Newly identified cytoskeletal components are associated with dynamic changes of podocyte foot processes

Jing Miao¹, Qingfeng Fan¹, Qinghua Cui², Han Zhang¹, Lihong Chen³, Suxia Wang⁴, Na Guan¹, Youfei Guan³ and Jie Ding¹

¹Department of Pediatrics, Peking University First Hospital, Beijing 100034, ²Department of Medical Informatics, Peking University Health Science Center, Beijing 100083, ³Department of Physiology and Pathophysiology, Peking University Health Science Center, Beijing 100083 and ⁴Department of Electron Microscopy, Peking University First Hospital, Beijing 100034, China

Correspondence and offprint requests to: Jie Ding; E-mail: djnc_5855@126.com

Abstract

Background. Proteinuria, one of the main manifestations of nephrotic syndrome, is an important risk factor for the progression of renal diseases. Podocyte foot processes (FPs) injury induces proteinuria in most renal diseases. The podocyte cytoskeleton plays important roles in maintaining the normal morphology of FPs. However, the underlying cytoskeletal component that initiates and regulates the dynamic changes of FPs is still unclear. Here, the involved podocyte cytoskeletal molecules were explored on different days in puromycin aminonucleoside nephropathy rats.

Methods. Microarray analysis of isolated glomeruli was performed at Day 2, Day 10 and Day 15 in puromycin aminonucleoside nephropathy rats. Cytoskeletal genebank was established by sorting with the keyword ‘cytoskeleton’ from PUBMED genebank to identify the differential cytoskeletal genes. Microarray results were further confirmed by real-time PCR, western blot and double immunolabelling to validate their localizations.

Results. Nine different cytoskeletal genes were found to be involved in the dynamic changes of FPs in puromycin aminonucleoside nephropathy rats, including six up-regulated (Tagln, Actr2, Dnm3, Arc, Vcl and Birc5) and three down-regulated (Krt2–7, Neb1 and Tnncl1). The differential expression of transgelin, survivin, arp2, cytokeatin7 and vinculin was verified by real-time PCR and western blot. Double immunolabelling revealed that five cytoskeletal proteins indeed colocalized with podocyte specific markers synaptopodin or α-actinin-4. In addition, similar expression and distribution changes were detected in patients with proteinuric renal diseases and puromycin aminonucleoside-treated podocytes.

Conclusions. We identified five novel podocyte cytoskeletal proteins and found that they were associated with the dynamic changes of FPs in podocyte injury.

Keywords: microarray analysis; podocyte cytoskeleton; proteinuria; puromycin aminonucleoside nephropathy

Introduction

Proteinuria, one of the most important manifestations of nephrotic syndrome (NS), is a major risk factor for the progression of renal diseases to end-stage renal failure [1]. Glomerular filtration barrier, especially the final layer podocyte slit diaphragm (SD), plays an important role in the occurrence of proteinuria [2]. The identifications of SD molecular components nephrin [3], podocin [4] and P-cadherin [5] further confirmed its critical role in the development of proteinuria. Podocyte, a differentiated glomerular visceral epithelial cell, has complex cell morphology, which displays as a large body extending major processes and foot processes (FPs). The inter-digitating podocyte FPs form the vital filter barrier SD [6]. The normal morphology of podocyte and FPs is indispensable for SD integrity. It is revealed that podocyte FPs display a dynamic and entirely reversible character in some proteinuric renal diseases. Human minimal change nephrotic syndrome (MCNS) is characterized with massive proteinuria and FPs effacement, which can be restored entirely within days of initiating glucocorticoid therapy [7]. Similarly, puromycin aminonucleoside (PAN)-induced nephrosis mimics human MCNS, in which FPs and proteinuria could spontaneously recover [8].

The highly dynamic FPs consist of actin-based contractile apparatus [9]. A single cytoskeletal gene mutation, such as ACTN4 encoding an actin-filament cross-linking protein α-actinin-4, leads to hereditary NS [10]. Further studies revealed that podocyte cytoskeleton links to the critical SD molecules such as nephrin via a few adaptors including CD2AP [11], ZO-1 [12] and Nck [13]. Although the derangement of podocyte cytoskeletons and intercellular junctional architecture can cause the injury of podocyte FPs, it is still unclear how the cytoskeletal molecules are involved in the FPs dynamic changes, and whether there are some key cytoskeletal molecules. These studies might help to understand the molecular mechanisms of proteinuria and to further develop new targets for dealing with proteinuria.
Here, five novel cytoskeleton proteins, transgelin, survivin, arp2, cytokeratin7 and vinculin, were identified, and demonstrated to be involved in the dynamic changes of FPs and proteinuria in PAN rats. More importantly, similar expressions and distribution changes were also observed in patients with proteinuric renal diseases, as well as in PAN-treated podocytes.

**Subjects and methods**

**Animal model**

Male Sprague-Dawley rats \((n = 36, 120–140\, \text{g})\) were purchased from the Experimental Animal Center at Peking University Health Science Center, and divided into control rats \((n = 6)\) and experimental rats \((n = 30)\). Experimental rats were given a single intra-peritoneal injection of PAN (Sigma-Aldrich, USA) \((15\, \text{mg/100 g weight})\), and six rats for each time point were sacrificed at Days 2, 5, 10, 15 and 20, respectively. Control rats were injected with 2 ml of normal saline and sacrificed after one day. The animal studies were approved by the Animal Research Review Board of Peking University.

**Urinary collection and tissue preparation**

Twenty-four hour urine was collected from control and PAN rats at Days 2, 5, 10, 15 and 20 for measuring urinary protein on an automatic biochemical analyser (7170A, Hitachi, Japan) using a Pyrogallol red-molybdate dye-binding method.

At the designated time points, the rats were sacrificed and the kidneys were removed. One kidney was used for isolating glomeruli using the conventional sieving method [14]. The renal cortex of the other kidney was further fixed in 1% osmium tetroxide, followed by dehydration in graded ethanol and embedding in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (JEM-1230, JEOL, Japan).

**Glomerular cytoskeleton gene expression profiling analysis**

Total RNA was extracted from each rat glomeruli using an RNeasy Micro Kit (Qiagen, Germany). Equal amounts of RNA from two rat glomeruli were pooled together for each time point. The gene expression profile was examined using a rat Affymetrix chip with 31,100 probe sets (AFF-900408, USA).

Raw data were analysed by the MASS condensing algorithm, and quality control was performed using GeneData Expressionist (GeneData AG, Basel, Switzerland). The rat gene ontology (GO) data were obtained from NCBI. A bank of cytoskeleton genes was constructed using the keyword “cytoskeleton” based on the rat gene GO terms. As a result, 411 rat cytoskeleton genes were identified, which corresponded to 558 probes in the chip. The differentially expressed cytoskeleton probe sets with at least a 2.0-fold change and false discovery rate (FDR) \(q\)-value not >0.25 were identified using ANOVA and the SAM software package [15]. All \(P\)-values were corrected for the FDR.

**Reverses transcription reaction and quantitative real-time polymerase chain reaction**

Two micrograms of RNA extracted from renal cortices with Trizol (Invitrogen, USA) were reverse transcribed to cDNA for quantitative real-time PCR with SYBR Green (TaKaRa, Japan). The primers for cytoskeleton genes and the podocyte-specific gene, NPHS2, encoding podocin are shown in Table 1. PCR reactions were performed on a GeneAmp 7300 (PE Biosystems, USA) at 95°C for 10 s, followed by 40 cycles of 95°C for 5 s, 61°C for 31 s, with a dissociation stage at 95°C for 15 s, 60°C for 1 min and then 95°C for 5 s. The quantity of expression of all the cytoskeleton genes messenger RNA (mRNA) was corrected by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression in the same specimen.

**Human kidney specimens**

Kidney specimens were obtained from children with MCNS, focal segmental glomerulosclerosis (FSGS) and membranous nephropathy (MN) \((n = 7, 5\) and 5, respectively), all diagnosed with clinically heavy proteinuria and corresponding typical pathological features, as revealed by light microscopy and electron microscopy. Five normal specimens from nephrectomized kidneys were used as controls under the approval of the Ethical Committee of Peking University First Hospital.

**Podocyte cultures**

Mouse podocyte clones were cultured at 33°C in RPMI1640 with a 10% fetal bovine serum (Gibco, USA) and 10 units/ml of γ-interferon for propagation. Cells were reseeded in 6-well plates with glass coverslips coated with 10 µg/ml of type-I collagen (Sigma-Aldrich, USA) for immunostaining and cultured ~10 days at 37°C by removal of γ-interferon for differentiation. When they were at 60% confluence, the podocytes were treated with 75 µg/ml PAN for 48 h.

**Indirect immunofluorescence staining**

Five micrometer cryosections were fixed in ice-cold acetone, subsequently permeablized and blocked with 0.3% Triton X-100 and 10% goat serum. The following primary antibodies were used: rabbit anti-podocin (1:800, a gift from Professor Corinne Antignac), rabbit anti-α-actinin, transgelin, arp2, cytokeratin7 (1:100, Santa Cruz, USA), survivin (1:800, Cell Signaling, USA), mouse anti-vinculin (1:500, Abcam, USA) and mouse anti-synaptopodin (ready to use, Progen, Germany). After three washes, the slides were incubated with FITC goat anti-rabbit IgG and TRITC goat anti-mouse IgG. For cells, the coverslips were fixed with 4% paraformaldehyde, followed by permeabilization and blocking with 0.3% Triton X-100 and 5% bovine serum albumin. Primary and secondary antibodies, and Hoechst nuclear dye were applied. The slides were mounted with 15% Mowiol (Sigma-Aldrich, USA). Stained images for each antibody at the same light exposure were obtained by confocal laser-scanning microscopy (Zeiss Lsm510 Meta, Germany). Photographs of glomeruli stained with each antibody were selected randomly and analysed by a person who was blinded to the study groups.

**Table 1. Primers and PCR product size of differential cytoskeletal genes, NPHS2 and GAPDH**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Primers sequences</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acn2</td>
<td>Forward GCTGGCCTTAAGAGACGACG</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>Reverse AAGCAATTCGACAAACCACACAA</td>
<td></td>
</tr>
<tr>
<td>Arc</td>
<td>Forward CCGCTCTAGAGGAGTTCTTA</td>
<td>178</td>
</tr>
<tr>
<td></td>
<td>Reverse GTACGTGTCACAGATCATCTCA</td>
<td></td>
</tr>
<tr>
<td>Krf2-7</td>
<td>Forward GCAGGATGTTGGAAGAAGTT</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td>Reverse CGTTAAGGGCTTGAAGGAGAAG</td>
<td></td>
</tr>
<tr>
<td>Vcl</td>
<td>Forward CGCTGTCAGTTGTTTACGTA</td>
<td>171</td>
</tr>
<tr>
<td></td>
<td>Reverse CAAATACGGCTTACATCT</td>
<td></td>
</tr>
<tr>
<td>Birc5</td>
<td>Forward AACTTCAGAAGCTGCCCTT</td>
<td>186</td>
</tr>
<tr>
<td></td>
<td>Reverse AGGGAGTGCTCTTCTATGCT</td>
<td></td>
</tr>
<tr>
<td>Nefh</td>
<td>Forward GAAGATGGTCGCAAGAAGTCA</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Reverse TCAGCTCCGAGACTGCTA</td>
<td></td>
</tr>
<tr>
<td>Tnc1</td>
<td>Forward GAAGTCTGGAGAGGAGCTGTCG</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>Reverse TTCATAGGCCTCTTGGATGTC</td>
<td></td>
</tr>
<tr>
<td>Tagln</td>
<td>Forward GAAAAGATATGGCAGCAGTGG</td>
<td>143</td>
</tr>
<tr>
<td></td>
<td>Reverse TTGACTGTCTGTGAACTCCC</td>
<td></td>
</tr>
<tr>
<td>Dnm3</td>
<td>Forward CGTGAGGCGAGAAGGAAGACG</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td>Reverse CCCATTCTTCATGCTGAG</td>
<td></td>
</tr>
<tr>
<td>NPHS2</td>
<td>Forward ACGACGGAGTCAACCTTGGTG</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>Reverse AAAACCCTACACCTCCTCACA</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward AAAACCACCATACACCTCCTCACA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse GTGTTGCAACCCATCACACAA</td>
<td></td>
</tr>
</tbody>
</table>

**Transmission electron microscopy**

The renal cortex kept in 3% gluteraldehyde was further fixed in 1% osmium tetroxide, followed by dehydration in graded ethanol and washing in acetone, and finally embedding in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (JEM-1230, JEOL, Japan).
Newly identified cytoskeletal components are associated with dynamic changes of podocyte foot processes

2399

Ultrastructural changes

FPs of normal rats were long and thin. Two days after PAN injections, podocytes appeared to swell, and a few FPs became broad. Five days after PAN injections, FPs broadened noticeably and showed segmental effacement. At Day 10, FPs were lost and presented with diffuse and widespread fusion. Fifteen days after PAN injections, FPs lesions began to recover. At Day 20, typical FPs were observed again (Figure 2).

Cytoskeleton gene expression profiles

The exact amount of glomeruli obtained by sieving was verified by light microscopy; >90% purity was obtained with little tubular attachment (Supplementary Figure S1). Based on the ultrastructural changes of FPs, gene profiling was performed at Days 2, 10 and 15 in PAN rats. Kendall’s coefficients of concordance for the three gene chips each time point were 0.97 (P = 0.00), 0.96 (P = 0.00), 0.96 (P = 0.00) and 0.97 (P = 0.00), respectively, indicating consistency. In all, nine cytoskeletal genes, including six up-regulated (Tagln, Actr2, Dnm3, Arc, Vcl and Birc5) and three down-regulated (Krt2–7, Neb1 and TnnC1), were identified (Table 2).

mRNA and protein expressions of five novel cytoskeletons

Real-time PCR showed that Tagln, Actr2, Vcl, Birc5 and Krt2–7 increased significantly at Day 10 in PAN rats compared to controls, and that Arc and Neb1 displayed no differences between control and PAN rats. NPHS2 decreased significantly at Day 2 in PAN rats compared to controls (Supplementary Figure S2). The specific protein bands for the five genes Tagln (transgelin), Actr2 (arp2), Vcl (vinculin), Birc5 (survivin) and Krt2–7 (cytokeratin7), were detected at sizes of 22, 43, 116, 17 and 54 kDa, respectively, and quantitated in relation to GAPDH (36 kDa). Compared to controls, all cytoskeleton proteins increased significantly in PAN rats at Day 10, followed by an obvious down-regulation at Day 15 (Figure 3). Similarly, the relative expression of five cytoskeleton proteins also increased in PAN-treated podocytes compared to control cells (Figure 6).

Localization of five novel cytoskeletal molecules

Rat kidneys. The staining of transgelin, survivin, arp2, cyto
terin7 and vinculin was very weak in normal glomeruli, and displayed a linear pattern in normal glomeruli. Fluores
cence intensities increased from Day 2 to Day 10, and then decreased to a level slightly above normal at Day 15. A low-power image of the five cytoskeleton proteins in the kid
ey clearly shows these intensive changes in the glomeruli (Supplementary Figure S3). Obvious granular spots of sur
vivin and cytokeratin7 were observed along the glomerular capillary loops at Day 10 in PAN rats. Synaptopodin and α-actinin-4 are considered as podocyte specific markers. Double-labelling assays showed that transgelin, survivin, arp2 and cytokeratin7 colocalized well with synaptopodin and vinculin colocalized completely with α-actinin-4. The
**Table 2.** Differential cytoskeletal genes sorted by Affymetrix assay

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene full name</th>
<th>Gene ID</th>
<th>Control: gene level</th>
<th>Gene expression level</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;/FC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Gene expression level</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;/FC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Gene expression level</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;/FC&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actr2</td>
<td>ARP2 actin-related protein 2 homolog (yeast)</td>
<td>289820</td>
<td>121.8 ± 45.8</td>
<td>161.7 ± 15.8</td>
<td>0.622/1.33</td>
<td>330.5 ± 133.9</td>
<td>0.032/2.71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>180.6 ± 85.0</td>
<td>0.115/1.48&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Arc</td>
<td>Activity regulated cytoskeletal-associated protein</td>
<td>54323</td>
<td>717.3 ± 314.5</td>
<td>725.7 ± 126.8</td>
<td>0.622/1.01</td>
<td>1486.9 ± 374.2</td>
<td>0.032/2.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1841.1 ± 217.2</td>
<td>&lt;0.000/2.57&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Togln</td>
<td>Transgelin</td>
<td>25123</td>
<td>667.5 ± 169.5</td>
<td>401 ± 365.7</td>
<td>0.993/0.74</td>
<td>3004.9 ± 1107.4</td>
<td>&lt;0.000/4.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>998.0 ± 368.4</td>
<td>0.115/1.5</td>
</tr>
<tr>
<td>Vcl</td>
<td>Vinculin</td>
<td>305679</td>
<td>578.3 ± 30.1</td>
<td>584.1 ± 156.3</td>
<td>0.622/1.01</td>
<td>1197.2 ± 179.5</td>
<td>&lt;0.000/2.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>875.2 ± 189.1</td>
<td>0.041/1.51</td>
</tr>
<tr>
<td>Birc5</td>
<td>Baculoviral IAP repeat-containing 5</td>
<td>64041</td>
<td>126.4 ± 45.8</td>
<td>117.6 ± 34.6</td>
<td>0.622/0.93</td>
<td>260.1 ± 35</td>
<td>0.032/2.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>155.7 ± 21.3</td>
<td>0.115/1.23</td>
</tr>
<tr>
<td>Dnm3</td>
<td>Dynamin 3</td>
<td>171574</td>
<td>35.2 ± 15.2</td>
<td>48.5 ± 18.1</td>
<td>0.622/1.38</td>
<td>87.2 ± 26.0</td>
<td>0.032/2.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47.2 ± 8.0</td>
<td>0.115/1.34</td>
</tr>
<tr>
<td>Krt2–7</td>
<td>Keratin complex 2, basic, gene 7</td>
<td>300242</td>
<td>3 584.2 ± 242.6</td>
<td>4 646.4 ± 1 085.7</td>
<td>0.622/1.3</td>
<td>2282.2 ± 776.1</td>
<td>0.066/0.64</td>
<td>1 787.4 ± 105.2</td>
<td>&lt;0.000/0.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nefl</td>
<td>Nebulette (predicted)</td>
<td>307189</td>
<td>5 882.7 ± 527.3</td>
<td>2 423.5 ± 796.6</td>
<td>&lt;0.000/0.41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3695.2 ± 1340.6</td>
<td>0.066/0.63</td>
<td>5 910.6 ± 886.8</td>
<td>0.115/1</td>
</tr>
<tr>
<td>Tnnc1</td>
<td>Troponin C type 1 (slow)</td>
<td>290561</td>
<td>15 830 ± 2 446.5</td>
<td>13 958.8 ± 609.4</td>
<td>0.982/0.88</td>
<td>6483.3 ± 4513.5</td>
<td>0.066/0.41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9 854.6 ± 3 245.4</td>
<td>0.047/0.62</td>
</tr>
</tbody>
</table>

<sup>a</sup>Compared with control.

<sup>b</sup>Up-regulation ≥2.0.

<sup>c</sup>Down-regulation ≥2.0.

P: the P-values, which are corrected by false discovery rate (FDR) FC: fold change.
Newly identified cytoskeletal components are associated with dynamic changes of podocyte foot processes

Fig. 2. Ultrastructural changes of podocyte foot processes in PAN rats. (A) The foot processes were long and thin in normal rats. (B) Two days after PAN injections, a few foot processes became broad. (C) Five days after PAN injections, foot processes broadened noticeably and showed segmental effacement. (D) Ten days after PAN injections, foot processes were effacement. (E) Fifteen days after PAN injections, foot processes began to rebuild. (F) Twenty days after PAN injections, foot processes recovered obviously. Bar = 1 µm.

Fig. 3. mRNA and protein expression of novel cytoskeletal molecules in PAN rats. In relation to GAPDH, the mRNA and protein expression of Tagln (transgelin) (A, A'), Birc5 (survivin) (B, B'), Actr2 (arp2) (C, C'), Krt2–7 (cytokeratin7) (D, D') and Vcl (vinculin) (E, E') increased significantly at Day 10, then decreased at Day 15 in PAN rats compared to controls (Con). Data are presented as mean ± SD. N = 6. *P < 0.05 versus Con; **P < 0.01 versus Con.

distribution of synaptopodin and α-actinin-4 showed no significant changes in PAN rats (Figure 4).

Human kidneys. In normal human kidneys, staining for transgelin and cytokeratin7 was negative in glomeruli, but it was intense in human MCNS, FSGS and MN glomeruli. In normal human kidneys, arp2 was negative in glomeruli and positive in partial tubules, but positive in MCNS and MN glomeruli. Transgelin, arp2 and cytokeratin7 all colocalized well with synaptopodin in MCNS, FSGS and MN glomeruli. Low-power images also displayed intensive arp2 and cytokeratin7 staining in MCNS, FSGS and MN tubules (Supplementary Figure S4). The staining of vinculin was very weak in normal glomeruli and intense in MCNS, FSGS and MN glomeruli, and showed complete colocalization with the podocyte-specific molecule podocin. We noted that
Fig. 4. Immunofluorescence staining of novel cytoskeletal molecules in PAN rats. Transgelin (A), survivin (D), arp2 (G) and cytokeratin7 (J) were labelled green and showed a weak staining in normal rat glomeruli (Control). Double-labelling assays showed that transgelin (C), survivin (F), arp2 (I) and cytokeratin7 (L) colocalized with podocyte marker synaptopodin, labelled red (Synap: B, H, K), displaying a linear pattern along the glomerular capillary loops. Vinculin (M), labelled red, showed colocalization with the podocyte molecule α-actinin-4, labelled green (N), displaying a dotted, linear pattern in glomeruli (O). Bar = 20 µm.

Survivin was negative in normal glomeruli and weakly positive in normal tubules, and mainly showed prominent perinuclear distribution in the glomeruli and tubules of MCNS, FSGS and MN (Figure 5).

Cultured mouse podocytes. In normal cultured podocytes, the staining of transgelin, arp2, cytokeratin7 and survivin was weak and evenly distributed in the cytoplasm, whereas their fluorescence intensity increased markedly, especially along the cell membrane of PAN-treated podocytes. Although the fluorescence intensity did not change after PAN treatment, the distribution of vinculin was obviously altered, displaying a significant dotted- and plaque-like condensation in the cell membrane (Figure 6).

Discussion

Recently, more and more studies have demonstrated that podocytes have complex cytoskeletons and that the fine regulation plays an important role in maintaining the normal morphology of podocytes [16]. However, the molecular mechanisms of the reorganization of podocyte cytoskeleton remain to be explored. In this study, we focused on the involved cytoskeletal molecules in the dynamic alterations of podocyte FPs in PAN nephrosis rats, in order to further understand the pathogenesis of proteinuria. Therefore, all of rat cytoskeleton genes were sorted from Genebank using the keyword ‘cytoskeleton’. A total of 411 rat cytoskeleton genes were found, which corresponded to 558 probes in our chip. To specifically identify the cytoskeleton molecules that localize to glomerular podocyte, rat glomeruli were isolated by conventional sieving methods in order to avoid tubuli and other interstitial tissues contamination. The microarray analysis of glomeruli was performed at different time points, including the occurrence, development and recovery of proteinuria in PAN rats.

Compared to controls, nine differential cytoskeletal genes were identified in PAN rats, including six up-regulated (Tagln, Actr2, Dnm3, Arc, Vcl and Birc5) and three down-regulated (Krt2–7, Neb1 and Tmec1). Furthermore, real-time PCR and western blot confirmed that five
Newly identified cytoskeletal components are associated with dynamic changes of podocyte foot processes

Fig. 5. Immunofluorescence staining of novel cytoskeletal molecules in human kidneys. Transgelin (A1, a1, a4, a7), arp2 (C1, c1, c4, c7), and cytokeratin7 (D1, d1, d4, d7) are green. Vinculin is red (E1, e1, e4, e7). Their staining was intense in the glomeruli of patients with MCNS (a1, c1, d1, e1), FSGS (a4, d4, e4) and MN (a7, c7, d7, e7) as compared to normal glomeruli (Control: A1, C1, D1, E1), except for arp2 in FSGS glomeruli (c4). Transgelin, arp2, and cytokeratin7 colocalized with the podocyte marker synaptopodin, labelled red, in glomeruli of MCNS (a3, c3, d3), FSGS (a6, d6) and MN (a9, c9, d9). Vinculin showed complete colocalization with the podocyte molecule podocin, labelled green in control (E3), MCNS (e3), FSGS (e6) and MN (e9). The staining of survivin was negative in glomeruli of normal controls (B1), but intensive staining was revealed in MCNS (b1), FSGS (b4) and MN (b7), and mainly distributed to the perinuclear area of glomeruli and tubules of MCNS (b3), FSGS (b6) and MN (b9). Bar = 20 µm.

Confocal laser scanning images further showed that the five cytoskeleton molecules all displayed a linear or dotted-linear pattern along the glomerular capillary loops. Moreover, their fluorescence intensity changed dynamically, increasing from Day 2 and persisting to Day 10, then decreasing to a level slightly above normal at Day 15 in PAN rats. Double immunolabelling revealed that the five cytoskeletal proteins indeed colocalized with podocyte specific markers synaptopodin or α-actinin-4, which implied that they might be associated with the dynamic changes of FPs.

Subsequently, we confirmed that the five cytoskeletal proteins were involved in human proteinuric renal diseases, and similar up-regulation and expression patterns of the five cytoskeletal proteins were detected in MCNS, FSGS and MN patients. We noted that arp2 showed a strong staining in podocytes and tubules of MCNS, weak staining in MN, and was negative in glomeruli of normal and FSGS. Arp2, a dynamic actin-associated protein, plays multiple roles in cell morphology, cell motility and vesicle trafficking [17]. Recent studies showed that arp2/3-mediated regulation of the actin cytoskeleton is crucial to myoblast fusion in the fly [18]. We postulated that differential expression of arp2 in podocytes might be involved in the different pathological types. In fact, in MCNS, arp2 shows prominent expression in diffuse fusional podocytes FPs. Interestingly, survivin showed a pronounced perinuclear expression in podocytes and tubules of MCNS, FSGS and
Fig. 6. (A) Distribution of novel cytoskeletal molecules in cultured podocytes. Cell nuclei were stained blue with Hoechst. In normal podocytes (Control: a–e), the staining of transgelin, survivin, arp2 and cytokeratin7 was weak and mainly distributed in cytoplasm, whereas their fluorescence intensity increased, especially along the cell membrane, in puromycin aminonucleoside-treated podocytes (PAN: f–j). The distribution of vinculin was obviously altered, displaying a dotted- and a plaque-like pattern along cell membranes. Bar = 30 µm. (B) Protein expression of novel cytoskeletal molecules in cultured podocytes. Transgelin (k), survivin (l), arp2 (m), cytokeratin7 (n) and vinculin (o) increased in PAN-treated cells compared to controls. Data are presented as mean ± SD. N = 3. Values were normalized to GAPDH. *P < 0.05 versus Con.

MN. Likewise, Lechler et al. [19] also observed the translocalization of survivin from the membrane, to cytoplasmic and perinuclear, in the acute renal failure model induced by cisplatin intoxication. Although cytokeratin7 is regarded as a marker of parietal epithelial cells, we found that it was highly expressed in podocytes both in PAN nephropathy rats and in MCNS, FSGS and MN patients. Some studies also reported that the expression of cytokeratin increased significantly in podocytes in primary FSGS patients [20]. Cytokeratins (K), belonging to the family of intermediate filament proteins, are selectively localized in epithelial cells. Generally, the subfamily K7 and K19 proteins are co-expressed. Our results showed that Krt2–19 mRNA did not change in PAN rats, implying that the balance between Krt2–7 and Krt2–19 might be important for maintaining the normal cell shape. Transgelin, encoded by the gene Tagln and also designated SM22α, is a 22 kDa actin-associated protein. Some previous studies indicated that transgelin is involved in the phenotypic modulation of modulates vascular smooth muscle cell from contractile to proliferating in the sclerotic artery [21]. In this study, the inducible expression of the novel podocyte cytoskeleton transgelin implied phenotypic changes or trans-differentiation of injured podocytes. In anti-GBM crescentic glomerulonephritis rats, Ogawa et al. [22] also found inducible expression of transgelin in both the glomerular visceral and parietal cells. Most recently, Hauser et al. [23] reported that transgelin showed significant up-regulation in podocytes of passive Heymann nephritis (experimental membranous nephropathy) rats.

To further confirm their localization in podocytes, the distributions of the five cytoskeletons were analysed in cultured podocyte cells. As seen for the in vivo expression, up-regulation was detected in the in vitro PAN-treated podocytes. The distribution of vinculin was obviously altered, showing a significant dotted- and plaque-like condensation along the cell membrane after PAN treatment. Vinculin is an important composition of cell adhesion junctions. Friedrich et al. [24] also found that vinculin was present in focal adhesions between cultured podocytes. In the podocyte–glomerular basement membrane interface, vinculin and the Arp2/3 complex not only transmit ‘outside-in’ signalling but also provide ‘inside-out’ signalling to modulate actin polymerization, cell morphology and motility [25].

Although we identified five novel podocyte cytoskeletal proteins and found that they were associated with the dynamic changes of FPs and podocyte injury, their functional significance and their contribution to the underlying mechanisms in the occurrence and development of proteinuria remain unclear.

Acknowledgements. We are grateful to Professor Peter Mundel (USA) for the podocyte clones, and Professor Corinne Antignac (France) for podocin antibody. We thank Lixia Yu and Guohong Wu for the technical
Newly identified cytoskeletal components are associated with dynamic changes of podocyte foot processes

support in tissue sectioning. We thank Sen Zhu and Jing Li for their help during the animal experiment. This study was supported by the National Nature Science Foundation of China (30830105, 30170992, 30672259) and Nature Science Foundation of Beijing (7072080).

Conflict of interest statement. None declared.

Supplementary data

Supplementary data are available online at http://ndt.oxfordjournals.org.

References


Received for publication: 28.2.09; Accepted in revised form: 19.6.09