrane and with lower enrichment at the lamellipodia tips. There was no significant difference in cell shape among the three different groups. a: Control without any plasmid transfection. b: Negative control transfected with an empty plasmid. c: Myo1e overexpressing podocytes transfected with the Myo1e-plasmid. Image is representative of > three experiments (400×).

TABLE I. Myo1e Expression in Podocytes by Real-Time PCR Among Control, Negative Control, and Overexpression Group

	n	Mean RQ (mean ± SD)
Control	3	0.4865 ± 0.0147
Overexpression	3	1.0092 ± 0.1716*
Negative control	3	0.4446 ± 0.1833

By ANOVA, there are significant difference in Myo1e expression among overexpression and control groups (overexpression vs. control, $t = 5.2567$, $P = 0.033$; overexpression vs. negative control, $t = 3.8948$, $P = 0.044$). No significant difference found between control and negative control ($t = 0.3944$, $P = 0.731$). *$P < 0.05$.

TABLE II. Myo1e Expression in Podocytes by Western Blot Among Three Groups

	n	Myo1e/β-actin (mean ± SD)
Control	3	0.6940 ± 0.0121
Overexpression	3	0.8117 ± 0.0146
Negative control	3	0.6775 ± 0.0141

By ANOVA, there are significant difference in Myo1e expression among overexpression and control groups (overexpression vs. control, $t = 10.7392$, $P = 0.04$; overexpression vs. negative control, $t = 11.4226$, $P = 0.038$). No significant difference found between control and negative control ($t = 1.5356$, $P = 0.1994$).

chemoattractant was placed in the well below. The cell suspension was aspirated, and the membranes were fixed with paraformaldehyde. The cells were removed from the upper surface of the membranes, rinsed in water, and stained for 10 min in crystal violet. The membranes were then detached from the inserts and mounted for microscopic examination. Cell nuclei on the underside of each membrane were counted in 12 random high-power fields (200 ×) under a light microscope. Podocyte migration in the experimental groups was expressed as the mean number of migrated cells per high-power field.

ENDOCYTOSIS

Cells were incubated with 50 μg/ml FITC-transferrin (Molecular Probes) in PBS for 15 min at 37°C. Coverslips were washed twice with ice-cold PBS. To remove cell surface transferrin, the cells were incubated for 2 min at 37°C in a pH 4.6 citrate buffer and re-equilibrated with two additional ice-cold PBS washes. The coverslips were fixed in a 4% formaldehyde solution at room temperature for 15 min. Excess formaldehyde was removed with 3–5 min washes in PBS. The fixed cells were observed under a fluorescence microscope.

ETHICAL CONSIDERATIONS

This study was approved by the ethical committees of The Children's Hospital of Zhejiang University School of Medicine, China.

RESULTS

MYO1E AND F-ACTIN DOUBLE STAINING IN NORMAL GLOMERULAR TISSUES AND CULTURED PODOCYTES

Staining of human kidney sections with antibodies against Myo1e revealed that in the kidney, Myo1e is predominantly expressed in the glomeruli (Fig. 1A). Myo1e partially co-localized with F-actin in podocyte foot processes.

Staining of cultured mouse podocytes with antibodies against Myo1e revealed that Myo1e is strongly expressed in the peri-nuclear cytoplasm as a spot-like pattern, meanwhile, Myo1e is also expressed in the lamellipodia tips and cellular ridge structure of podocytes though in a lower intensity (Fig. 1B,C; Figs. S1 and S2).

MYO1E OVEREXPRESSION IN PODOCYTES DETERMINED BY REAL-TIME PCR

Myo1e overexpression was confirmed by real-time PCR (Fig. 2A and Table I). According to ANOVA analysis, Myo1e expression was significantly different between the overexpression and control groups (overexpression vs. control, $t = 5.2567$, $P = 0.033$; overexpression vs. negative control, $t = 3.8948$, $P = 0.044$). No significant difference was found between the control and negative control groups ($t = 0.3944$, $P = 0.731$).

MYO1E OVEREXPRESSION DETERMINED BY WESTERN BLOT

Myo1e overexpression was confirmed by Western blotting (Fig. 2B and Table II). According to ANOVA analysis, Myo1e expression was significantly different between the overexpression and control groups (overexpression vs. control, $t = 10.7392$, $P = 0.04$; overexpression vs. negative control, $t = 11.4226$, $P = 0.038$). No significant difference was found between the control and negative control groups ($t = 1.5356$, $P = 0.1994$).

MYO1E OVEREXPRESSION IN PODOCYTES CULTURED FOR DIFFERENT TIMES MEASURED USING ELISA

The expression of Myo1e was sustained for longer in the over-expression group than in the other two groups (Fig. 2C and Table III). Compared with the results from the control and negative control groups, expression of Myo1e was more stable in the Myo1e overexpression group after 48 h of podocyte culture. At the 48 h

TABLE III. OD Values of Myo1e in Three Different Groups by ELISA Measurement

Time	12 h	24 h	36 h	48 h
Control	0.2682 ± 0.0101	0.2568 ± 0.0160	0.1898 ± 0.0046	0.1600 ± 0.0094
Overexpression	0.2270 ± 0.0048	0.2250 ± 0.0029	0.2203 ± 0.0035	0.2178 ± 0.0022
Negative control	0.2600 ± 0.0018	0.2382 ± 0.0056	0.2005 ± 0.0026	0.1803 ± 0.0233

Compared with the results from control and negative control, the expression of Myo1e was more stable in Myo1e overexpression group after 48 h culture of podocytes. At 48 h time-point, Myo1e expression was more abundant in overexpression group than control ($P < 0.001$) and negative control ($P = 0.005$) by ANOVA and LDS test.

time point, Myo1e expression was more abundant in the over-expression group than in the control ($P < 0.001$) and negative control ($P = 0.005$) groups by both ANOVA and LSD analyses.

MYO1E OVEREXPRESSION IN PODOCYTES DETERMINED BY IMMUNOFLUORESCENCE

The effect of Myo1e overexpression was also confirmed by immunofluorescence in differentiated, cultured podocytes using antibodies against mouse Myo1e (Fig. 2D).

CELL ADHESION WAS INCREASED IN MYO1E OVEREXPRESSING PODOCYTES

Cell-cell contacts and the adherence of podocytes to the extracellular matrix of the GBM are crucial for podocyte function. Adhesion assays were performed to investigate the effect of Myo1e overexpression on podocyte anchorage to the GBM. Adhesion assays were performed to investigate the effect of *MYO1E* overexpression on podocyte anchorage to the GBM. Figure 3 and Table IV demonstrated that more podocytes was attached to the bottom of the plate in *MYO1E* overexpression group than control and negative control, which demonstrated that Myo1e overexpression significantly increased the adherence of podocytes.

OVEREXPRESSION OF MYO1E INHIBITED PAN-INDUCED PODOCYTE DETACHMENT

We compared the detachment of cultured podocytes in the control and Myo1e overexpression groups. A significant difference in cell detachment after 48 h ($P < 0.01$, $n = 6$) was noted in the present study. The average numbers of podocytes from four different fields in three independent sets of experiments were analyzed by student's *t*-test. Figure 4 and Table V shows that the cell number attached to the bottom of the culture bottle was significantly increased in the *MYO1E* overexpression than control and negative control after PAN treatment, which demonstrated that over

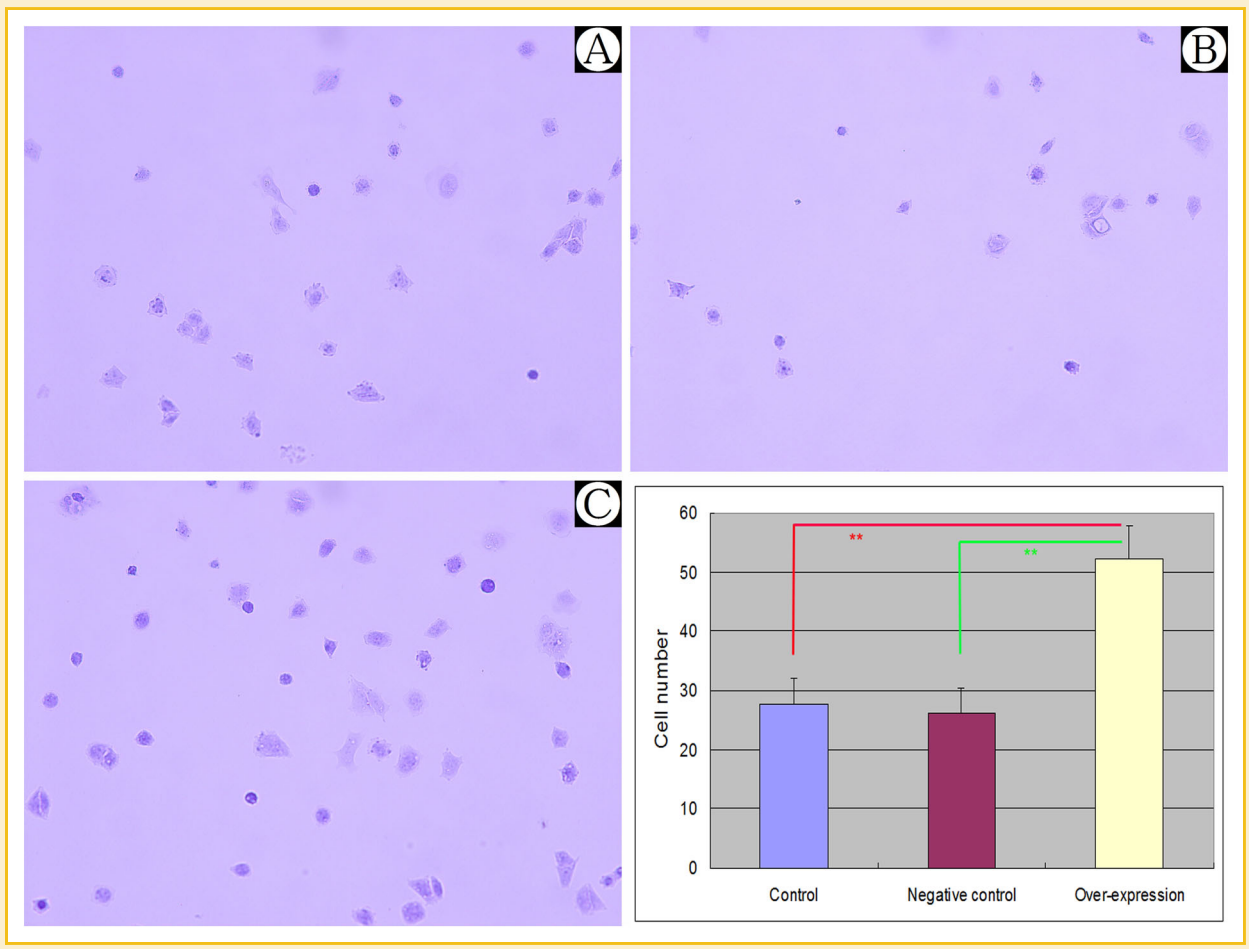

Fig. 3. Myo1e overexpression significantly increased the adherence of podocytes. Adhesion assays were performed to investigate the effect of *MYO1E* overexpression on podocyte anchorage to the GBM. Figure 3 demonstrated that more podocytes was attached to the bottom of the plate in *MYO1E* overexpression group than control and negative control. A: Control without any plasmid transfection. B: Negative control transfected with an empty plasmid. C: Myo1e overexpressing podocytes transfected with the Myo1e-plasmid (400×). According to ANOVA analysis, cell adhesion was significantly different between the overexpression and control groups (overexpression vs. control, $t = 11.3199$, $P = 0.005$; overexpression vs. negative control, $t = 12.0570$, $P = 0.0006$). No significant difference was found between the control and negative control groups ($t = 0.8523$, $P = 0.4032$).

TABLE IV. Podocyte Adhesion in Different Groups

	n	Mean
Control	3	27.6 ± 4.3
Overexpression	3	$52.1 \pm 5.7^{**}$
Negative control	3	26.1 ± 4.3

By ANOVA, there are significant difference in cell adhesion among overexpression and the other groups (overexpression vs. control, $t = 11.3199$, $P = 0.005$; overexpression vs. negative control, $t = 12.0570$, $P = 0.0006$). No significant difference found between control and negative control ($t = 0.8523$, $P = 0.4032$). $^{**}P < 0.01$.

regulation of Myo1e slow-down the PAN-induced podocyte detachment. These data suggest an attachment supporting effect of Myo1e as a key component of the cytoskeleton in cultured mouse podocytes.

TABLE V. The Number Of Podocytes Remained on the Bottom of the Culture Plate in the Three Different Groups After Treatment of Puromycin Aminonucleoside

	n	Mean (cell number)
Control	3	59.4 ± 4.6
Overexpression	3	$80.6 \pm 4.5^{**}$
Negative control	3	56.8 ± 3.6

By ANOVA, there are significant difference in cell number remained on the bottom of the culture plate among overexpression and control groups (overexpression vs. control, $t = 7.3060$, $P = 0.0001$; overexpression vs. negative control, $t = 9.1947$, $P < 0.001$). No significant difference found between control and negative control ($t = 0.9971$, $P = 0.3479$). $^{*}P < 0.01$.

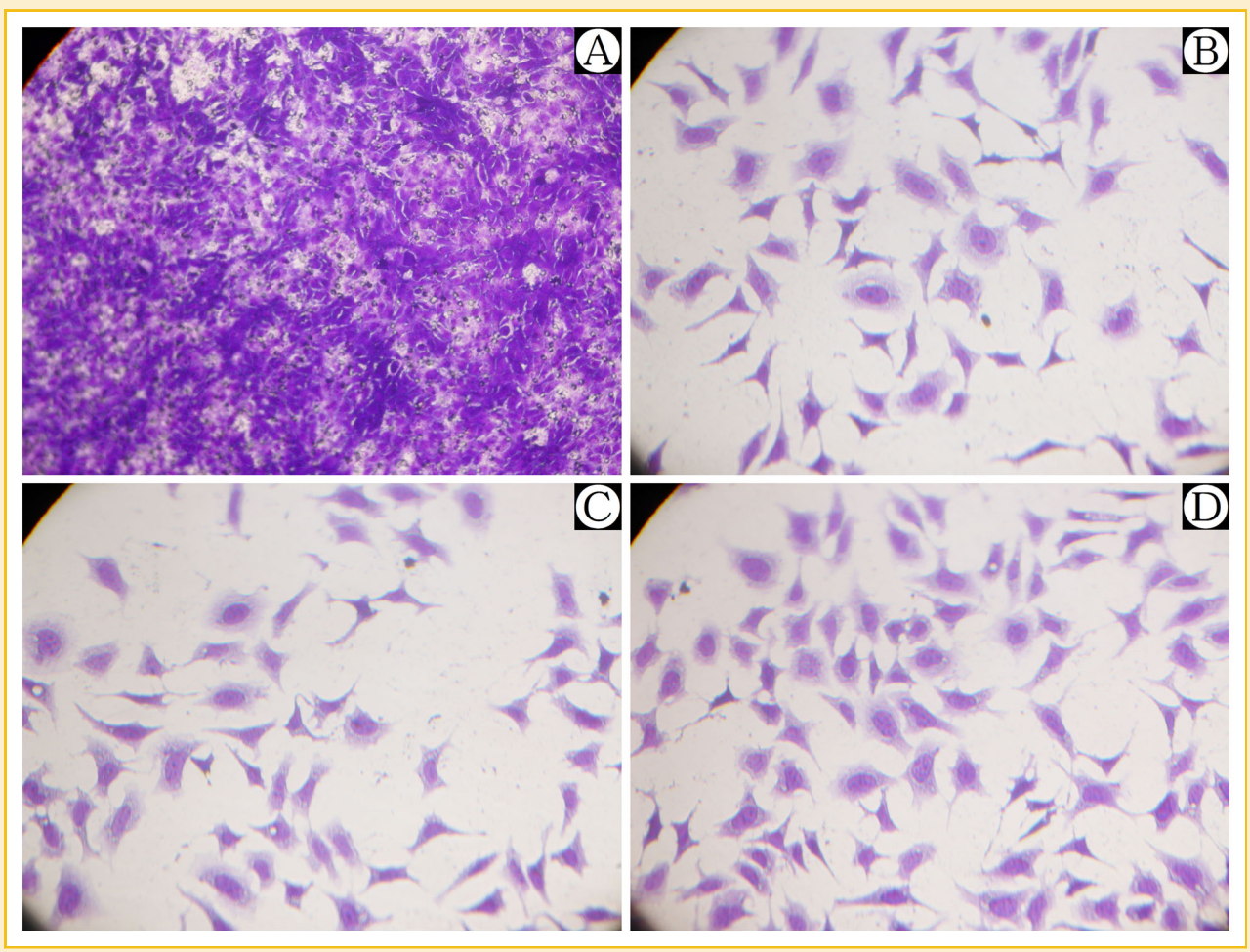

Fig. 4. Detachment assay: number of attached podocytes remaining after treatment with puromycin aminonucleoside (PAN). The average numbers of podocytes from four different fields in three independent sets of experiments were analyzed by student's *t*-test. Figure shows that the cell number attached to the bottom of the culture bottle was significantly increased in the *MYO1E* overexpression than control and negative control after PAN treatment, which demonstrated that over regulation of Myo1e slow-down the PAN-induced podocyte detachment. A: Cultured podocytes before treatment with PAN. B: Control podocytes without plasmid transfection. C: Negative control podocytes transfected with an empty plasmid. D: Myo1e overexpressing podocytes transfected with Myo1e-plasmids (400×). According to ANOVA analysis, there were significant differences in the number of cells remaining on the bottom of the culture plate between the overexpression and control groups (overexpression vs. control, $t = 7.3060$, $P = 0.0001$; overexpression vs. negative control, $t = 9.1947$, $P = 0.000$). No significant difference was found between the control and negative control groups ($t = 0.9971$, $P = 0.3479$).

CELL MIGRATION WAS INCREASED IN MYO1E OVEREXPRESSING PODOCYTES

The foot processes of podocytes are highly flexible and dynamic structures and play a key role in withstanding the continuous filtration pressure in the kidneys. In the event of damage or loss of single podocyte, the motility of the podocytes is important to repair wounds on the capillary loop. The increased motility of podocytes may present an adaptive response to the podocytopenia by genetic abnormality or environmental injury. These previous studies have shown that these processes highly depend on re-arrangements of the actin cytoskeleton [Asanuma et al., 2006]. In present study podocyte motility was measured by the transwell migration assay, and Figure 5 showed that the cell number migrated from upper chamber to lower chamber through transwell filter is more abundant in *MYO1E* overexpression group than control and negative control, which demonstrated that Myo1e overexpression significantly increased the cellular motility of podocytes.

INTERNALIZATION OF FITC-TRANSFERRIN WAS INCREASED IN MYO1E OVEREXPRESSING PODOCYTES

The mechanisms of glomerular protein handling have received much attention in the study of nephrotic syndrome following the recognition of proteinuria as an independent risk factor for both renal failure and cardiovascular disease. Protein endocytosis by podocytes may represent a useful, measurable phenotypic characteristic against which potentially injurious or beneficial interventions can be assessed [Eyre et al., 2007]. To analyze the effect of overexpression of Myo1e on endocytosis by podocytes, we used FITC-transferrin to study protein endocytosis through a direct quantitative assay and fluorescence microscopy. After co-incubation of podocytes with FITC-transferrin for 15 min, the number of cells with FITC-positive vesicles in their soma dramatically increased in the Myo1e overexpressing group relative to the control groups (Fig. 6).

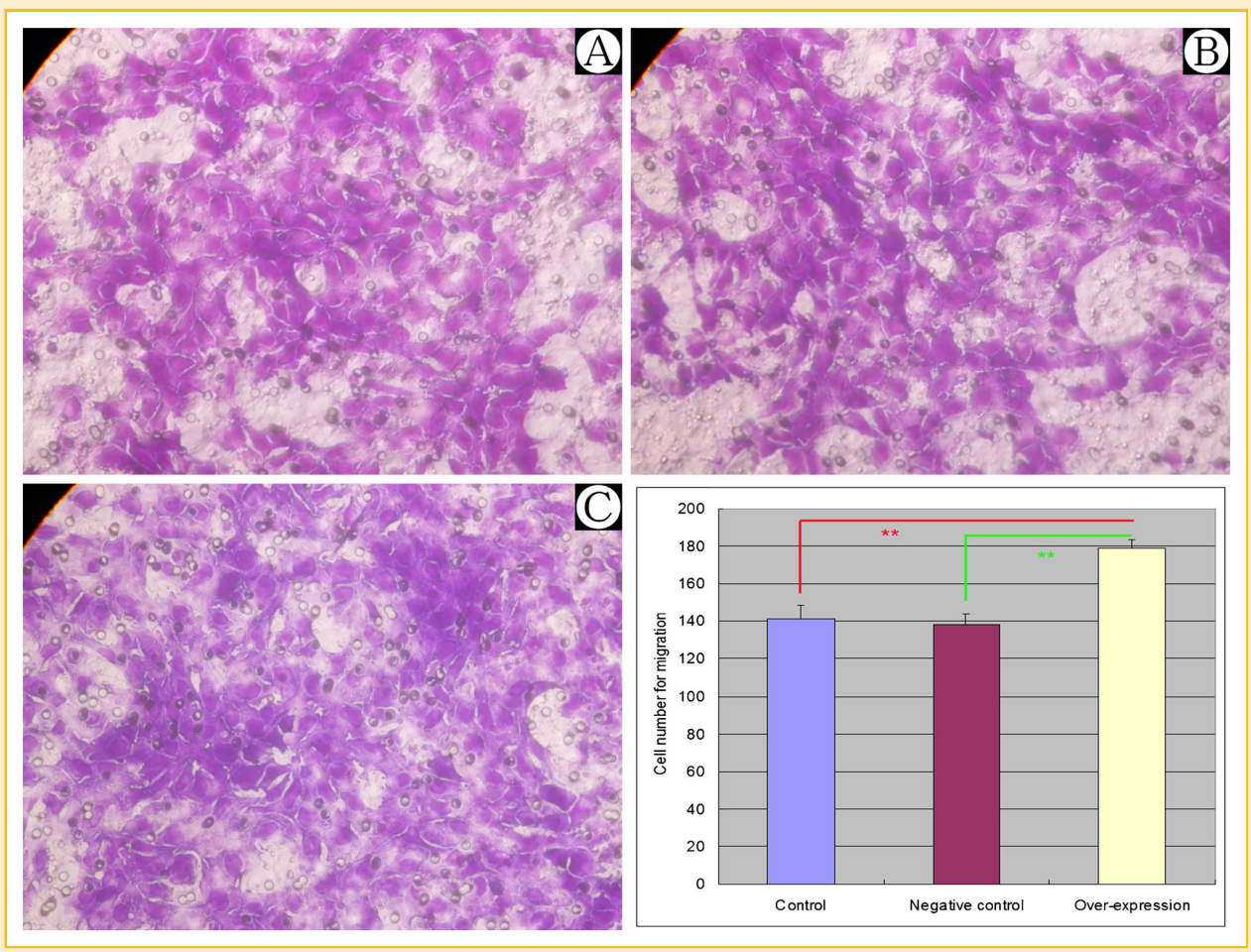

Fig. 5. *MYO1E* overexpression stimulates podocyte motility as evaluated by a transwell assay. In the event of damage or loss of single podocyte, the motility of the podocytes is important to repair wounds on the capillary loop. The increased motility of podocytes may present an adaptive response to the podocytopenia by genetic abnormality or environmental injury. These processes highly depend on rearrangements of the actin cytoskeleton. Podocyte motility was measured by the transwell migration assay, and Figure showed that the cell number migrated from upper chamber to lower chamber through transwell filter is more abundant in *MYO1E* overexpression group than control and negative control, which demonstrated that Myo1e overexpression significantly increased the cellular motility of podocytes. a: Control podocytes without plasmid transfection. b: Negative control transfected with an empty plasmid. c: Myo1e overexpressing podocytes transfected with Myo1e-plasmids (400×).

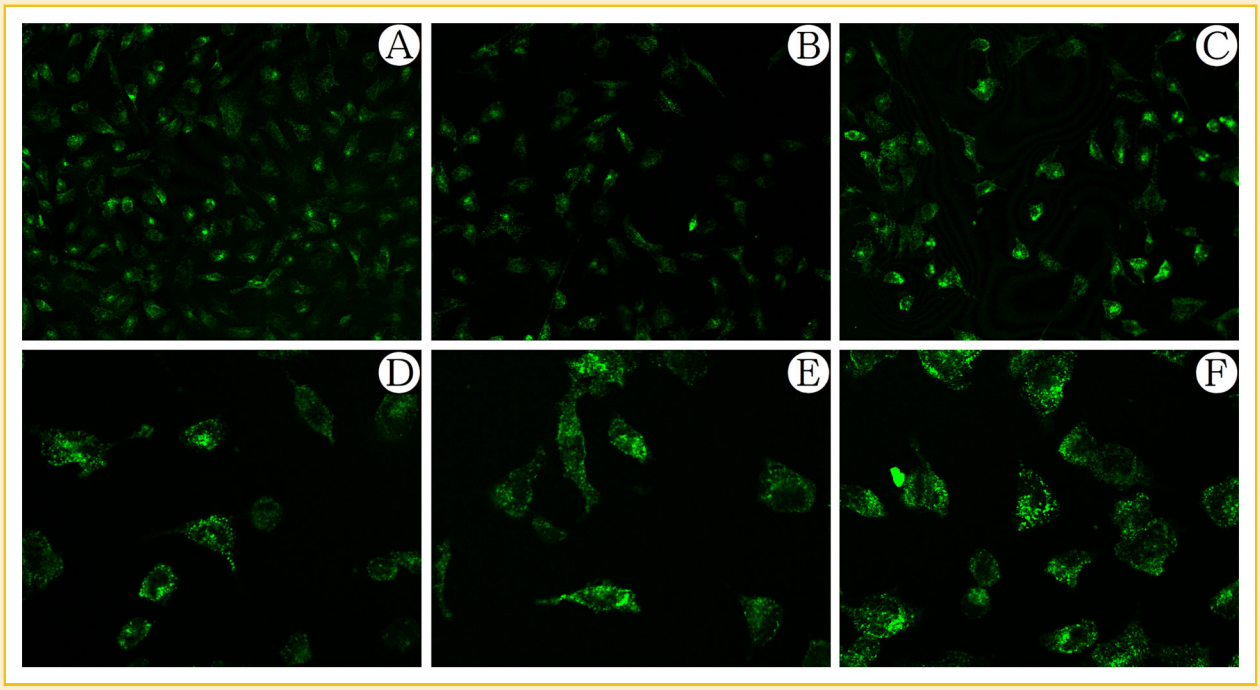

Fig. 6. FITC-transferrin endocytosis of podocytes visualized by immunofluorescence. A: Control podocytes without plasmid transfection (200×). B: Negative control podocytes transfected with an empty plasmid (200×). C: Myo1e-overexpressing podocytes transfected with the Myo1e-plasmid (200×). D: Control podocytes without plasmid transfection (400×). E: Negative control podocytes transfected with an empty plasmid (400×). F: Myo1e-overexpressing podocytes transfected with the Myo1e-plasmid (400×). After podocytes were co-incubated with FITC-transferrin for 15 min, the number of cells with FITC-positive vesicles within their soma dramatically increased in the Myo1e overexpression group relative to the control and negative control groups.

DISCUSSION

In this study, using overexpression of Myo1e in cultured podocytes, we demonstrated that podocyte migration, internalization, and adhesion to a model glomerular basement membrane (GBM) were dramatically increased relative to the podocytes in the control groups. The results of the present study indicate that restoring Myo1e expression in podocytes may increase their functional integrity against environmental and mechanical injury.

It has been reported that podocytes can endocytose proteins, including albumin, immunoglobulin, and transferrin, in a receptor-mediated manner [Abbate et al., 2002; Eyre et al., 2007; Akilesh et al., 2008]. A wealth of new evidence indicates that endocytosis and recycling are master regulators of diverse cellular functions, such as nutrient uptake and metabolism, proliferation, differentiation and polarity, reprogramming, migration, and adhesion [Andersson, 2012; Sigismund et al., 2012]. Such dynamic organization of membrane trafficking requires diverse protein networks that include non-conventional progressive motors, cytoskeletal proteins, and their regulators [Grant and Donaldson, 2009; Canton and Battaglia, 2012]. In a previous study, we used FITC-transferrin to study protein endocytosis through direct quantitative assay and fluorescence microscopy. After co-incubation of podocytes with FITC-transferrin, the number of cells with FITC-positive vesicles in their soma in the Myo1e knockdown group was dramatically decreased relative to the control groups, which suggests that Myo1e depletion may reduce the rate of endocytosis of FITC-transferrin in podocytes. In the present study, FITC-transferrin was observed in abundant large vesicles within podocyte cell bodies, especially in podocytes overexpressing Myo1e. Together with our previous study, our present results indicate that Myo1e may play an important role in podocyte endocytosis by regulating the actin cytoskeleton.

As they are exposed to permanent trans-capillary filtration pressure, podocytes must adhere tightly to the underlying glomerular basement membrane (GBM) to withstand the continuously high trans-capillary filtration pressure [Sachs and Sonnenberg, 2013]. At the molecular level, cell-matrix adhesions involve extracellular ligands within the GBM (e.g., laminins), transmembrane podocyte adhesion receptors (e.g., integrins), and intracellular linker proteins (e.g., integrin-linked kinase) that couple to the podocyte cytoskeleton. In cultured human podocytes, Myo1e localizes close to the cytoplasmic membrane, with enrichment at the lamellipodia tips [Mele et al., 2011]. In our previous study, Myo1e knockdown significantly reduced the adherence of podocytes to the extracellular matrix of the GBM, and downregulation of Myo1e accelerated PAN-induced podocyte detachment. In contrast, in the present study, overexpression of Myo1e inhibited PAN-induced podocyte detachment, which suggests an attachment-supporting effect of Myo1e as a key component of the cytoskeleton in cultured mouse podocytes. From this perspective, Myo1e may function as an intracellular linker protein to couple the podocyte cytoskeleton to transmembrane podocyte adhesion receptors.

Podocytes are a contractile and motile cell type [Noris and Remuzzi, 2012], and their movement and migration, which requires

a rearrangement of the actin cytoskeleton, is important for adaptation to environmental changes [Lasagni et al., 2013]. Renal biopsies of patients with proteinuria and kidney disease often present podocyte foot process effacement [Kalluri, 2006], which may also represent a motile event that results in the spreading of the foot process on the GBM [Wei et al., 2008]. Thus, we studied podocyte motility before and after knockdown and overexpression of Myo1e. In a previous study with Myo1e knockdown, Myo1e depletion significantly decreased podocyte motility in a transwell migration assay. In the present study, compared with the control group, Myo1e overexpression significantly increased podocyte motility. Michaud et al. [2006] reported that podocyte-specific expression of an FSGS-associated a-actinin-4 mutant (K256E) reduced podocyte migration in vitro and caused proteinuria in mice. Together, these findings indicate that the function of non-muscle myosins is important for maintaining podocyte structure and motility and in regulating the crosstalk between podocytes and their matrix [Noris and Remuzzi, 2012].

Overexpression of Myo1e in the present study restored podocyte motility, endocytosis, and adhesion to the GBM, which confirms a critical role for Myo1e in renal physiology and pathophysiology. In this context, Myo1e could be a new molecular target for functional rescue of podocytes and proteinuria therapy.

REFERENCES

Abbate M, Zoja C, Morigi M, Rottoli D, Angioletti S, Tomasoni S, Zanchi C, Longaretti L, Donadelli R, Remuzzi G. 2002. Transforming growth factor-beta1 is up-regulated by podocytes in response to excess intraglomerular passage of proteins: A central pathway in progressive glomerulosclerosis. Am J Pathol 161:2179–2193.

Akilesh S, Huber TB, Wu H, Wang G, Hartleben B, Kopp JB, Miner JH, Roopenian DC, Unanue ER, Shaw AS. 2008. Podocytes use FcRn to clear IgG from the glomerular basement membrane. Proc Natl Acad Sci USA 105:967–972.

Andersson ER. 2012. The role of endocytosis in activating and regulating signal transduction. Cell Mol Life Sci 69:1755–1771.

Asanuma K, Yanagida-Asanuma E, Faul C, Tomino Y, Kim K, Mundel P. 2006. Synaptopodin orchestrates actin organization and cell motility via regulation of RhoA signalling. Nat Cell Biol 8:485–491.

Canton I, Battaglia G. 2012. Endocytosis at the nanoscale. Chem Soc Rev 41:2718–2739.

Chase SE, Encina CV, Stolzenburg LR, Tatum A, Holzman LB, Krendel M. 2012. Podocyte-specific knockout of myosin 1e disrupts glomerular filtration. Am J Physiol Renal Physiol 303:F1099–F1106.doi: 10.1152/ajprenal.00251.2012

Eyre J, Ioannou K, Grubb BD, Saleem MA, Mathieson PW, Brunskill NJ, Christensen EI, Topham PS. 2007. Statin-sensitive endocytosis of albumin by glomerular podocytes. Am J Physiol Renal Physiol 292:F674–F681.

Grant BD, Donaldson JG. 2009. Pathways and mechanisms of endocytic recycling. Nat Rev Mol Cell Biol 10:597–608.

Greka A, Mundel P. 2011. Cell biology and pathology of podocytes. Annu Rev Physiol 74:299–323.

Kalluri R. 2006. Proteinuria with and without renal glomerular podocyte effacement. J Am Soc Nephrol 17:2383–2389.

Krendel M, Kim SV, Willinger T, Wang T, Kashgarian M, Flavell RA, Mooseker MS. 2009. Disruption of Myosin 1e promotes podocyte injury. J Am Soc Nephrol 20:86–94.

Lasagni L, Lazzeri E, Shankland SJ, Anders HJ, Romagnani P. 2013. Podocyte mitosis—A catastrophe. Curr Mol Med 13:13–23.

Mathieson PW. 2011. The podocyte as a target for therapies—New and old. Nat Rev Nephrol 8:52–56.

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, Schaefer F, Macciardi F, Ozaltin F, Emre S, Ibsirlioglu T, Benigni A, Remuzzi G, Noris M. 2011. MYO1E mutations and childhood familial focal segmental glomerulosclerosis. N Engl J Med 365:295–306.

Michaud JL, Chaisson KM, Parks RJ, Kennedy CR. 2006. FSGS-associated alpha-actinin-4 (K256E) impairs cytoskeletal dynamics in podocytes. Kidney Int 70:1054–1061.

Noris M, Remuzzi G. 2012. Non-muscle myosins and the podocyte. Clin Kidney J 5:94–101.

Patrakka J, Xiao Z, Nukui M, Takemoto M, He L, Oddsson A, Perisic L, Kaukinen A, Szigyarto CA, Uhlen M, Jalanko H, Betsholtz C, Tryggvason K. 2007. Expression and subcellular distribution of novel glomerulus-associated proteins dendrin, ehd3, sh2d4a, plekhh2, and 2310066E14Rik. J Am Soc Nephrol 18:689–697.

Ruotsalainen V, Patrakka J, Tissari P, Reponen P, Hess M, Kestila M, Holmberg C, Salonen R, Heikinheimo M, Wartiovaara J, Tryggvason K, Jalanko H. 2000. Role of nephrin in cell junction formation in human nephrogenesis. Am J Pathol 157:1905–1916.

Sachs N, Sonnenberg A. 2013. Cell-matrix adhesion of podocytes in physiology and disease. Nat Rev Nephrol 9:200–210.

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, Palomero T, Ghiggeri GM, 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.

Sigismund S, Confalonieri S, Ciliberto A, Polo S, Scita G, Di Fiore PP. 2012. Endocytosis and signaling: Cell logistics shape the eukaryotic cell plan. Physiol Rev 92:273–366.

Wei C, Moller CC, Altintas MM, Li J, Schwarz K, Zacchigna S, Xie L, Henger A, Schmid H, Rastaldi MP, Cowan P, Kretzler M, Parrilla R, Bendayan M, Gupta V, Nikolic B, Kalluri R, Carmeliet P, Mundel P, Reiser J. 2008. Modification of kidney barrier function by the urokinase receptor. Nat Med 14:55–63.

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