Biochemical and Biophysical Research Communications 399 (2010) 31–36

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Angiopoietin-like protein 3 regulates the motility and permeability of podocytes by altering nephrin expression *in vitro*

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ARTICLE INFO

*Article history:*
Received 5 July 2010
Available online 13 July 2010

*Keywords:*
Angiopoietin-like protein 3
Podocyte
Motility
Permeability
Nephrin

ABSTRACT

It is well known that podocyte injury plays a vital role in massive proteinuria. The increase of podocyte motility results in podocyte foot process (FP) effacement, a typical form of podocyte injury. Our previous studies demonstrated that glomerular podocytes can express angiopoietin-like protein 3 (ANGPTL3) and that the increase of ANGPTL3 in dysfunctional glomerulus is correlated with podocyte FP effacement. Little is known, however, about the role of ANGPTL3 in podocyte injury. In this study, we investigated ANGPTL3's effect on the motility and permeability of podocytes and on the expression of nephrin, a key molecule in podocytes. By scrape-wound and transwell migration assay, we found that ANGPTL3 over-expression significantly increased podocyte motility, whereas after ANGPTL3 knockdown by RNA interference, motility remained the same as that of the control group. Adriamycin (ADR) treatment significantly promoted podocyte motility. However, the same dose of ADR treatment could not promote motility after the knockdown of ANGPTL3. In addition, we assayed the diffusion of FITC-BSA across the podocytes' monolayer to investigate whether ANGPTL3 could promote protein loss by means of an increase in podocyte motility. The results showed that the changes in the FITC-BSA permeability of the podocytes corresponded to changes in motility. Furthermore, we found that ANGPTL3 over-expression dramatically increased the expression of nephrin but that the up-regulation of nephrin induced by ADR was significantly inhibited when ANGPTL3 was diminished by RNAi. In conclusion, we found ANGPTL3 to be capable of regulating the motility and permeability of podocytes and that the mechanism of ANGPTL3's regulation could be associated with the altered expression of nephrin.



## 1. Introduction

Podocytes cover the outer aspect of the glomerular basement membrane (GBM). Thus, they form the final barrier to protein loss, which explains why podocyte injury is typically associated with marked proteinuria [1]. The mechanism of podocyte injury, however, is not well understood. Recent studies have reported that the kidney glomerulus could express angiopoietins (Angs) such as angiopoietin 1 (Ang1) and angiopoietin 2 (Ang2). Further studies demonstrated that Ang1 and Ang2 were involved in podocyte injury [2,3].

Recently, a family of proteins structurally similar to the angiopoietins was identified and designated "angiopoietin-like proteins" (ANGPTLs). As a new member of this family, angiopoietin-like protein 3 (ANGPTL3) has the same structure as the C-terminal fibrinogen (FBN)-like domain (FLD) and N-terminal coiled-coil domain (CLD), among others. According to previous reports, ANGPTL3 was mainly expressed in liver cells and was only weakly expressed in the kidney [4]. In different regions, ANGPTL3 exhibited varying functions, including C-terminal FLD-induced angiogenesis when bound to integrin αVβ3 and increased plasma triglyceride levels in mice when bound to the N-terminal CLD [5,6]. Because of its powerful inhibition of lipoprotein lipase activity, ANGPTL3 is thought to play an important role in lipid metabolism [4,5,7,8]. To date, however, the relationship between ANGPTL3 and proteinuria has not been well clarified.

It is known that podocytes are highly specialized epithelial cells with a complex cellular organization consisting of a cell body, major processes, and foot processes (FPs). Podocyte FPs form a characteristic interdigitating pattern with FPs of neighboring podocytes, creating filtration slits that are bridged by the glomerular slit diaphragm (SD). Proteinuria kidney diseases are typically associated with FP effacement. FP effacement is considered to be a motile event, a characteristic that explains the spread of podocyte FPs on the GBM [9].

Nephrin, which belongs to the Ig super family, plays a key role in the SD and binds adjacent podocyte FPs. In addition to its

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0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.07.027

structural role, nephrin acts as a signaling receptor molecule. Thus, it could influence podocyte motility by interacting through cytoskeleton protein signal molecules [10,11].

In our previous studies, we observed that ANGPTL3 was up-regulated in nephrotic kidney tissues using a gene chip technique [12,13]. Through laser micro-cutting techniques, we further detected that ANGPTL3 was only expressed in the kidney glomerulus and not in the kidney tubules [12]. In addition, by using immunohistochemistry, we found that ANGPTL3 was concentrated in the glomerular podocytes of humans and rats [13]. Furthermore, the altered expression of ANGPTL3 in the glomerulus was associated with proteinuria and FP effacement in kidney diseases [14]. These results suggested that ANGPTL3 could be involved in proteinuria development and in podocyte injury.

To better understand ANGPTL3's function in podocytes, we investigated ANGPTL3's effect on podocyte motility as well as the role of nephrin in podocyte motility changes regulated by ANGPTL3. In this study, we found that ANGPTL3 over-expression may contribute to the motility of the podocytes. No effect was observed in those podocytes in which there had been knockdown of ANGPTL3. In addition, adriamycin (ADR) treatment failed to promote the podocyte-directed motility of those cells in which the knockdown of ANGPTL3 had occurred. Our data suggested that nephrin was involved in the signaling mechanism for ANGPTL3-mediated motility in the podocytes.

## 2. Materials and methods

### 2.1. Antibodies and reagents

The antibodies and reagents used in this study are listed with their sources in parentheses as follows: monoclonal antibody to glyceraldehydes-phosphate dehydrogenase (GAPDH); rabbit polyclonal antibody to nephrin (Santa Cruz Biotechnology, Santa Cruz, CA); polyclonal antibody to ANGPTL3 (R&D Systems, Minneapolis, USA); adriamycin (Pfizer Inc., USA); fluorescein-isothiocyanate-labeled bovine serum albumin (FITC-BSA, Sigma–Aldrich, St. Louis, USA).

### 2.2. Podocyte culture and treatment

The cultured, immortal mouse podocytes originated in the laboratory of Prof. Mundel P. in the USA. The culture management was performed according to the standards outlined in Prof. Mundel's review [15]. Briefly, the growing immortalized podocytes, which were small and polygonal, were cultured for 5 days at 33 °C, 5% $CO_2$ in permissive conditions with INF-γ and then for 14 days at 37 °C, 5% $CO_2$ in restrictive conditions without INF-γ. By the 14th day, the podocyte morphology had changed into spindled cells with small branches. The stable expression of nephrin produced by the podocytes was confirmed by Western blots.

The cells were 70–80% confluent prior to ADR treatment. When podocytes were well differentiated 14 days later, a 2 μl ADR stock solution was added into each well to a final concentration of 0.5 μmol/l. The cells were then cultured for another 24 h and harvested for the next assays.

### 2.3. Plasmids construction

The pcDNA3.1-ANGPTL3-cDNA plasmid was designated by the Yueda Biotech Laboratory. We purchased the pcDNA™6.2-GW/EmGFPmiR from Invitrogen (Carlsbad, CA). It was produced by the BLOCK-iT™ Pol II miR RNAi Expression Vector Kits and fully complements the mouse ANGPTL3 site and cleaves its mRNA [16]. We used the oligo sequence 5′-TGC TGT ATA GAT GTT CCC TCC AGG AAG TTT TGG CCA CTG ACT GAC TTC CTG GAG AAC ATC TATA-3′, 5′-CCT GTA TAG ATG TTC TCC AGG AAG TCA GTC AGT GGC CAA AAC TTC CTG GAG GGA ACA TCT ATAC-3′.

### 2.4. Gene transient transfection

In this study, the differentiated podocytes were trypsinized and plated in six-well plates 24 h prior to transfection in RPMI-1640 containing 10% fetal bovine serum. Four micrograms of DNA was transfected with 10 μl of a Lipofectamine 2000 reagent.

### 2.5. Western blots

The cells were washed with PBS (0.1 M Tris–HCl, pH 7.4; 0.15 M NaCl) and lysed in a buffer containing 50 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, phosphate inhibitors (100 mM $Na_3VO_4$, 10 mM NaF) and protease inhibitors (1 mM PMSF), and the samples were quantified using the Lowery protein assay. For the total proteins, the cells were lysed in 1× SDS lysis buffer (50 mM Tris–HCl, pH 6.8; 2% SDS; 10% glycerol; 1 mM PMSF; and 1 mM $Na_3VO_4$). An equal amount of lysates (50 μg) were loaded on 10% SDS–PAGE and blotted onto PVDF membranes (Millipore Corp.). The samples were blocked in PBS-Tween (PBST) (0.1 M Tris–HCl, pH 7.4; 0.15 M NaCl; 0.1% Tween-20) with 5% non-fat dry milk, and the membranes were incubated with primary antibodies at appropriate dilutions in PBST with 5% milk overnight at 4 °C. Subsequently, the membranes were washed three times with the PBST solution, followed by incubation with horseradish peroxidase-linked secondary antibody (1:3000) in PBST with 5% milk. The results were visualized by fluorography using an enhanced chemiluminescence system (Perfect Biotech, Shanghai, China).

### 2.6. Quantitative real-time PCR assays

Quantitative real-time PCR was conducted as described by Vemuganti et al. [17]. The PCR analysis was conducted as described in the instruction manual for real-time PCR Master Mix (TOYOBO Co., Ltd., Osaka, Japan). For each transcript, quantitative real-time PCR was conducted three times in duplication using each of the cDNA samples. The amplified transcripts were quantized with the comparative CT method using β-actin as the internal control. The primers were designed using the Primer Express software (Primer Premier 5.0) based on the GenBank accession numbers, and the sequences were listed: β-actin, 5′-CCT CTA TGC CAA CAC AGT GC-3′, 5′-ATA CTC CTG CTT GCT GAT CC-3′; ANGPTL3, 5′-ACA TGT GGC TGA GAT TGC TGG-3′, 5′-CCT TTG CTC TGT GAT TCC ATG TAG-3′; nephrin, 5′-GCT GGA CGT GCA TTA TGC T-3′, 5′-CTC CTC GTC TTC CCC CAG T-3′.

### 2.7. Transwell migration assay

Transwell cell culture inserts (pore size 8 μm; Costar Corporation, Corning, NY) were coated with a type-I collagen, rinsed once with PBS and placed in an RPMI 1640 medium in the lower compartment. For each experiment, $1 \times 10^4$ of the differentiated podocytes were seeded in the inserts and allowed to migrate for 18 h while being incubated at 37 °C. Non-migratory cells were removed from the upper surface of the membrane, and migrated cells were fixed with cold methanol and stained with Crystal Viole Solution (Sigma–Aldrich). The number of migrated cells was counted using phase contrast microscopy with a 10× objective on an ECLIPSE TS 100 microscope (Nikon, Tokyo, Japan) in the center of a membrane (one field). Data represent the means ± SEM of six independent experiments.

### 2.8. Scrape-wound assay

The differentiated podocytes (each $5 \times 10^5$) were seeded overnight on type-I collagen-coated coverslips in six-well plates. Each coverslip was then scratched with a sterile 200-μl pipette tip, washed with PBS and placed into fresh medium. After 18 h, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 in PBS and the cell nuclei were stained with DAPI. Images were captured by phase-contrast microscopy under a 10× objective on an ECLIPSE TS 100 microscope (Nikon) at 0 and 18 h after scratching. And the rate of cell migration was measured as the percentage of invaded area with respect to the initial wound area.

### 2.9. Filtering ratio of podocytes assays

In accordance with the filtering ratio of the podocytes assays previously reported [18], the transwell filters were seeded with $5 \times 10^5$ podocytes/filter and cultured under differentiating or permissive conditions for 10–14 days. Their podocyte differentiation was confirmed with nephrin expression using Western blots. Subsequently, the cells were washed twice with PBS supplemented with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ to preserve the cadherin-based junctions. The mediums in the inserts were replaced by 400 μl ECM containing 0.5 mg/ml FITC-BSA, and those in the well were replaced by 600 μl ECM containing 0.5 mg/ml unlabeled BSA (Sigma–Aldrich). Three hours later, 200-μl aliquots were removed to measure their fluorescence using a Fluor spectrometer at 492 nm and to calculate their concentration based on a set of standard dilutions.

### 2.10. Statistical analysis

The values expressed as means ± SEM were subjected to one-way ANOVA testing among the groups, with *P*-values of <0.05 considered statistically significant.

## 3. Results

### 3.1. In vitro synthesis of ANGPTL3 by cultured podocytes

After being cultured *in vitro* at 37 °C for 10–14 days, the immortalized mice podocytes were well differentiated and matured. The differentiated podocytes were divided into six groups according to treatment: ANGPTL3 over-expressed via transient gene transfection (ANGPTL3-cDNA); ANGPTL3 knocked down via RNAi (RNAi); ADR stimulation after ANGPTL3 knock down (RNAi + ADR); transfected negative controls (MOCK); MOCK treated with ADR at 0.5 μmol/l for 24 h (MOCK + ADR) and wild type.

To test the expression of ANGPTL3 in podocytes *in vitro*, we used real-time PCR and Western blots to separately analyze ANGPTL3 mRNA and proteins. We found that only a small amount of ANGPTL3 was expressed by the wild-type podocytes *in vitro* (Fig. 1A–C). The ADR treatment dramatically increased ANGPTL3 expression in cultured podocytes. These findings were in accordance with our previous data concerning ADR-treated and normal rats (Fig. 1A–C).

In combination with these experiments, we tested the transfection efficiency in this study. The results showed that the ANGPTL3 mRNA of the RNAi + ADR group was significantly decreased (44.5 + 2.5%) compared with that of the MOCK + ADR group ($P < 0.05$) (Fig. 1A). Furthermore, the podocytes transiently transfected with ANGPTL3 cDNA significantly increased ANGPTL3 mRNA ($7.35 \times 10^3$ ANGPTL3/β-actin fold) compared with the controls. The Western blot data further confirmed the changes in ANGPTL3 mRNA levels (Fig. 1B and C).

### 3.2. The influence of ANGPTL3 on podocyte motility

In our previous study, we found that the changes in ANGPTL3 expression in the glomerulus were correlated with the degree of FP effacement [13]. The increasing motility of podocytes *in vitro* is known to generally indicate the FP effacement *in vivo*. To further

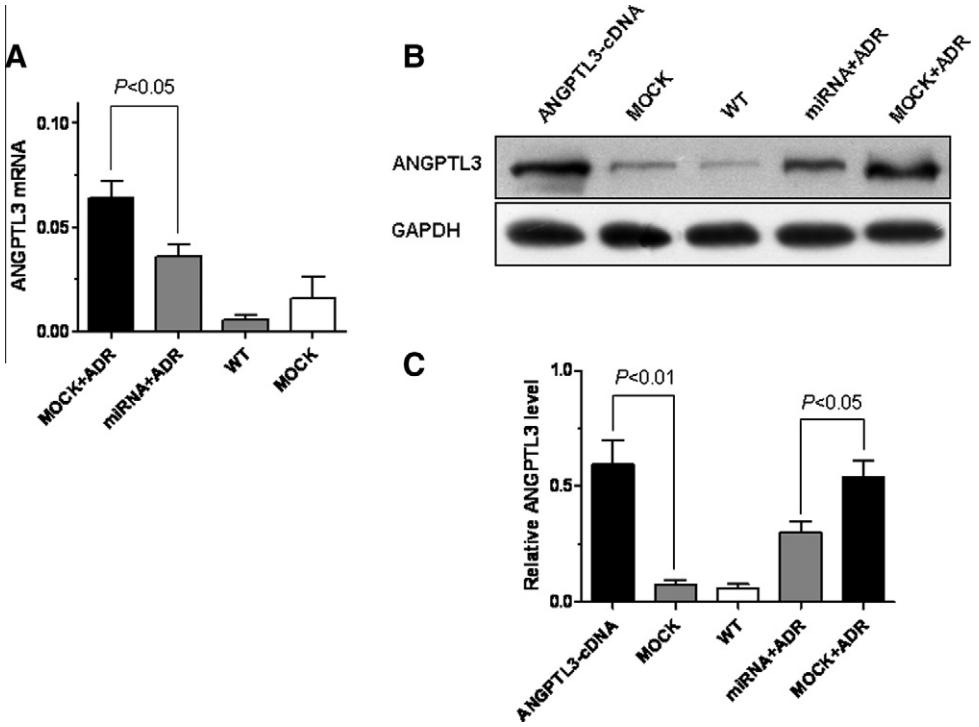


**Fig. 1.** *In vitro* synthesis of ANGPTL3 by cultured podocytes. (A) Quantitative real-time PCR used to quantify the cDNA copies in different podocyte groups; after ADR treatment, ANGPTL3 mRNA was significantly increased ($P < 0.01$); real-time PCR results confirming that RNAi gene knockdown was successful. (B) Western blot results showing weaker ANGPTL3 expression in MOCK podocytes than in the ADR group; ANGPTL3-cDNA group showing increased expression of ANGPTL3. (C) Relative ANGPTL3/GAPDH levels. ANGPTL3-cDNA: over-expression of ANGPTL3 by gene transfection; MOCK: negative transfect control; MOCK + ADR: MOCK podocytes treated with ADR for 24 h; RNAi + ADR: treated with ADR after the knockdown of ANGPTL3; WT: wild type.

investigate ANGPTL3's role on FP effacement, we used the modified scrape-wound assay and transwell migration assay to test the motility of the cultured podocytes with different treatments.

From the scrape-wound assays, the ability of wound repair in the ANGPTL3 over-expressing podocytes was found to be significantly enhanced compared with that in the MOCK (Fig. 2A and B). Accordingly, it was also found that their immigrations were higher than those of the MOCK by transwell assays (Fig. 2C).

To further investigate the effect of ANGPTL3 on motility, we knocked down the ANGPTL3 of the podocytes using RNAi to examine the possible changes. The data regarding wound repair ability and immigration showed that the knockdown of ANGPTL3 failed to change podocyte motility compared with the transfection of negative control cells (Fig. 2A–C).

Extensive FP effacement is the principle pathologic change in minimal change disease (MCD) nephropathy. ADR is often used to induce MCD in rats. In this study, we observed that ADR treatment significantly enhanced cultured podocyte motility and promoted its migration (Fig. 2A–C). The results suggested that the podocyte motility promoted by ADR is one of mechanisms of FP effacement in ADR-treated nephrotic animal models.

Furthermore, we evaluated the potential effect of ANGPTL3 on the alteration of motility by ADR *in vitro*. Surprisingly, the podocytes with knocked down ANGPTL3 inhibited significantly enhanced motility as a result of ADR treatment, as indicated by the reduced number of immigrating cells.

### 3.3. ANGPTL3 associated with the increasing podocyte permeability induced by ADR

As an ultra-filtration system, the glomerular barrier guarantees against protein loss. The dysfunction of podocyte permeability is considered to be a critical step on the way to proteinuria. To investigate the effect of ANGPTL3 on podocyte permeability, we used BSA-labeled FITC (FITC-BSA) to examine the permeability of the monolayer podocytes in different groups. Our results showed that the FITC-BSA permeability in the over-expressing ANGPTL3 podocytes was significantly higher than that in the controls, suggesting that ANGPTL3 promotes the development of proteinuria (Fig. 3).

Furthermore, the data also confirmed that ADR treatment could significantly promote the FITC-BSA permeability of podocytes [19]. However, the ADR stimulation could not powerfully increase the FITC-BSA permeability of podocytes after knockdown of ANGPTL3 (Fig. 3).

### 3.4. ANGPTL3 orchestrating the expression of nephrin in podocytes

To explore the possible mechanism of ANGPTL3 in podocyte injury, we examined nephrin expression in cultured podocytes with different treatments. Quantitative real-time PCR and Western blots confirmed that a weak expression of nephrin occurred in the immortalized mice podocytes *in vitro* [15,20]. Compared with the controls, the over-expressing ANGPTL3 podocytes displayed a

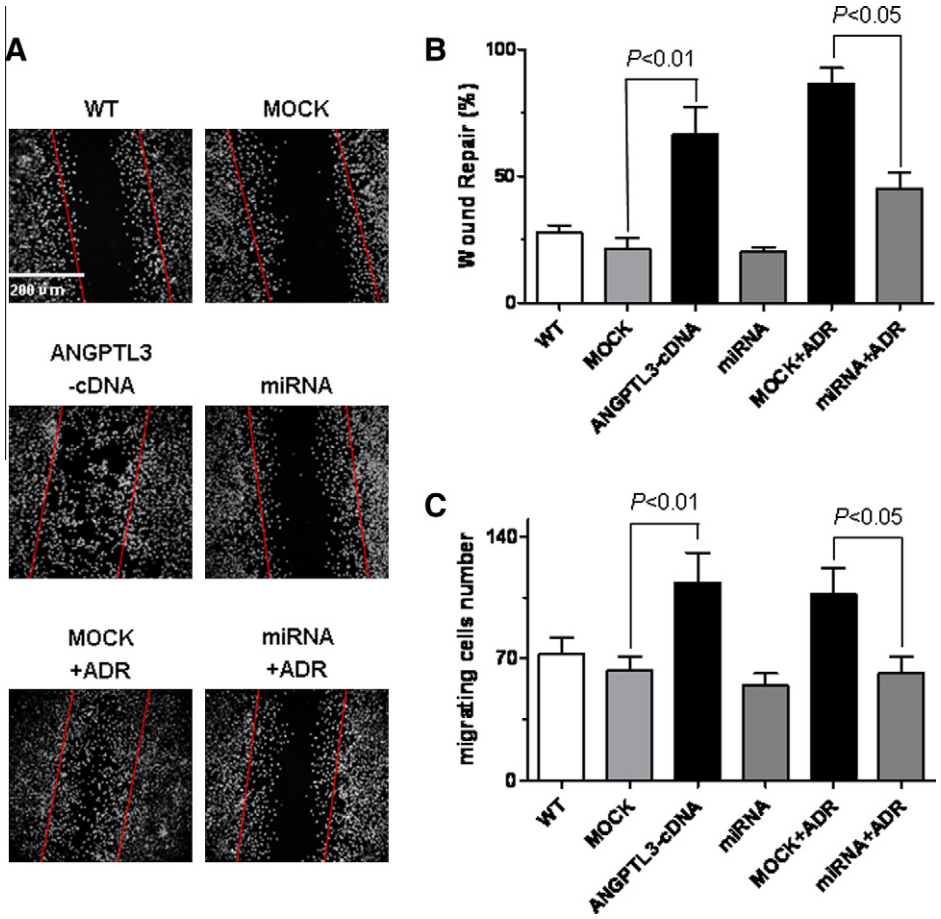


**Fig. 2.** The influence of ANGPTL3 on podocyte motility. (A) Scrape-wound assay: MOCK podocytes starting to migrate into the wound track 18 h after the podocyte layer was scraped; the ANGPTL3 gene transfect promoted podocyte migration; the knockdown of ANGPTL3 by RNAi failed to alter podocyte migration, as reflected by fewer cells having migrated into the wound track; treatment with ADR significantly promoted the migration of MOCK podocytes, and knockdown of ANGPTL3 by RNAi strongly reduced podocyte migration. Scale bar, 200 μm. (B) The percent of wound repair in different groups. (C) Transwell tests analyzing the migration of podocytes. The results of transwell tests showing that ADR treatment significantly elevated the number of migrating podocytes; in contrast, the knockdown of ANGPTL3 significantly decreased the migration after ADR treatment. RNAi: ANGPTL3 gene-knockdown podocytes.

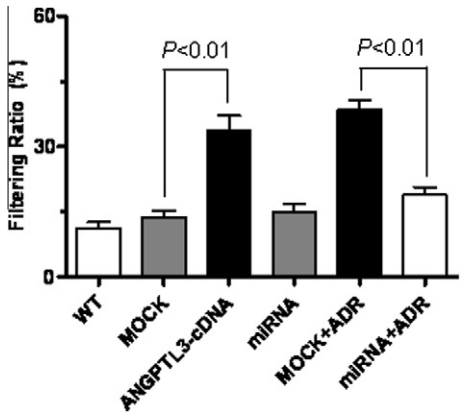


**Fig. 3.** ANGPTL3 mediated the filtering ratio of podocytes. BSA labeled by FITC used to test the filtering ratio of podocytes; ADR treatment enhanced the filtering ratio of FITC-BSA; the absence of ANGPTL3 significantly reduced the filtering ratio.

dramatic increase in nephrin expression (Fig. 4A–C). Moreover, the expression of nephrin was found to be unremarkable in the podocytes with ANGPTL3 knockdown 24 h later (Fig. 4A–C).

In this study, we repeatedly observed a significant increase of nephrin in the cultured podocytes with ADR treatment for 24 h (Fig. 4A–C). ADR stimulation failed to enhance nephrin expression in podocytes with the knockdown of ANGPTL3.

## 4. Discussion

Our data demonstrated that ANGPTL3 could be expressed by cultured podocytes *in vitro* and that its expression was significantly increased by the ADR treatment. Further, the over-expression of ANGPTL3 significantly promoted the motility and permeability of podocytes to an extent similar to ADR treatment. The knockdown of ANGPTL3 powerfully inhibited the increased motility and permeability in the podocytes treated by ADR.

In this study, we confirmed the effect of ANGPTL3 on the motility and permeability of podocytes. ANGPTL3 was found to strongly promote wound repair and cellular migration in podocytes, suggesting that it might aggravate FP effacement *in vivo*. The permeability assay on the ANGPTL3 over-expressing podocyte further suggested that ANGPTL3 could be involved in the development of proteinuria. The knockdown ANGPTL3 results suggested that absence of ANGPTL3 did not impact the motility of podocytes and the permeability ratio of the podocyte monolayer under normal conditions. These data might partly explain why ANGPTL3 knockdown mice failed to develop proteinuria and glomerular dysfunction [6]. In conclusion, ANGPTL3 does not safeguard the normal function of podocytes and is more likely to play a pathologic role.

ADR treatment uses classical reagents to induce podocyte injury *in vivo/in vitro*. In the current study, our results suggested that ANGPTL3 could play a vital role in the motility and the permeability of podocytes promoted by ADR stimulation. Therefore, we hypothesized that podocytes damaged by reactive oxygen species (ROS) could improve the output of ANGPTL3, which might then aggravate the podocyte injury. The most recent reports support a new evaluation in which podocytes are not only passive but also provocative in proteinuria glomerular pathology. Our results might provide evidence for this position [21].

To explore the mechanism of ANGPTL3's effects on podocyte motility, we investigated its association with nephrin *in vitro*. The results showed that ANGPTL3 over-expression significantly increased the expression of nephrin in the cultured podocytes (Fig. 4A–C) and suggested that ANGPTL3 may enhance podocyte motility by affecting the expression of nephrin, which might be

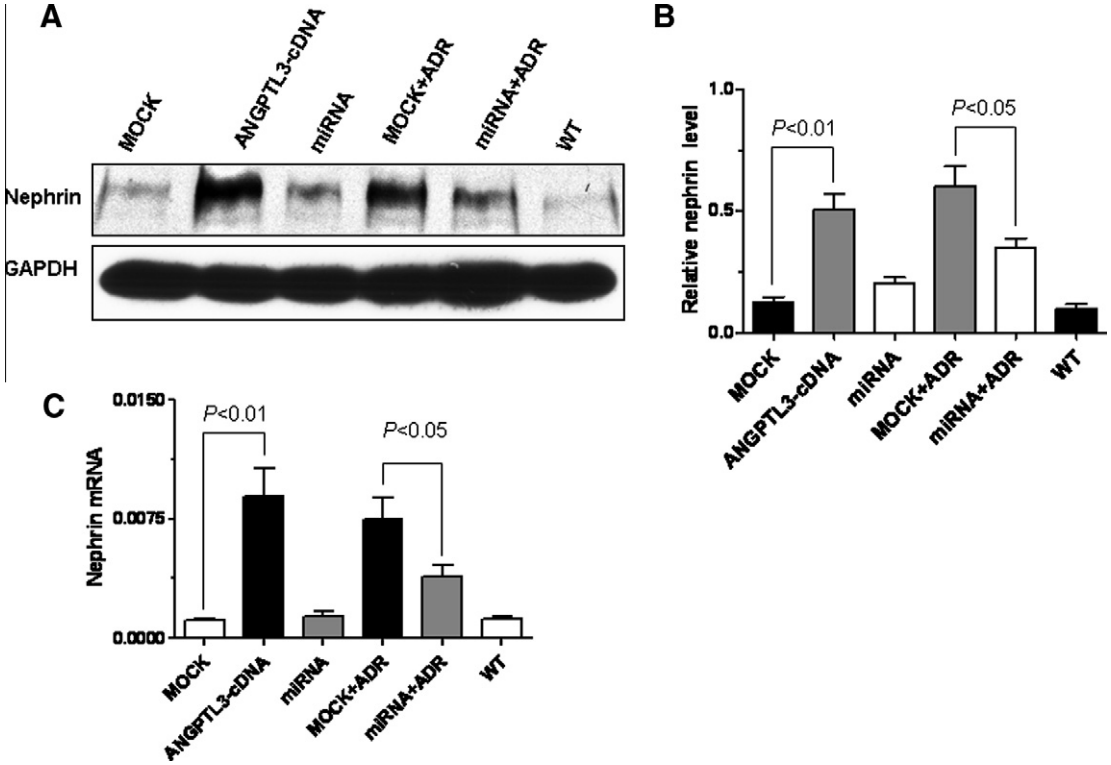


**Fig. 4.** ANGPTL3 orchestrated the expression of nephrin and podocin in podocytes. (A) ANGPTL3 orchestrated the expression of nephrin; Western blots suggesting that ADR treatment induced a significant improvement of nephrin protein of podocytes, as in the case of ANGPTL3-cDNA podocytes; after knockdown, ADR treatment failed to increase the expression of nephrin. (B) Relative nephrin/GAPDH level. (C) Quantitative real-time PCR assessing the mRNA of nephrin in different groups.

involved in podocytes FP effacement and in the development of proteinuria *in vivo*. Previous results have shown that the expression of nephrin is significantly increased in ADR-induced nephrotic glomerular [22,23], but there are few reports on ADR-treated podocytes *in vitro*. In the present study, we demonstrated that ADR could also increase the expression of nephrin *in vitro*. Interestingly, the knockdown of ANGPTL3 podocytes may significantly inhibit the enhanced expression of nephrin by ADR. In light of this result, it could be concluded that the podocyte injury induced by ROS was related to the alteration in nephrin and that ANGPTL3 plays an important role in regulating the expression of nephrin in pathologic conditions.

This study might be helpful in understanding the mechanism of podocyte injury. It is a meaningful attempt to explore the function of ANGPTL3 in kidney diseases.

## 5. Conclusion

ANGPTL3 could promote the motility and permeability of podocytes. ANGPTL3 played a vital role in ADR-treated podocyte injury. As a candidate signaling molecule, nephrin was involved in ANGPTL3's mechanism.

## Acknowledgment

This work was supported by the National Natural Science Foundation of China (Grant No. 30971375). The study was also is sponsored by the Program of Shanghai Subject Chief Scientist (Grant No. 10XD1400700). We thank all the staff in the Department of Nephrology at the Children's Hospital of Fudan University for their technical assistance, and we are also grateful to Prof. Xiliang Zha for his helpful discussions. This study also received assistance from the Fudan University Outstanding Doctoral Research Fund.

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