The relationship among nephrin, podocin, CD2AP, and α-actinin might not be a true 'interaction' in podocyte

Q Fan[1,2], Y Xing[1,2], J Ding[1], N Guan[1] and J Zhang[1]

[1]Department of Pediatrics, Peking University First Hospital, Beijing, Peoples Republic of China

The abnormality of a single podocyte molecule, caused by a single gene mutation, such as *NPHS1*, *NPHS2*, *CD2AP*, and *ACTN4*, can lead to the hereditary/congenital nephrotic syndromes (NS). Further studies suggested that more than one podocyte molecule were together involved in acquired or experimental NS. However, we do not know much on the relationship among these podocyte molecules, and the molecular response induced by the change of each podocyte protein to the remaining ones. We respectively knockdown the nephrin, podocin, CD2AP, or α-actinin-4 mRNA by using reconstructed RNA interference vector – psiRNA-hH1GFPzeo in mouse podocyte clone. The molecular behavior or response was revealed by the quantitative expression both at mRNA and protein levels with RT-PCR and Western blot, and by the molecular distribution detected with confocal microscopy. With nephrin knockdown, only CD2AP increased, whereas podocin showed no change. Contrarily, with podocin or CD2AP knockdown, nephrin decreased, while CD2AP or podocin increased. Nephrin, podocin, or CD2AP knockdown did not change the expression of α-actinin-4, whereas α-actinin-4 knockdown begetted the reduction of nephrin, and the increment of podocin and CD2AP. The redistributions of nephrin, podocin, and CD2AP were revealed around a predominant nuclear staining compared with the membrane surface staining in the control podocytes. Our data imply that the response between the four podocyte molecules is very complicated and evidently different. There is not always an interaction between podocyte molecules. The normal localization of podocyte molecules would depend on their normal expression quantity and the molecular reactions between them.

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Correspondence: *J Ding, Department of Pediatrics, Peking University First Hospital, No.1, Xi An Men Da Jie, Beijing 100034, Peoples Republic of China. E-mails: djnc_5855@126.com or jieding@public.bta.net.cn*

[2]These authors contributed equally to the work.

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Podocytes are highly terminally differentiated cells with major processes and foot processes interlinked by ultrathin slit diaphragms (SD). The SD may represent a modified adhesion junction and is primarily responsible for the size selectivity of the glomerular filter.[1,2] However, the molecular components of the SD complex are still poorly understood for many years. Recently, the identification of several novel podocyte molecules and the description of their physical and functional interactions brought new insights into the components of the SD complex and the regulation of the glomerular filtration barrier. Nephrin, encoded by *NPHS1*, the gene mutated in congenital nephrotic syndrome (NS) of the Finnish type, has been suggested to form the SD by either homo- or heterophilic interactions.[3] Two other genes involved in proteinuria have also been identified. Of them, *NPHS2*, the causative gene for autosomal recessive steroid-resistant NS, encodes an integral membrane-associated protein, podocin, which is proposed to function as a scaffolding protein and capable of modulating nephrin-signaling activity.[4,5] The other gene, *ACTN4*, has been shown to cause hereditary familial focal segmental glomerulosclerosis, and encodes an actin-filament cross-linking protein, α-actinin-4.[6] In addition, the mice completely knockout of the gene *CD2AP* encoding CD2-assosiated protein (CD2AP), which localizes to the cytoplasmic face of the SD, develop the congenital NS and die of the massive proteinuria soon after birth.[7] Moreover, NEPH1, another IgG superfamily protein,[8] and FAT, the member of the protocadherin superfamily proteins,[9] and P-cadherin[2] have been shown to localize to the SD. Targeted deletion of the *FAT* or *NEPH1* gene in mice also leads to NS.[8,9] These studies suggested that these podocyte proteins play an essential role in maintaining the structural and functional integrity of the glomerular filtration barrier.

A single gene mutation, such as *NPHS1*, *NPHS2*, and *ACTN4*, can lead to the hereditary or familial NS, implying that the abnormality of a single podocyte molecule caused by a single gene mutation, might be enough to induce NS. Further studies from acquired NS[10–14] and experimental NS[15–18] suggested that many podocyte molecules were together involved in maintaining the structural and functional integrity of the SD. Although we are starting to understand the role of podocyte molecule from the view of a

single molecule, we do not know much on the relationship among these molecules in maintaining the integrity of the SD and in the occurrence and development of proteinuria, and we also do not know much on the molecular effects caused by the change of each podocyte proteins on the remaining ones. So, it is necessary to explore the function of a single podocyte molecule, especially the three important proteins nephrin, podocin, and CD2AP, and the cytoskeletal protein α-actinin-4, in order to discover the potential sites or molecular target(s) for the intervention of proteinuria.

In this study, we investigated the molecular behavior or response by the respective knockdown (KD) of nephrin, podocin, CD2AP, or α-actinin-4 mRNA in mouse podocyte clone 5[19] with a novel gene block technique – RNA interference (RNAi), which is widely used to eliminate gene activity by degrading the specific mRNAs with complementary sequence.[20]

RESULTS

Immunofluorescence staining

In control group, the staining for podocin and nephrin distributed in perinuclear and mainly on the cell membrane surface in a filamentous pattern. CD2AP was evenly localized in the cytoplasm and on the cell membrane surface. The α-actinin staining pattern was different from that of podocin, nephrin, and CD2AP. α-Actinin mainly distributed in the cytoplasm in a filamentous pattern and also extended to the podocyte processes (Figure 1).

In podocin KD group (siPod 966 and siPod 54), the intensities of podocin and nephrin were distinctly lower than those in control, and the staining for them was localized predominantly around nuclei with a loss of surface distribution. The intensity of CD2AP increased significantly, and its staining mainly distributed around nuclei with a normal membrane expression. However, the intensity and distribution of α-actinin did not change (Figure 2).

In nephrin KD group (siNep 492), the changes of the intensities and distributions of nephrin and CD2AP were the same as those in podocin KD group, whereas that of podocin and α-actinin did not change (Figure 3). In siNep 1031 group, the intensities and distributions of nephrin, podocin, CD2AP, and α-actinin showed no change (data not shown).

In CD2AP KD group (siCda 744 and siCda 21), the intensities of CD2AP and nephrin were obviously lower than those in control, and the staining for them predominantly distributed around nuclei with a loss of surface expression. Compared with control, the intensity of podocin was distinctly higher, and its staining was mainly localized around nuclei with the normal expression of the cytoplasm and the membrane. No change was observed for α-actinin (Figure 4).

In α-actinin-4 KD group (siAct 1790 and siAct 319), the intensity of α-actinin was distinctly lower with normal distribution. All other proteins changed with distribution and expression alteration, that is, nephrin decreased with a loss of surface expression, whereas the perinuclear staining for podocin and CD2AP increased evidently with a normal expression of the membrane surface (Figure 5).

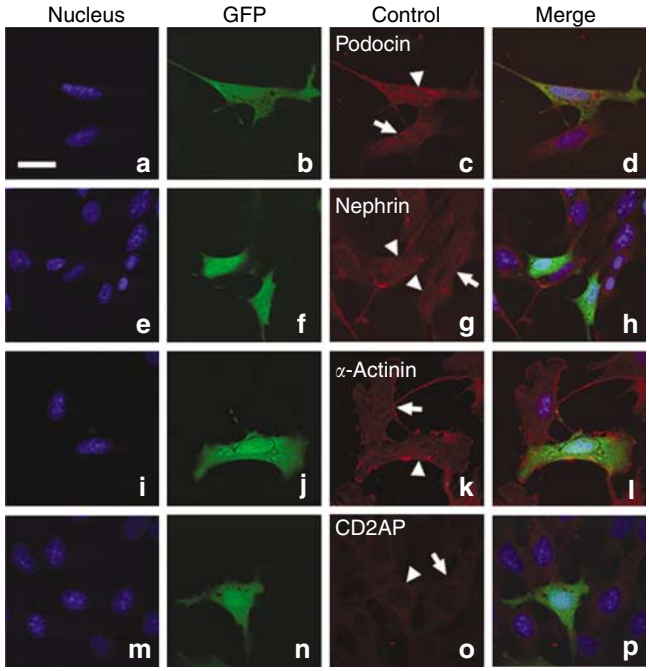

Figure 1 | Expressions of podocin, nephrin, α-actinin, and CD2AP in mouse podocytes transfected with control RNA interference vector. (**a**, **e**, **i**, and **m**) The nuclei were stained in blue with Hoechst . (**b**, **f**, **j**, and **n**) The podocytes transfected with control RNA interference vector were indicated in green with green fluorescence protein and (**c**, **g**, **k**, and **o**) marked by the arrowhead. (**c**, **g**, **k**, and **o**) The expressions of podocin, nephrin, α-actinin, and CD2AP were shown in red, respectively, in which arrows indicate the podocytes not transfected with control RNA interference vector. (**d**, **h**, **l**, and **p**) The merged photos from **a–c**, **e–g**, **i–k**, and **m–o**, respectively. Both in the transfected cells and the non-transfected cells, podocin (**c**) and nephrin (**g**) all showed a perinuclear and mainly continuous membrane staining pattern, α-actinin (**k**) showed a cytoplasmic filamentous staining extending to the processes, and CD2AP (**o**) was weakly diffuse in the cytoplasm and on the membrane. Bar = 50 μm.

Semiquantitative reverse transcriptase-PCR

The specific DNA bands of podocin, nephrin, CD2AP, α-actinin-4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were observed with the size of 193, 211, 191, 277, and 201 bp, respectively, and the quantitative results in relation to GAPDH at mRNA level were shown in Table 1 and Figure 6.

Western blotting

The specific protein bands of podocin, nephrin, CD2AP, α-actinin, and GAPDH were detected with the size of 44, 180, 80, 100, and 36 kDa, respectively, and the quantitative results at protein level in relation to GAPDH were shown in Table 2 and Figure 7.

DISCUSSION

The massive proteinuria is the most important clinical manifestation of NS. The SD plays a crucial role in preventing