Mesangial Medium from IgA Nephropathy Patients Induces Podocyte Epithelial-to-mesenchymal Transition through Activation of the Phosphatidylinositol-3-kinase/Akt Signaling Pathway

Cheng Wang¹, Xun Liu¹, ZunFu Ke², Ying Tang³, Cui-Cui Li¹, Can-Ming Li¹, ZengChun Ye¹, Jun Zhang¹ and Tanqi Lou¹

¹Division of Nephrology, Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, Guangdong;

²Department of Pathology, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong;

³Division of Nephrology, Second Affiliated hospital of Sun Yat-Sen University, Guangzhou, Guangdong

Key Words

IgA1 • IgA nephropathy • Epithelial-to-mesenchymal transition • Podocyte • Mesangial cell

Abstract

Background: Podocyte injury plays an important role in glomerulosclerosis in IgA nephropathy (IgAN). Epithelial-to-mesenchymal transition (EMT) caused by different factors is the main reason for podocyte damage. This study hypothesized that conditioned mesangial medium may induced EMT process of podocytes and thereby lead to glomerular injury or sclerosis. **Materials and Methods:** Podocytes were incubated in medium from mesangial cells incubated with aggregated IgA1 (aIgA1) isolated from IgAN patients. Wortmannin were used to inhibit phosphatidylinositol-3-kinase (PI3-K) in podocytes. **Results:** Western blot analysis, real-time PCR and confocal fluorescent microscopy demonstrated that reduced expression of P-Cadherin, Zonula occludens-1 (ZO-1) and podocin, increased expression of fibroblast-specific protein (FSP-1), α -smooth muscle action (α -SMA) and desmin in podocytes exposed to medium from mesangial cells incubated with aIgA1 isolated from IgAN patients compared with podocytes

cultured in RPMI 1640 medium containing 0.5% fetal bovine serum (FBS) ($p < 0.05$). Mesangial medium resulted in a greater albumin influx across the podocyte monolayer ($p < 0.05$). Phosphorylation of Akt increased with this medium, as indicated by an increase in the p-Akt/Akt ratio. Treatment with wortmannin partly restored the changes in epithelial and mesenchymal markers and albumin influx. IgAN patients with massive proteinuria showed remarkable α -SMA and FSP-1 expression in podocytes. **Conclusion:** Our findings indicated that mesangial medium from cells incubated with aIgA1 isolated from IgAN patients induced EMT in podocytes and the PI3-K/Akt-signaling pathway was involved in the process.

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Introduction

Immunoglobulin A nephropathy (IgAN) is the most common primary glomerulonephritis worldwide [1]. Although the pathophysiologic mechanisms in IgAN are poorly understood, increasingly, studies indicate that deposition of undergalactosylated IgA1 in mesangial areas

might contribute to the pathogenic mechanisms of IgAN. This is attributed to specific binding to mesangial cells, inducing the production of a number of cytokines [2, 3]. Recent studies have revealed that the amount of aberrantly glycosylated IgA1 correlates significantly with the severity of glomerular histological lesions and with IgAN prognosis [4].

Podocytes are unique glomerular epithelial cells that comprise the outermost layer of the glomerular filtration barrier (GFB). Injury to podocytes that disrupts the structural and functional integrity of the slit diaphragm eventually leads to defective glomerular filtration, thereby causing proteinuria. Loss of podocytes is a hallmark of progressive kidney diseases, including IgAN. Reduced podocyte number in kidneys is an important factor in the progression of glomerulosclerosis and tubulointerstitial injury in IgAN patients [5]. Normopodocytic IgAN patients showed a significantly lower proteinuria progression rate than podocytopenic IgAN patients regardless of the comparable clinical features at biopsy and treatment regimen between the two groups. Podocyte injury is involved in development of proteinuria, and loss of podocytes predicts progression of proteinuria in IgAN [6]. More serious is that podocyte damage propagates injury by triggering secondary damage of “remnant” intact podocytes, even when the primary insult is short-lived. This transmission of podocyte injury may form a vicious cycle leading to accelerated podocyte deterioration and glomerulosclerosis [7]. Therefore, podocyte injury is considered the most important early event in initiating glomerulosclerosis, resulting in renal failure in various animal models and humans [8].

Recently, apoptosis has been proposed after podocyte injury under different conditions, emphasizing the importance of podocyte depletion resulting from apoptosis as a causative factor for the onset of proteinuria and glomerulosclerosis [9]. In this hypothesis, the reduced podocyte number in glomeruli is attributed to the apoptotic death of these cells. However, recent studies have shown that the detached podocytes might be alive and could be present in the urine of some patients with chronic kidney disease (CKD) [10]. Therefore, another hypothesis for podocyte injury proposes that injured podocytes could attain motile ability, facilitating their detachment from the glomerular basement membrane (GBM). In this context, emerging evidence suggests that podocytes could undergo an epithelial-to-mesenchymal transition (EMT) process when challenged by different injurious stimuli, such as transforming growth factor- β 1 (TGF- β 1), high glucose, and adriamycin [11].

EMT is a phenotypic conversion of epithelial cells, leading to the loss of epithelial cell-cell-basement membrane contacts and structural/functional polarity, and the acquisition of a fibroblastic phenotype [12]. Emerging evidence has established EMT as a major mechanism of tubulointerstitial fibrosis and glomerulosclerosis [13]. We previously reported that medium from mesangial cells incubated with IgA1 from IgAN patients can inhibit podocyte adhesive capacity and suppress the expression of nephrin [14, 15], indicating that this medium can change the podocyte phenotype.

Phosphorylated lipids are key mediators in diverse intracellular signaling pathways controlling cell growth, cell migration, endocytosis, and cell survival [16]. The conversion at the inner leaflet of the plasma membrane is catalyzed by phosphoinositide 3-OH kinases (PI3-K). Class Ia-PI3-K are heterodimers of regulatory and catalytic subunits. A major downstream mediator of PI3K activity is the serine-threonine kinase Akt. The binding of PI3-K-generated phospholipids to the PH domain of Akt leads to the translocation of Akt to the inner surface of the plasma membrane and induces conformational changes that are required for the proper phosphorylation and activation of Akt. Relocalization of Akt to the plasma membrane allows the close proximity of Akt to regulatory kinases that phosphorylate Akt at two regulatory sites, threonine-308 and serine-473, resulting in Akt activation [17]. Increasingly, results are finding that the PI3-K /Akt signaling pathway is involved in the regulation of nephrin and CD2AP [18], apoptosis [19] and other biological behavior [20].

The present study hypothesized that the conditioned mesangial medium may induce EMT process of podocytes and thereby leads to glomerular injury or sclerosis, and PI3-K/Akt signaling pathway plays a role in the EMT process. To test this hypothesis, we performed a series of experiments.

Materials and Methods

Cell culture and Treatment

Mouse podocytes and mesangial cells were provided and cultured as previously described [14, 15]. In the following experiments, cells were first cultured to 80% confluence and then growth was arrested with RPMI-1640 culture medium containing 0.5% fetal bovine serum (FBS) for 18–24 h.

Wortmannin, 200nM (Sigma, Saint Louis, MO, USA) were used to inhibit PI3-K for 1h.

Growth-arrested podocytes were cultured with medium from arrested mesangial cells exposed to aIgA1 from IgAN

patients and RPMI 1640 medium containing 0.5% FBS at a 1:9 (v/v) ratio for 48 h; the ratio and time were determined in a pilot study[14, 15]. Other podocytes were incubated with medium from mesangial cells incubated with aIgA1 from healthy controls and RPMI 1640 medium containing 0.5% FBS in a 1:9 (v/v) ratio or aIgA1 (10 µg/ml) from IgAN patients separately. Podocytes cultured in RPMI 1640 medium containing 0.5% FBS were used as controls. Podocytes incubated with recombinant mouse TGF-β1 (Cell Signaling Technology, Beverly, MA, USA) at 2 ng/ml were used as positive controls.

Patients and controls

We enrolled were 22 IgAN patients for the current study. IgAN was diagnosed by granular IgA deposits, mainly in the glomerular mesangium and occasionally along the peripheral capillary basement membrane using immunofluorescence examination, and by the presence of electron-dense deposits in the mesangium using ultrastructural examination. All patients were symptomatic for more than 12 months before examination. Control subjects were 15 healthy volunteers of comparable age and race and without microscopic hematuria or proteinuria. Kidney samples from renal biopsy of the three IgAN patients were collected and kept at ~70°C.

Informed written consent was recorded and signed for each subject before collecting blood samples and kidney biopsy tissues. The study was approved by the Ethics Committees of the Third Hospital, Sun Yat-sen University.

Purification of IgA1 by jacalin-agarose affinity chromatography

IgA1 was isolated from pooled serum from IgAN patients and healthy donors separately by jacalin affinity chromatography as previously described [14, 15]. Because the amount of polymeric IgA1 (pIgA1) recovered from the purification process was not sufficient for further analysis, we incubated monomeric IgA1 (mIgA1) at 63°C for 150 min to obtain algA1 as described previously [14, 15]. The transition from mIgA1 to algA1 was monitored using a Sephacryl S-200 column, and a single peak was observed after incubation at 63°C. The purified IgA1 was identified by Western blotting and stored at ~70°C for further analysis.

RNA extraction, cDNA synthesis and real-time PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad CA, USA) according to the manufacturer's instructions. Total RNA (500 ng) from each group was reverse transcribed using reverse transcriptase (RT) from a SYBR Premix Ex Taq kit (Perfect Real Time; TaKaRa, Otsu, Shiga, Japan). Reactions were carried out at 37°C for 15 min and 85°C for 5 s.

The primers are listed in Table 1. Using ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) and a SYBR Premix Ex Taq (Perfect Real Time; TaKaRa, Otsu, Shiga, Japan), polymerase chain reaction (PCR) was performed three times as previously reported [14, 15].

		Sequence (5'→ 3')
Podocin	Sense	TCCGACTGGGACATCTGCTTC
	Antisense	CAAGGTCTGGAGACGGAGATCAA
Zo-1	Sense	GCCGCTAAGAGCACAGCAA
	Antisense	TCCCCACTCTGAAAATGAGGA
P-cadherin	Sense	CTGGAGCCGAGCCAAGTTC
	Antisense	GGAGTGCATCGCATCCTTCC
FSP-1	Sense	TGAGCAACTTGGACAGCAACA
	Antisense	CTTCTTCCGGGGCTCCTTATC
Desmin :	Sense	GTGGATGCAGCCACTCTAGC
	Antisense	TTAGCCGCGATGGTCTCATAC
α-SMA	Sense	CCCAGACATCAGGGAGTAATGG
	Antisense	TCTATCGGATACTTCAG CGTCA
GAPDH	Sense	TGTGTCCGTCGTGGATCTGA
	Antisense	TTGCTGTTGAAGTCGCAGGAG

Table 1. Sequences of primers

Indirect immunofluorescent staining

Podocytes were grown to confluency on glass coverslips (Fisher Scientific, Pittsburgh, PA, USA). After stimulation, cells were rinsed briefly with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.25% Triton X-100 in PBS for 8 min, and blocked with 10% normal goat serum in PBS for 60 min at room temperature. Cells were incubated with polyclonal rabbit anti-podocin (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-P-cadherin (1:100, Cell Signaling Technology, Beverly, MA, USA), anti-ZO-1 (1:100; Invitrogen, Carlsbad, CA, USA), or anti-FSP-1 (1:100; AbCam Inc, Cambridge, MA, USA); monoclonal rabbit anti-desmin (1:100, Cell Signaling Technology, Beverly, MA, USA); or monoclonal mouse anti-α-SMA (1:100, Sigma, Saint Louis, MO, USA) in a humidified chamber overnight at 4°C. After incubation with primary antibody, cells were washed three times with PBS and co-incubated with AlexaFluor546 goat anti-rabbit (1:1000; Molecular Probes, Eugene, OR, USA) or DyLight 488 goat anti-mouse (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 60 min at room temperature. Nuclei were stained with Hoechst 33342 for 5 min. Coverslips were examined on a confocal laser scanning microscope (Zeiss 510 Metaseries; Carl Zeiss Microimaging, Thornwood, NY, USA).

Western blot

Cell proteins were extracted by the addition of lysis buffer (Cell Signaling Technology, Beverly, MA, USA) at 4°C. The suspension was centrifuged at 14,000 x g and media containing cellular proteins was collected. For Western blotting, sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) were run under standard conditions with 80 µg protein in each lane. Gels were placed in transfer buffer for 15 min and transfer to polyvinylidene fluoride membranes at 200 mA for 1 h. Membranes were rinsed in Tris-buffered saline and blocking buffer (5% milk powder) for 5 min, then immersed in blocking buffer for 1 h before incubation with primary antibodies: polyclonal rabbit anti-podocin (1:500, Santa Cruz

Biotechnology, Santa Cruz, CA, USA), anti-P-cadherin (1:2000, Cell Signaling Technology, Beverly, MA, USA), anti-ZO-1 (1:1000; Invitrogen, Carlsbad, CA, USA), anti-FSP-1 antibody (1:500; Abcam inc, Cambridge, MA, USA); monoclonal rabbit anti-desmin (1:1000, Cell Signaling Technology, Beverly, MA USA); monoclonal mouse anti- α -SMA (1:1000, Sigma, Saint Louis, MO, USA); monoclonal rabbit anti-phosphorylated-Akt (p-Akt) (1:1000, Cell Signaling Technology, Beverly, MA, USA) or anti-Akt (pan) (1:1000, Cell Signaling Technology, Beverly, MA, USA); or polyclonal rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:2000, Cell Signaling Technology, Beverly, MA, USA). After rinsing in wash buffer, horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit or goat anti-mouse, Pierce, New York, USA) was used for 1 h. After final washing, membranes were developed using ECL Chemiluminescence Reagent (Pierce, New York, USA). Examinations were repeated three times.

Albumin influx assay

Albumin influx assay was used to evaluate the filtration barrier function of podocyte monolayers. Briefly, podocytes (5×10^3) were seeded onto collagen-coated trans-well filters (3 μ m pores; Corning, New York, USA) and cultured under different conditions. After 10 days, podocytes were serum-starved overnight and treated with different conditioned culture media. Cells were washed twice with PBS supplemented with 1 mmol/L MgCl₂ and 1 mmol/L CaCl₂ to preserve cadherin-based junctions. The top chamber was refilled with 0.15 ml of RPMI 1640 and the bottom chamber with 1 ml of RPMI 1640 supplemented with 40 mg/ml of bovine serum albumin and incubated at 37°C. At different time points, 100 μ l of media from the top chamber was collected and albumin concentration determined using a BCA protein assay kit (KeyGEN, Nanjing, China). The examinations were repeated three times.

Double-immunofluorescent staining

Double-immunofluorescent staining was performed using renal biopsy specimens from IgAN patients. After fixation, slides were incubated with mouse anti-WT (1: 500, Sigma, St. Louis, MO, USA), followed by incubation with Alexa 555-labeled goat anti-mouse secondary antibody. Polyclonal rabbit anti-FSP-1 antibody (1:500; AbCam Inc, Cambridge, MA, USA) or monoclonal rabbit anti- α -SMA (1:500, Epitomics, Inc., California, USA) was used to treat the slides overnight at 4°C. After washing, slides were incubated with AlexaFluor546 goat anti-rabbit antibody (1:1000; Molecular Probes, Eugene, OR, USA). Slides were mounted and subjected to examinations using on a confocal laser scanning microscope (Zeiss 510 Metaseries; Carl Zeiss Microimaging, Thornwood, NY, USA).

Statistical analysis

All data are expressed as mean with standard deviation (SD) unless otherwise specified. Statistical differences were assessed using multivariate analysis of variance (ANOVA) for repeated measures. All values quoted are two-tailed, and significance was defined as $p < 0.05$.

Results

Medium from mesangial cells incubated with IgAN aIgA1 inhibits expression of podocin in podocytes

Similar to TGF- β 1, medium from mesangial cells incubated with aIgA1 from IgAN patients inhibited podocin expression, which was significantly lower than controls ($p < 0.05$) and podocytes exposed to medium from mesangial cells incubated with aIgA1 from healthy controls or aIgA1 (10 μ g/ml) from IgAN patients ($p < 0.05$). Podocin mRNA and protein expression are shown in Fig. 1.

Medium from mesangial cells incubated with IgAN aIgA1 suppresses epithelial podocin P-cadherin and ZO-1 in podocytes

We examined the expression of P-cadherin and ZO-1 in podocytes after different stimulations. We found that the mesangial medium suppressed P-cadherin and ZO-1 expression compared to the control group or podocytes exposed to medium from mesangial cells incubated with aIgA1 from healthy controls or aIgA1(10 μ g/ml) from IgAN patients ($p < 0.05$). TGF- β 1 showed similar inhibition of P-cadherin and ZO-1 expression. The results are shown in Fig. 1.

Medium from mesangial cells incubated with IgAN aIgA1 induces mesenchymal markers in podocytes

We next investigated whether the mesangial medium induced mesenchymal conversion in podocytes. The expression of FSP-1, desmin and α -SMA was examined in podocytes after treatment. As illustrated in Fig. 1, the mesangial medium significantly stimulated expression of the three markers compared to the control group, or to podocytes exposed to medium from mesangial cells incubated with aIgA1 from healthy control or aIgA1 (10 μ g/ml) from IgAN patients ($p < 0.05$).

Medium from mesangial cells with IgAN aIgA1 impairs the filtration barrier of podocytes

As shown in Fig. 2, compared with controls or podocytes exposed to medium from mesangial cells incubated with aIgA1 from healthy control or aIgA1 (10 μ g/ml) from IgAN patients, the mesangial medium resulted in a greater albumin influx across the podocyte monolayer ($p < 0.05$).

Fig. 1. Effect of different media on podocin, ZO-1 and P-cadherin expression in podocyte cultures. A. Results of real time PCR (Data were normalized to GAPDH mRNA and controls. Column c (controls) was arbitrarily set to 1. Error bars represent the SD). B. Western-blot. C. Indirect immunofluorescent staining (ZO-1 original magnification 1000 $\times$, others 400 $\times$). Column 1: podocytes exposed to culture medium from mesangial cells cultured with aIgA1 (100 μ g/mL) isolated from IgA nephropathy mixed 1:9 (v/v) with RPMI 1640 containing 0.5% FBS. Column 2: podocytes exposed to culture medium from mesangial cells cultured with aIgA1 (100 μ g/mL) isolated from healthy controls mixed 1:9 (v/v) with RPMI 1640 containing 0.5% FBS. Column 3: podocytes exposed to aIgA1 (10 μ g/mL) isolated from IgA nephropathy. Column 4: podocytes exposed to RPMI 1640 containing 0.5% FBS. Column 5: podocytes pretreated with wortmannin (200 nM) and exposed to supernatant from mesangial cells cultured with aIgA1 (100 μ g/mL) isolated from IgAN patients diluted 1:9 (v/v) with RPMI 1640 containing 0.5% FBS. Column 6: podocytes exposed to recombinant mouse TGF- β 1 (2 ng/ml). \$P < 0.05 vs. column 1, #P < 0.05 vs. column 5, *P < 0.05 vs. column 4, n = 3.

Medium from mesangial cells with IgAN aIgA1 activates phosphatidylinositol-3-kinase/Akt signaling

As seen in Fig. 3, culture medium from mesangial cells incubated with aIgA1 from IgAN patients induced Akt activity, which is associated with Akt phosphorylation, as indicated by the increase in p-Akt/Akt ratio, compared with the control group or podocytes exposed to medium from mesangial cells incubated with aIgA1 from

healthy control or aIgA1 (10 μ g/ml) from IgAN patients (p<0.05).

Wortmannin partly restores podocin, P-cadherin and ZO-1 expression

The expression of podocin, P-cadherin and ZO-1 in podocytes pretreated with Wortmannin before exposure to medium from mesangial cells incubated with aIgA1 from IgAN patients was higher than in podocytes ex-

Fig. 2. Results of albumin influx assay of podocytes cultured with different media. Column 1: podocytes exposed to culture medium from mesangial cells cultured with aIgA1 (100 μ g/mL) isolated from IgA nephropathy mixed 1:9 (v/v) with RPMI 1640 containing 0.5% FBS. Column 2: podocytes exposed to culture medium from mesangial cells cultured with aIgA1 (100 μ g/mL) isolated from healthy controls mixed 1:9 (v/v) with RPMI 1640 containing 0.5% FBS. Column 3: podocytes exposed to aIgA1 (10 μ g/mL) isolated from IgA nephropathy. Column 4: podocytes exposed to RPMI 1640 containing 0.5% FBS. Column 5: podocytes pretreated with wortmannin (200 nM) and exposed to supernatant from mesangial cells cultured with aIgA1 (100 μ g/mL) isolated from IgAN patients diluted 1:9 (v/v) with RPMI 1640 containing 0.5% FBS. Column 6: podocytes exposed to recombinant mouse TGF- β 1(2 ng/ml). (&P<0.05 vs. column1, SP<0.05 vs. column 2, #P<0.05 vs. column 3, *P<0.05 vs. column 4, n=3).

Fig. 3. Expression of p-akt and Akt in podocytes cultured with different media. Column 1: podocytes exposed to culture medium from mesangial cells cultured with aIgA1 (100 μ g/mL) isolated from IgA nephropathy mixed 1:9 (v/v) with RPMI 1640 containing 0.5% FBS. Column 2: podocytes exposed to culture medium from mesangial cells cultured with aIgA1 (100 μ g/mL) isolated from healthy controls mixed 1:9 (v/v) with RPMI 1640 containing 0.5% FBS. Column 3: podocytes exposed to aIgA1 (10 μ g/mL) isolated from IgA nephropathy. Column 4: podocytes exposed to RPMI 1640 containing 0.5% FBS. Column 5: podocytes pretreated with wortmannin (200 nM) and exposed to supernatant from mesangial cells cultured with aIgA1 (100 μ g/mL) isolated from IgAN patients diluted 1:9 (v/v) with RPMI 1640 containing 0.5% FBS. (&P<0.05 vs. column1, SP<0.05 vs. column 2, #P<0.05 vs. column 3, *P<0.05 vs. column 4, n=3).

posed to the mesangial medium ($P < 0.05$), but lower than the control group ($p < 0.05$). The results are shown in Fig. 1.

Wortmannin partly lowers mesenchymal markers

Pretreatment with Wortmannin decreased expression of FSP-1, desmin and α -SMA in podocytes cultured with medium from mesangial cells incubated with aIgA1 from IgAN patients ($p < 0.05$). These were also lower than control group ($p < 0.05$). The results are shown in Fig. 1.

Wortmannin partly reverses impaired filtration barrier function of podocytes

Treatment with Wortmannin lowered the increased albumin influx across the podocyte monolayer induced

by culture medium from mesangial cells incubated with aIgA1 from IgAN patients, which was higher than the control group ($p < 0.05$). The results are shown in Fig. 2.

Expression of FSP-1 and α -SMA in human biopsy kidney tissues

To assess the possibility of podocyte EMT *in vivo*, we examined the expression of EMT markers in the glomeruli of IgAN patients. Three IgAN nephropathy patients with the same Oxford classification (M1S0E0T0) expressed different amounts of FSP-1 and α -SMA in podocytes. Baseline parameters were shown in Table 2. Patients with massive proteinuria showed more α -SMA expression in podocytes compared with patients with less proteinuria. The results were shown in Fig. 4.

Fig. 4. Common pathological changes and expression of fibroblast-specific protein-1 and α -Smooth muscle actin in podocytes in human IgA Nephropathy. A. Common pathological changes(HE: hematoxylin-eosin staining; Masson: masson's trichrome staining; PAS: Periodic Acid Schiff Staining; PASM: Periodic Schiff-Methenamine Silver staining; original magnification is 400 $\times$). B. Fibroblast-specific protein-1 expression in podocytes in these three human IgA Nephropathy patients (original magnification is 400 $\times$). C. α -Smooth muscle actin expression in podocytes in these three human IgA Nephropathy patients (original magnification is 400 $\times$).

Discussion

The goal of this study was to determine whether mesangial medium from mesangial cells incubated with aIgA1 from IgAN patients induced EMT in podocytes. We found that mesangial medium decreased the expression of epithelial markers (P-cadherin and ZO-1), but increased the expression of mesenchymal markers (FSP-1 and α -SMA) and impaired the filtration barrier function of podocytes *in vitro*. This indicated that certain mesangial media can induce EMT in podocytes.

	Patients A	Patients B	Patients C
Age(years)	22	21	26
BMI(Kg/m ²)	19.1	18.5	22.2
Proteinuria(g/24h)	0.18	6.98	5.81
Serum Albumin(g/L)	36.6	21.2	16.4
Serum Globulin (g/L)	21.9	25.4	20.1
Serum Urea nitrogen (mmol/L)	3	4.66	3.92
Serum Creatinine (umol/L)	63.8	59.3	73.9
Serum Cystatin C(mmol/L)	0.6	0.91	0.78
eGFR ml/min/1.73m ²	107	118	118
Oxford classification	M1S0E0T0	M1S0E0T0	M1S0E0T0

Table 2. Clinical parameters of three patients.

Podocyte EMT offers a new explanation for how glomerular mesangial injury causes podocyte dysfunction, leading to impairment of GFB and proteinuria. In response to various injurious stimuli, podocytes undergo a series of adaptive changes, including hypertrophy, ded-

ifferentiation, detachment, and apoptosis, depending on the severity and duration of the injury [21, 22]. If the injury is progressive, podocytes will undergo EMT to escape from apoptosis, which results in the loss of highly specialized podocyte features and acquisition of new mesenchymal markers. This leads to an impaired GFB, ensuring the onset of proteinuria. More severe and/or longer injury induces podocyte detachment from GBM and/or apoptosis, resulting in podocyte loss, which exacerbates proteinuria and leads to glomerulosclerosis. We found a greater albumin influx across the podocyte monolayer induced by certain media accompanying phenotypic alterations of podocytes. This helps explain EMT as a potential pathway leading to podocyte dysfunction and represents an early cellular event causing defective glomerular filtration and proteinuria.

Podocytes and tubular epithelial cells are developmentally derived from the same origin (metanephric mesenchyme) [23]. Podocytes, similar to tubular epithelial cells, may undergo a phenotypic conversion, namely, EMT, under specific injurious conditions. Several *in vivo* studies demonstrated podocyte EMT in kidney diseases [21, 24–26]. In human biopsy samples of diabetic nephropathy (DN) and focal-segmental glomerulosclerosis (FSGS), loss of nephrin and ZO-1 expression in glomerular podocytes is a common feature, where those cells express mesenchymal markers such as desmin, FSP-1 and α -SMA. TGF- β 1, a potent fibrogenic cytokine, was first reported as a potent trigger for podocyte EMT [21]. We used recombinant mouse TGF- β 1 as positive control, inducing podocyte EMT *in vitro* in accordance with previous reports [21]. Hyperhomocysteinemia induces podocytes to undergo EMT through the activation of NADPH oxidase [27]. To our knowledge, this is the first report that mesangial medium from mesangial cells incubated with aIgA1 from IgAN patients induces EMT in podocytes. Cytokines such as TGF- β 1 and Angiotensin-II (Ang-II) are detected in the medium of mesangial cells when they are cultured in the presence of aIgA1 [28, 29]. Some of these cytokines such as TGF- β 1 and Ang-II, induce podocyte injury, which helps explain why our mesangial media induced EMT in podocytes.

The PI3K/Akt pathway is a critical signaling pathway in the regulation of cell survival. Akt, generally considered a main effector of PI3-K, is best known for its anti-apoptotic/prosurvival action; P-Akt/Akt levels were used to detect the activation of Akt. Some studies suggest that hypercholesterolemic serum

changes the distribution of ZO-1 and increases the permeability in endothelial cells through the PI3K signaling pathway. In our *in vitro* study, we found that our mesangial medium increased the p-Akt/Akt ratio compared to the control group, which indicated that this medium can induce Akt activity. When the PI3-K specific inhibitor Wortmannin was used to pretreat podocytes, the p-Akt/Akt ratio was significantly lowered. All these results suggested that our medium activated the PI3-K/Akt signaling pathway. Wortmannin can partly preserve podocin, P-Cadherin and ZO-1 expression, however mesenchymal markers (FSP-1, Desmin and α -SMA) expression and restore the impaired filtration barrier function of podocytes induced by medium from mesangial cells incubated with aIgA1 from IgAN patients. This indicated that the PI3-K/Akt signaling pathway played a role in podocyte EMT induced by our medium. However, several intracellular signal transduction pathways such as TGF- β /Smad [21], integrin-linked kinase (ILK) [30, 31] and Wnt/ β -catenin [32] signaling are essential in controlling EMT. We found the PI3-K/Akt-signaling pathway only partly contributed to the process. We propose that TGF- β 1/Smad and ILK pathway also contribute to the process induced by the medium in considering of ILK activation [14] and stimulation of TGF- β 1 synthesis [29]. We will explore these pathways in future studies.

IgAN is characterized by pIgA1 deposition in glomerular mesangium, but the small amount of pIgA1 in serum limits its use *in vitro*. Aggregated IgA1 must be used by heating mIgA1, which is similar to IgA immune complexes and commonly used *in vitro* studies [13, 14].

Podocyte EMT also occurs in proteinuric kidney diseases in human biopsy. In samples of DN and FSGS, loss of nephrin and ZO-1 expression in glomerular podocytes is a common feature, although these cells express mesenchymal markers such as desmin, FSP1 and matrix metalloproteinase-9 (MMP-9) [25]. However, no studies report podocyte EMT in IgAN patients. In our study, we found that these patients show FSP-1 and α -SMA expression in podocytes, which indicated podocytes in IgAN might show EMT. Patients with more proteinuria showed more α -SMA expression than those with less proteinuria even if they have the same Oxford classification. This shows that the transition of podocytes after injury might be critical in causing podocyte dysfunction and ultimately leading to defective glomerular filtration and proteinuria. These *in vivo*

results correlated with our *in vitro* study, which helps to explain why patients with same Oxford classification have different degrees of proteinuria symptoms.

In summary, this study demonstrated that the PI3-K/Akt signaling regulated the integrity of podocyte structure and function and that activation of the pathway resulted in EMT, causing glomerular injury.

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