Angiopoietin-like protein 3 modulates barrier properties of human glomerular endothelial cells through a possible signaling pathway involving phosphatidylinositol-3 kinase/protein kinase B and integrin αVβ3

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Podocytes can influence glomerular endothelial cell (GEnC) barrier properties and take part in the development of proteinuria by some molecules. Angiopoietin-like protein 3 (Angptl3), secreted by podocytes, is a member of the angiopoietin-like protein family that has important biological functions in endothelial cells. In our previous studies, we showed that mRNA expression of Angptl3 increased significantly in kidneys of children with minimal change nephrotic syndrome. And the mRNA level of Angptl3 was increased in the glomerulus of adriamycin rats with the development of proteinuria. It was also found that Angptl3 was expressed in the cytoplasm of cultured podocytes. Thus, Angptl3 might influence the biological functions of GEnCs in a paracrine manner. In this study, we found that Angptl3 could increase the permeability of GEnCs and increase the level of protein kinase B phosphorylation in cultured GEnCs *in vitro*. LY294002, a phosphatidylinositol-3 kinase inhibitor, could prevent the increase of permeability of GEnCs induced by Angptl3. Our results also indicated that the integrin αVβ3 antibody (LM609) could block the Angptl3-induced protein kinase B phosphorylation.

Keywords angiopoietin-like protein 3; glomerular endothelial cells; glomerular filtration barrier; PI3K/Akt pathway; integrin αVβ3

A number of studies have shown that some molecules produced by podocytes could influence glomerular endothelial function and contribute to the change of the glomerular filtration barrier through some mediators in the glomerulus [1]. Some of them also take part in the development of proteinuria, such as vascular endothelial growth factor and angiopoietin 1 [2,3]. These molecules can act as paracrine regulators of endothelial behavior and play essential and coordinate roles in physiologic regulation, vascular development, and maturation [4]. They are also key players in pathologic alteration of glomerular permeability and take part in the development of proteinuria through a complicated molecular network [5–7].

Angiopoietin-like protein 3 (Angptl3) was found to be expressed in a liver-specific manner [8]. However, in our previous studies, using GeneChip technology (Affymetrix, Santa Clara, USA), we found the mRNA level of Angptl3 was significantly increased in kidneys of children with minimal change nephrotic syndrome compared to the normal control [9]. The mRNA level of Angptl3 was also increased in the glomerulus of adriamycin rats along with the development of proteinuria, as shown by laser microdissection. The expression of Angptl3 in the cytoplasm of cultured podocytes was examined by immunofluorescence, reverse transcription, and Western blot analysis [10]. These preliminary data suggested that Angptl3 secreted by podocytes might regulate the biological function of the glomerular filtration barrier and could take part in the development of proteinuria.

Angiopoietin-like proteins and angiopoietins are structurally resemblant glycoproteins characterized by two domains, an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain [11]. However, co-immunoprecipitation experiments failed to detect the binding of Angptl3 to the angiopoietin receptor Tie2 [12], indicating that it might bind to a different receptor. Experiments confirmed that Angptl3 could bind to the integrin αVβ3 and induce integrin αVβ3-dependent biological activity of

endothelial cells [12]. Integrin $\alpha V\beta 3$ is one of the main integrin heterodimers that GEnCs express [13]. It was considered whether Angptl3 secreted by podocytes has effect on GEnCs through integrin $\alpha V\beta 3$ and what signaling pathway could be involved during this process.

The glomerular filtration barrier consists of the endothelium, glomerular basement membrane, and podocytes. Models indicate that all three layers have important contributions [14,15]. GEnCs are specialized cells that form a continuous inner layer of the glomerular capillaries and play important roles in physiological filtration and glomerular disease. GEnC is perforated by fenestrae up to 60–80 nm in diameter [16], which cover 20% of the endothelial surface. Previously, the fenestrations were thought to be less important and therefore provided little barrier to the passage of proteins. However, recently, some studies have shown that a glomerular glycocalyx of 200–400 nm in thickness covers the fenestrae and interfenestral domains [17,18]. The glycocalyx is likely to offer significant resistance to filtration of macromolecules. Although the exact nature of the glycocalyx is yet to be defined, these observations suggest that the contribution of GEnCs to the permeability barrier to proteins might have been underestimated. It is necessary to further understand the role of GEnCs in the glomerular filtration barrier. However, little is known about GEnCs because they are difficult to isolate and propagate in culture.

The present work is part of our effort to understand the linkage between Angptl3 secreted by podocytes and the biological functions of GEnCs. We developed a system for studying the permeability properties of GEnCs *in vitro* after treatment with Angptl3. We show here that Angptl3 could modulate human glomerular endothelial cell barrier properties through a possible signaling pathway involving phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt). Thus, Angptl3, the molecules associated with podocytes, should be relevant to the pathophysiology of the development of proteinuria.

Materials and Methods

Cell culture
GEnCs derived from decapsulated glomeruli isolated from normal human kidney (according to the supplier's data sheet) were obtained from ScienCell Research Laboratories (Carlsbad, USA). Cells were cultured in endothelial growth medium, made up of endothelial basal medium (ECM), 5% fetal bovine serum (FBS), antimicrobial agents, and endothelial cell growth supplement (ECGs). ECM, FBS, and ECGs were also purchased from ScienCell Research

Laboratories.

Culture of GEnCs in tissue-culture inserts
Polycarbonate supports (0.4 μm pore size, 0.6 cm^2 surface area) in tissue-culture inserts (1.2 cm diameter; Millipore, Billerica, USA) were seeded with GEnCs at passages 3–8 at 1×10^5 cells/cm^2. Inserts were placed in 24-well plates and used for experiments 3–4 d after seeding.

Measurement of transendothelial electrical resistance (TEER)
TEER was measured using the Millicell-ERS electrical resistance system (Millipore). Millicell-ERS is an accurate quantitative measurement of lower resistance monolayers. The symmetrically apposing electrodes were situated above and beneath the membrane, allowing a uniform current density to flow across the membrane. The electrodes were immersed so that the shorter electrode was in the Millicell culture insert and the longer electrode was in the outer well. The shorter electrode did not contact cells growing on the membrane. The resistance was recorded when the meter indicated a stable resistance. The resistance of the endothelial monolayer was calculated as the total resistance measured minus the mean resistance of control inserts, with a correction for surface area. Formation of a satisfactory monolayer for use in experiments was indicated by a TEER >30 Ω/cm^2.

Measurement of transendothelial protein passage
Transendothelial permeability to macromolecules was assessed by measuring the passage of fluorescein-isothiocyanate-labeled bovine serum albumin (FITC-BSA; Sigma-Aldrich, St. Louis, USA) across the monolayer. GEnCs were incubated in serum-free medium. After 1 h, the medium in the insert was replaced by 400 μl ECM containing 0.5 mg/ml FITC-BSA; the medium in the well was replaced by 600 μl ECM containing 0.5 mg/ml unlabeled BSA (Sigma-Aldrich). At the indicated time, 200 μl aliquots were removed. The fluorescence of the aliquots was measured using a fluorospectrometer at 492 nm and the concentration of FITC-BSA was calculated by reference to a set of standard dilutions.

Western blot analysis
The cultured cells were lysed in modified loading buffer containing 50 μM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol and protease inhibitor, 1 μM phenylmethanesulfonyl fluoride, 1 μM NaF, and 1 μM Na_3VO_4. The samples were boiled for 10 min, then centrifuged at 12,000 g for 10 min, and insoluble material

was removed. Protein concentrations were determined using a modified Lowry assay. Proteins (50 µg/lane) were separated using 10% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to polyvinylidene difluoride membranes for Western blotting. After blocking with 5% skimmed milk in phosphate-buffered saline containing 0.05% Tween-20, the membranes were probed with primary antibodies (phospho-Akt monoclonal antibody, 1:1000; Cell Signaling Technology, Danvers, USA) or glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH monoclonal antibody, 1:1000; Santa Cruz Biotechnology, Santa Cruz, USA), followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibody. Phospho-Akt membranes were subsequently stripped and reprobed with non-phosphorylation-specific Akt antibodies (1:500; Santa Cruz Biotechnology) as an additional loading control. Antibody complexes were detected by chemiluminescence using an ECL kit (Perfect Biotech, Shanghai, China). The bands were scanned and quantified by Total Lab version 2.01 software (Nonlinear Dynamics, Durham, USA), and normalized by that of GAPDH.

Statistics

All data were expressed as the mean±SEM and analyzed by Student's t-test or ANOVA, using SPSS 10.0 Statistical Package (SPSS, Chicago, USA). Statistical values were considered significant when $P<0.01$.

Results

Angptl3 could significantly increase permeability of GEnC monolayer

Cultured endothelial cell monolayers grown on tissue-culture inserts have been used extensively to study the barrier function of endothelial cells. TEER is a measurement of ion flux and is inversely related to the filtration area open to water and molecules across cell monolayer [19]. Measurement of the TEER of endothelial monolayers is considered a common method to determine the permeability of the monolayer. In the present study, the addition of Angptl3 (0.5 µg/ml; AdipoGen, Seoul, Korea) to the electrode system decreased the resistance of the GEnC monolayer by a maximum of 14.2±4.21 Ω/cm^2 (**Fig. 1**), relative to the controls.

To further identify whether Angpl3 could change the permeability of the GEnC monolayer, measurement of the transendothelial protein passage was carried out to detect the GEnC monolayer's passage of FITC-BSA. As shown in **Fig. 2**, we found that Angptl3 (0.5 µg/ml) significantly
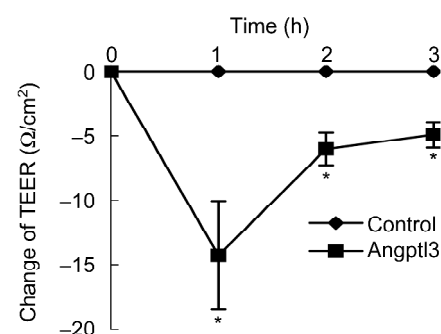

Fig. 1 Effect of angiopoietin-like 3 protein (Angptl3) on transendothelial electrical resistance (TEER) of glomerular endothelial cell monolayer The monolayer was starved with serum-free medium for 1 h, then treated with phosphate-buffered saline or Angptl3 (0.5 µg/ml). TEER was determined at 0, 1, 2, and 3 h. *$P<0.01$; $n=6$. Data are presented as the mean±SEM.
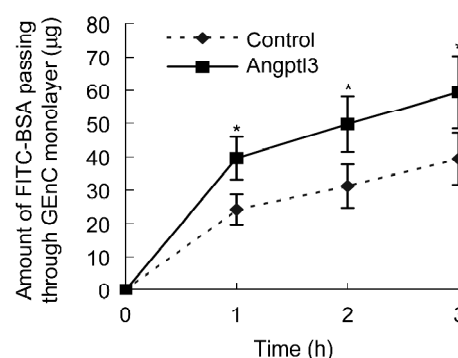

Fig. 2 Effect of angiopoietin-like protein 3 (Angptl3) on passage of fluorescein-isothiocyanate-labeled bovine serum albumin (FITC-BSA) of glomerular endothelial cell monolayer The monolayer was starved with serum-free medium for 1 h, then treated with phosphate-buffered saline or Angptl3 (0.5 µg/ml). The passage of FITC-BSA was determined at 0, 1, 2, and 3 h. *$P<0.01$; $n=6$. Data are presented as the mean±SEM.

increased the amount of FITC-BSA passing through the monolayer (148% at 3 h) compared to the control, consistent with the observed decrease in TEER. Thus, we concluded that Angptl3 could significantly increase the permeability of the GEnC monolayer.

Angptl3 influences permeability GEnC monolayer through PI3K/Akt pathway

Camenisch et al [12] found that Angptl3 can stimulate focal adhesion kinase, mitogen-activated protein kinase, and Akt phosphorylation. Integrin αVβ3 is also known to induce phosphorylation of Akt [12]. So we postulated that

Angptl3 could influence the GEnC monolayer through the PI3K/Akt signaling pathway. To understand the possible signaling pathway by which Angptl3 regulates the permeability of this monolayer, Western blot assay was used to analyze the levels of phospho-Akt and total Akt after Angptl3 stimulated GEnCs at indicated time points. As shown in **Fig. 3**, after GEnCs were treated with Angptl3 (0.5 μg/ml), a strong increase in the phosphorylation status of Akt at Ser473 was observed. The level of phosphorylation of Akt was 2.27-fold at 1 h and 1.97-fold at 2 h compared to the control (*P*<0.01). To further confirm the role of the PI3K/Akt pathway during this process, we examined whether LY294002, a PI3K inhibitor, could affect the barrier properties of the Angptl3-induced GEnC monolayer. We incubated GEnCs with LY294002 (1 μM; Sigma-Aldrich) before the addition of Angptl3 (0.5 μg/ml), then observed the TEER and FITC-BSA crossing GEnCs. As showed in **Fig. 4**, we found that LY294002 pretreatment inhibited an Angptl3-induced decrease of TEER in GEnC monolayer. In LY294002 pretreatment, the Angptl3-
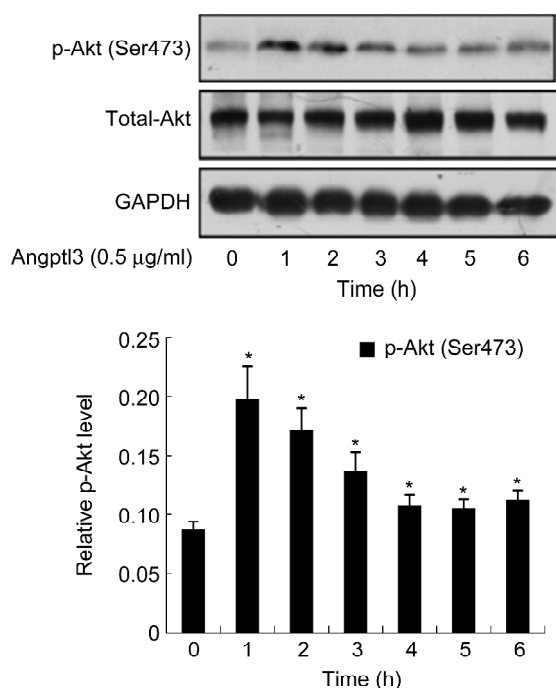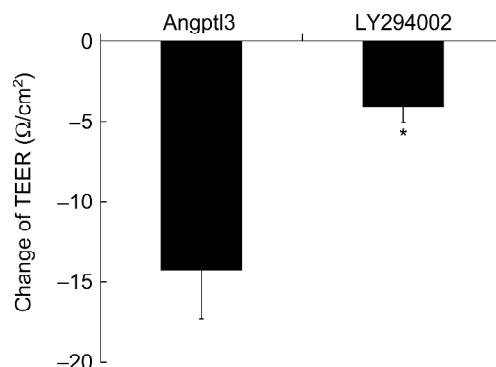

Fig. 4 LY294002 pretreatment blocked angiopoietin-like protein 3 (Angptl3)-induced decrease of transendothelial electrical resistance (TEER) glomerular endothelial cells (GEnCs) The GEnC monolayer was pretreated with phosphatidylinositol-3 kinase inhibitor LY294002 (1 μM) for 1 h, followed by stimulation with Angptl3 (0.5 μg/ml) for 1 h. TEER was measured at 1 h. *P<0.01; n=6.

induced decrease in TEER was suppressed by 71%. We also found LY294002 pretreatment inhibited an Angptl3-induced increase in the amount of FITC-BSA passing through the GEnC monolayer (**Fig. 5**). For instance, in LY294002 pretreatment, the Angptl3-induced increase in the amount of FITC-BSA was suppressed by 76% at 1 h. These data suggested that Angptl3 could influence the biological function of GEnCs through the PI3K/Akt signaling pathway.

Fig. 3 Time dependence of angiopoietin-like 3 (Angptl3)-induced protein kinase B (Akt) phosphorylation Glomerular endothelial cells were starved with serum-free medium for 1 h, then treated with Angptl3 (0.5 μg/ml) or phosphate-buffered saline for the indicated time. Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted onto a nitrocellulose membrane, and probed with phosphospecific Akt antibody or anti-Akt antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) bands confirm the loading of comparable amounts of protein. *P<0.01, n=6
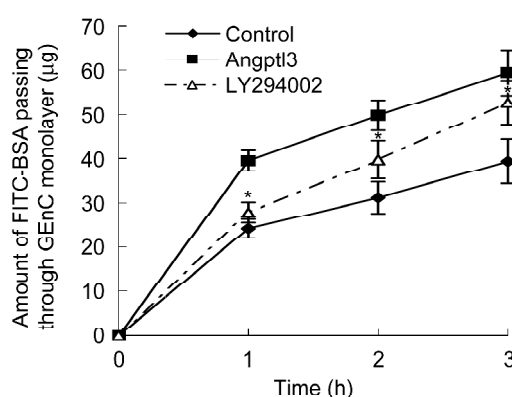

Fig. 5 LY294002 pretreatment blocked angiopoietin-like protein 3 (Angptl3)-induced increase of fluorescein-isothiocyanate-labeled bovine serum albumin (FITC-BSA) passage through glomerular endothelial cell monolayer The monolayer was pretreated with phosphatidylinositol-3 kinase inhibitor LY294002 (1 μM) for 1 h, followed by stimulation with Angptl3 (0.5 μg/ml) for the indicated time periods. FITC-BSA was measured at 1, 2, and 3 h. Data are presented as the mean±SEM. *P<0.01; n=6.

Integrin αVβ3 necessary for Angptl3-mediated Akt phosphorylation and modulation of GEnC permeability

Recently, Camenisch *et al* [12] found that Angptl3 affects endothelial cell adhesion and migration through Angptl3 binding to integrin αVβ3, and that integrin αVβ3 is one of the main integrin heterodimers that GEnCs express. Thus, we set out to determine whether integrin αVβ3 was involved in this Angptl3-mediated PI3K/Akt activation and change in the permeability of the GEnC monolayer. For this purpose, we pretreated GEnCs with LM609 (Chemicon, Temecula, USA)*,* a specific integrin αVβ3 antibody, before Angptl3 was added. As shown in **Fig. 6**, pretreatment with LM609 (1 μM) could significantly block Angptl3-induced Akt phosphorylation, suggesting that the activation of the PI3K/Akt pathway by Angptl3 is dependent on integrin αVβ3. To further confirm the role of integrin αVβ3 during this process, we examined whether LM609 could prevent Angptl3-induced effects on GEnC monolayer barrier properties. As showed in **Fig. 7**, we showed that LM609 pretreatment blocked Angptl3-induced decrease of TEER in GEnC monolayer. In LM609 pretreatment, the Angptl3-induced decrease in TEER was suppressed by 74%. We also showed LM609 pretreatment blocked an Angptl3-induced increase in the amount of FITC-BSA passing through the GEnC monolayer (**Fig. 8**). For instance, in LM609 pretreatment, the Angptl3-induced increase in the amount of FITC-BSA was sup-

Fig. 7 LM609 pretreatment blocked angiopoietin-like protein 3 (Angptl3)-induced decrease of transendothelial electrical resistance (TEER) in glomerular endothelial cell monolayer
The monolayer was pretreated with specific integrin αVβ3 antibody LM609 (1 μM) for 1 h, followed by stimulation with Angptl3 (0.5 μg/ml) for 1 h. TEER was measured at 1 h. *$P<0.01$; $n=6$.

Fig. 8 LM609 pretreatment blocked angiopoietin-like protein 3 (Angptl3)-induced increase of fluorescein-isothiocyanate-labeled bovine serum albumin (FITC-BSA) transport through glomerular endothelial cell monolayer The monolayer was pretreated with specific integrin αVβ3 antibody LM609 (1 μM) for 1 h, followed by stimulation with Angptl3 (0.5 μg/ml) for the indicated time. FITC-BSA was measured at 1, 2, and 3 h. Data are presented as the mean±SEM. *$P<0.01$; $n=6$.
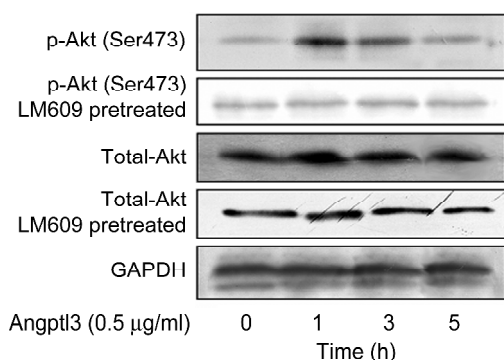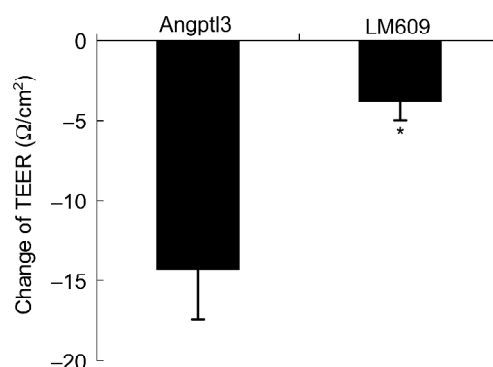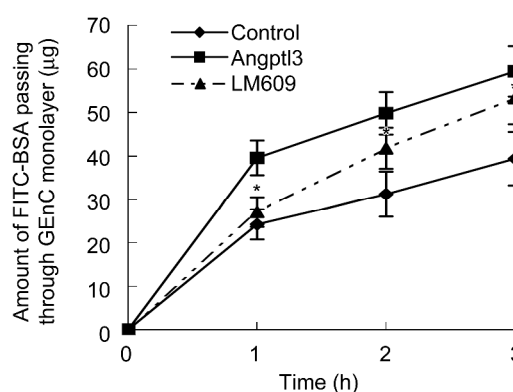

Fig. 6 LM609 pretreatment blocked angiopoietin-like protein 3 (Angptl3)-induced protein kinase B (Akt) phosphorylation Glomerular endothelial cells were pretreated with specific integrin αVβ3 antibody LM609 (1 μM) for 1 h, and treated with Angptl3 (0.5 μg/ml) for the indicated time periods. Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted onto a nitrocellulose membrane, and incubated with phosphospecific Akt antibody or total Akt antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) bands confirm the loading of comparable amounts of protein.

pressed by 77% at 1 h. These results showed that LM609 could block the function of Angptl3 on the GEnC monolayer and indicated that Angptl3 affects the function of GEnCs through binding to integrin αVβ3.

Discussion

In our previous studies, we showed that the expression of Angptl3 was significantly increased in kidneys of children with minimal change nephrotic syndrome and in the

glomerulus of adriamycin rats with the development of proteinuria [9]. But it remains to be investigated how Angptl3 works in renal disease. In these studies, we first found that Angptl3 could modulate GEnC barrier properties, leading to the increased permeability of the GEnC monolayer. These results suggested that Angptl3 produced by podocytes affects the barrier properties of GEnCs in a paracrine manner and takes part in the development of proteinuria in renal disease. But it remains to be seen whether Angptl3 is an important factor, and which factors can regulate the level of Angptl3 in the development of proteinuria.

The Angptl family of proteins has been identified as containing a coiled-coil domain and a fibrinogen-like domain, similar to angiopoietins. Seven members of this family have now been identified and have been designated as angiopoietin-like protein 1 to 7. Several early reports described members of the angiopoietin and Angptl families as angiogenic factors in the vascular system [20,21]. Recently, additional functions have been revealed, for example, Angptl3 can regulate serum lipid levels [22,23]. Several studies showed that angiopoietin 1, expressed by podocytes, is a potential regulator of glomerular vascular endothelial growth factors, and indicated that the action of angiopoietin 1 is important in glomeruli, including stabilization of capillary structure, promotion of endothelial survival, and reduction of endothelial permeability [2]. Co-immunoprecipitation experiments failed to detect binding of Angptl3 to the Tie2 receptor and found that recombinant Angptl3 bound to $\alpha V\beta 3$ [12], indicating that different signaling pathways might be involved.

Proteinuria is a characteristic feature in primary and secondary glomerulopathies. In the glomerular filtration barrier, all three layers have important contributions. The damage to the glomerular filtration barrier leads to the development of proteinuria. So it is of utmost importance to understand the underlying molecular mechanisms and structural changes that lead to changes in glomerular filtration. However, the exact molecular mechanism leading to change in the filtration barrier integrity remains unclear. An important mechanism is the change in charge and size permeability of the glomerular capillary wall, leading to the transglomerular passage of albumin and proteins of high molecular weight that usually do not cross the glomerular barrier. Size permeability is related to tight junctions between cells [24,25]. Specialized tight junctions maintain endothelial cell-cell contacts. Disruption of endothelial tight junctions leads to increase in endothelial permeability [26]. Angptl3 caused a decrease in TEER of a GEnC monolayer and a corresponding increase in protein passage, indicating a decrease in the integrity of the monolayer and widening of interendothelial gaps. The rapidly occurring changes might mediate action of second-messenger pathways, including the PI3K/Akt pathway. Studies have shown that PI3K can mediate phosphorylation of actin-binding protein, leading to alteration in cell shape and widening of interendothelial junctions. So we postulate that Angptl3 could enlarge the intercellular gap and increase the transport to macromolecules solute through integrin $\alpha V\beta 3$ and the PI3K/Akt pathway. But further ultrastructural study and molecular mechanisms should be examined.

Taken together, from our data we concluded that Angptl3 is considered to be a novel factor capable of regulating the biological function of GEnCs. These studies have implications for the functional role of Angptl3 in the development of proteinuria in renal disease and possible signaling pathways.

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