Effect of the Knockdown of Podocin mRNA on Nephrin and α-Actinin in Mouse Podocyte

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Recently, the novel podocyte proteins podocin, nephrin, and α-actinin-4 have been identified in three congenital/family nephrotic syndromes, respectively. Further studies showed that these podocyte proteins were involved in some acquired nephrotic syndromes and various experimental models of proteinuria. However, the molecular interactions among these podocyte proteins remain unclear. In this study, to investigate the molecular interactions among podocin, nephrin, and α-actinin-4, we reconstructed the RNA interference (RNAi) expression vector, p*Silencer* 2.1-U6, specifically targeting podocin mRNA, and it was transfected into the mouse podocyte clone (MPC5). Immunofluorescence staining, double-immunolabeling, confocal microscopy, semiquantitative reverse transcription polymerase chain reaction (RT-PCR), and Western blotting were used to detect the distribution and expression of podocin, nephrin, α-actinin-4, and glyseraldehyde-3-phosphate dehydrogenase (GAPDH)/β-actin. The fluorescence intensity of podocin and nephrin decreased obviously, along with the evident distribution change from the cell membrane surface to the nucleus circumference in podocyte. In relation to GAPDH, the mRNA reductions of podocin and nephrin were observed by about 65% and 70%, respectively. The expression of podocin protein was too low to be detected in the interference group. In relation to β-actin, the protein level of nephrin decreased by about 78%. The distribution and the mRNA and protein level of α-actinin showed no appreciable change. Alpha-actinin localized mainly in the cytoplasm and also extended to the processes. Thus, the significant decreased expression of nephrin along with the redistribution were detected with the knockdown of podocin mRNA, whereas the expression and distribution of α-actinin-4 showed no change. These results suggest that podocin may interact directly with nephrin, but not with α-actinin. Exp Biol Med 229:964–970, 2004

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Introduction

Selective permeability to plasma is one of the central functions of the kidney, occurring in a specialized filtration structure called the glomerular filtration barrier. The glomerular filtration barrier consists of the three layers in the glomerular capillary wall; that is, a single cell layer of the innermost highly fenestrated vascular endothelial cells, the glomerular basement membrane (GBM), and the final layer of the glomerular visceral epithelial cells known as podocytes. Podocytes are highly specialized, terminally differentiated cells with major processes and foot processes interlinked by ultrathin slit diaphragms (1, 2). The molecular composition of the slit diaphragm has been, however, poorly understood for a period of time. Until recent years, the podocyte proteins, such as the novel proteins nephrin and podocin, and another molecule, α-actinin-4, specifically expressed in podocyte, have been identified in three types of hereditary/congenital nephrotic syndrome (3–5). Besides the three molecules, zonula occludens-1 (ZO-1), CD2-associated protein (CD2AP), FAT, P-cadherin, and α-, β-, and γ-catenins have also been found in podocyte (6–10). Importantly, podocin, nephrin, P-cadherin, FAT, ZO-1, and catenins have been localized to the slit diaphragm (9, 10). Simultaneously, the abnormalities of the podocyte proteins have also been reported in some acquired nephrotic syndromes (11–14) and various experimental models of proteinuria (15–18), which indicated that more than one podocyte protein is involved in the occurrence of proteinuria. However, the precise molecular mechanism of the occurrence of proteinuria, the function of the podocyte proteins, and the molecular interactions among these podocyte proteins remain unclear.

In this study, we investigated the molecular interactions using a novel gene block technique—RNA interference (RNAi), which is a post-transcriptional process triggered by the introduction of small interfering RNA (siRNA; Ref. 19).

Three important proteins expressed solely in podocyte—podocin, nephrin, and α-actinin-4—were selected as the target molecules. The sequence-specific knockdown of podocin mRNA was carried out in the mouse podocyte clone (MPC5).

Materials and Methods

Podocin siRNA Design. We designed siRNA to interfere with the expression of podocin mRNA, referring to technical information (Ambion, Austin, TX). To avoid the strong secondary structure of potential interference target, we predicted the secondary structure of podocin mRNA by using the software RNA structure 3.71 (http://rna.chem.rochester.edu). One set of 19-mer oligonucleotides (718th~736th nt, according to the number from the first base of the start codon), just downstream of an AA dinucleotide, was selected as the interference target site from the podocin mRNA coding region (GenBank number NM130456). We confirmed that the selected oligonucleotide did not have homology to any other mouse genes by using a BLAST search (http://www.ncbi.nlm.nih.gov/blast/) to ensure that they would not interfere with other genes. The 19-mer sense siRNA sequence and antisense siRNA sequence were linked with a nine nucleotide spacer (TTCAAGAGA) as a loop. Six T bases and 6 A bases were added as an RNA pol III termination signal to the 3′ end of the forward oligonucleotides and 5′ end of the reverse oligonucleotides, respectively. Then five nucleotides corresponding to the *Bam*HI (GATCC) and *Hin*dIII (AGCTT) restriction sites were added to the 5′ end of the forward and reverse oligonucleotides, respectively. The basic p*Silencer* 2.1-U6 vector map, the forward and reverse oligonucleotides, and the transcript-hairpin siRNA are shown in Figure 1.

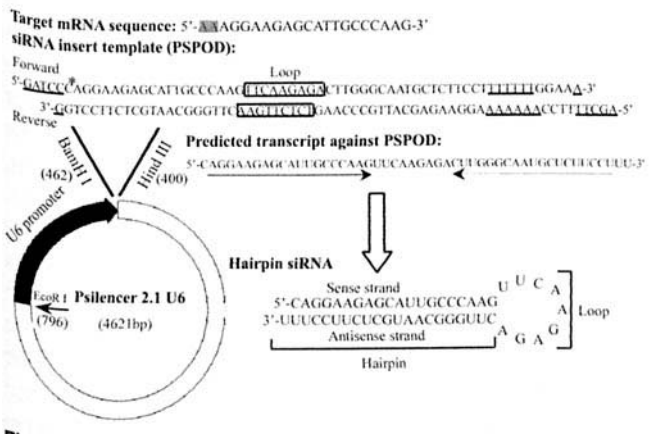

Figure 1. A vector-based suppression of podocin gene expression. The siRNA insert with a loop sequence (TTCAAGAGA) and a termination signal (6T) was cloned between *Bam*HI (GATCC) and *Hin*dIII (AGCTT) sites, immediately downstream of a U6 promoter. The transcript-hairpin siRNA consists of a sense strand, a loop, and an antisense strand. *The purpose of this additional CG base pair is to provide a G residue as the first nucleotide of the siRNA transcript because RNA pol III prefers to initiate transcription with a purine. This extra nucleotide C in the sense strand has no effect on the activity of the hairpin siRNA.

Cloning and Recombinant Plasmid Constructs. Forward and reverse oligonucleotides were incubated in annealing buffer (100 m*M* potassium acetate, 30 m*M* HEPES KOH [pH 7.4] and 2 m*M* magnesium acetate) for 3 mins at 90°C, followed by incubation for 1 hr at 37°C. The annealed DNA for siRNA was ligated with linearized p*Silencer* 2.1-U6 siRNA expression vector (Ambion) at *Bam*HI and *Hin*dIII sites. After transformation, many clones were picked and digested with *Eco*RI and *Hin*dIII to confirm the presence of the insert, the sequence of which was further identified by sequencing from both sides. Then, the identified recombinant plasmid constructs were prepared with Wizard PureFection Plasmid DNA Purification System (Promega, Madison, WI) and quantitated with UV 2100 spectrophotomer (Shimadzu, Kyoto, Japan).

The vector, p*Silencer* 2.1-U6 Negative Control (Ambion), was used as the negative control plasmid in all experiments. The sequence of the "interference" target site is 5′>ACTACCGTTGTTATAGGTG<3′, which lacks homology to any coding sequences of mouse genes. Therefore, the transcript-hairpin siRNA was expected to have no interference on mouse genes.

Podocyte Culture and Transfection. Conditionally immortalized mouse podocyte clone (MPC5) was established by transfection with temperature-sensitive *SV40-T* gene (20). Podocytes were cultured in RPMI 1640 (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gibco), 100 units/ml of penicillin and 100 units/ml of streptomycin in a humidified atmosphere of 5% CO_2. To propagate cells, the podocytes were cultured at 33°C in the medium containing 10 units/ml of recombinant mouse γ-interferon (PEPRO, London, UK). Then, these cells were reseeded in 6-well plates (1.0×10^5 cells/well) with glass coverslips and 25-cm^2 flasks (3.0×10^5 cells/flask) coated with 10 μg/ml of type-I collagen (Sigma, St. Louis, MO), and cultured about 7 days at 37°C by removal of γ-interferon for immunostaining and isolating total cell RNA or protein.

When they were grown to about 60% confluence, podocytes were divided into three groups and transfected according to the manufacturer's instructions: the podocytes transfected with the above recombinant plasmid as interference group (MPC5 PSPOD), transfected with the Negative Control plasmid as negative control group (MPC5 PSNC), and transfected only with siPORT *XP-1* (Ambion) as blank control group (MPC5 BC). Cells were harvested after about 48 hrs to assay the activities of target genes. In this study, podocytes between passages 10 and 12 were used in all experiments. All experiments were repeated three times, and the results were also repeatable and credible.

Immunofluorescence (IF) Staining and Double-Immunolabeling. The following primary antibodies were used: rabbit polyclonal anti-mouse nephrin antibody (kindly supplied by Professor Karl Tryggvason, Sweden); rabbit

polyclonal anti-human podocin antibody (a kind gift of Professor Corinne Antignac, France); and mouse monoclonal anti-human α-actinin antibody (Chemicon, Temecula, CA). Alpha-actinin has four subtypes, which are α-actinin-1, -2, -3, and -4, respectively. In the renal glomerulus, only α-actinin-4 is expressed and localized to the podocytes (5), so we can detect the expression of α-actinin-4 with anti-α-actinin antibody in podocyte. The following secondary antibodies were used: fluorescein isothiocyanate conjugated (FITC) goat anti-rabbit IgG and tetramethylrhodamine isothiocyanate conjugated (TRITC) goat anti-mouse IgG (Santa Cruz Biotechnology, CA). Hoechst (Sigma) was used to stain the nuclei.

The immunostaining was done as previously described (20, 21) and revised properly. Briefly, the coverslips were fixed with ice-cold acetone for 15 mins, then permeated and blocked with 0.3% Triton X-100 and 5% bovine serum albumin for 30 mins. Primary and secondary antibodies were applied at the appropriate dilutions according to standard techniques, and the coverslips were mounted on glass slides with 15% Mowiol.

For double-immunolabeling, the coverslips were simultaneously incubated with rabbit anti-podocin antibodies and mouse anti-α-actinin antibodies, or rabbit anti-nephrin antibodies and mouse anti-α-actinin antibodies overnight at 4°C. After three washes with phosphate-buffered saline, the coverslips were further simultaneously incubated with FITC goat anti-rabbit IgG, TRITC goat anti-mouse IgG, and Hoechst for 45 mins at room temperature. Further washes and mounting were as above. Images were obtained by confocal laser-scanning microscopy using a Bio-Rad Radiance 2100 TM confocal system (Hercules, CA) attached to a Nikon TE 300 microscope (Nikon, Tokyo, Japan).

Reverse Transcription (RT) Reaction and Semi-quantitative PCR. Total RNA was isolated from the cultured cells by Trizol Reagent (Gibco) according to the manufacturer's instructions. RNA concentration and quality were assessed spectrophotometrically at wavelengths 260 and 280 nm. Two μg of total RNA were reverse transcribed in the presence of random primers and M-MLV reverse transcriptase (Promega) according to the following sequence: 70°C for 5 mins, followed by 37°C for 60 mins, and 75°C for 15 mins. Four μl of the resulting cDNA were used for the semiquantification of podocin, nephrin, and α-actinin-4 mRNA in relation to the housekeeping gene (GAPDH). Twenty-five μl of PCR reaction were performed in the GeneAmp 2400 PCR System (Perkin-Elmer, Shelton, CT) using 1.5 m*M* of $MgCl_2$, 1.25 units of *Tag* DNA polymerase (Promega), and 0.2 μ*M* of sequence-specific primers according to the following program: after the initial denaturation at 94°C for 5 mins, amplification was done using 30/28/26 cycles (94°C for 45 secs, 57°C for 30 secs, and 72°C for 45 secs), followed by a final extension at 72°C for 5 mins.

The sequence-specific primers (Table 1) were designed by online software Primer 3.0 (http://frodo.wi.mit.edu/) according to the mRNA sequences of mouse podocin, nephrin, α-actinin-4, and GAPDH from GenBank (podocin: NM130456; nephrin: AF191090; α-actinin-4: NM021895; GAPDH: NM008084).

Table 1. The Sequence-Specific Primers and the Size of PCR Products of Podocin, Nephrin, α-Actinin-4, and GAPDH

Gene	Primers sequence	Product size
Podocin	Forward: 5′>tgaggatggcggctgagat<3′	
	Reverse: 5′>ggtttggaggaacttgggt<3′	193 bp
Nephrin	Forward: 5′>cccaacactggaagaggtgt<3′	
	Reverse: 5′>ctggtcgtagattccccttg<3′	211 bp
α-Actinin-4	Forward: 5′>actaccacgcagcgaacc<3′	
	Reverse: 5′>tcccctgaaatgacctcc<3′	277 bp
GAPDH	Forward: 5′>ctcatgaccacagtccatgc<3′	
	Reverse: 5′>cacattgggggtaggaacac<3′	201 bp

The PCR products were separated on a 1.5% agarose gel. For the quantification, an image of the gel stained with ethidium bromide was captured, and the intensity of the bands was quantitated using the AlphaImager gel analysis system (Alpha Innotech, San Leandro, CA). In this study, the semiquantification analysis was performed with the PCR products of 28 cycles, which displayed distinct difference between the interference group and the control groups.

Protein Extraction and Western Blotting. 5.0×10^5 cells per sample were lysed in Tris lysis buffer (4% SDS, 150 m*M* Tris [pH 6.8], 100 m*M* dithiothreitol [DTT], 1 m*M* phenylmethylsulfonyl fluoride [PMSF], 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) (Sigma) on ice for 30 mins (21). Lysates were centrifuged at 12,000 *g* for 5 mins, and the supernatants containing the cellular protein were collected. Equal amounts of total protein were boiled, electrophoresed on 8% SDS-PAGE, and transferred to nitrocellulose membranes (Santa Cruz). The membranes were rinsed with washing buffer (Tris-buffered saline with 0.02% Tween-20) followed by immersing in blocking buffer (5% low-fat milk powder) for 2 hrs. Subsequently, the membranes were incubated with anti-podocin, anti-nephrin, anti-α-actinin, or anti-β-actin antibody at 4°C overnight. Mouse monoclonal anti-chicken β-actin antibody (Chemicon) was used in this study. After being rinsed three times in washing buffer, the membranes were incubated with 3 horseradish peroxidase–conjugated goat anti-rabbit/mouse IgG (Santa Cruz) for 1 hr at room temperature. Then, the membranes were developed with electrochemiluminescence reagent (Santa Cruz), and the specific protein bands were scanned and quantitated in relation to the housekeeping gene β-actin (43 kDa).

Results

Confirmation of the Recombinant Plasmid. The recombinant plasmid-bearing podocin siRNA insert was digested with *Eco*RI and *Hin*dIII. After the digestion

products were electrophoresed in 1% agarose gel, two DNA bands were detected with the size of 398 bp and 4,226 bp in accordance with the size of the actual products. The sequence of insert fragment was confirmed by further sequencing.

Immunostaining. The fluorescence intensities of podocin and nephrin were universally and distinctly lower in the interference group than in the control groups, whereas that of α-actinin showed no appreciable difference between the interference group and the control groups (Fig. 2).

Double-Immunolabeling. The distribution changes of podocin and nephrin were revealed by double-immunolabeling. The staining for podocin and nephrin was distributed around nuclei and mainly on the cell membrane surface in a filamentous pattern in the control groups, whereas their staining localized predominantly around nuclei in a dotted pattern with a loss of surface expression in the interference group. The distribution of α-actinin showed no difference between the interference group and the control groups, which localized mainly in the cytoplasm with a filamentous pattern and also extended to the processes (Fig. 3).

Semiquantitative RT-PCR. The PCR products were electrophoresed in 1.5% agarose gel, and the specific DNA bands of podocin, nephrin, α-actinin, and GAPDH were observed with the sizes of 193 bp, 211 bp, 277 bp, and 201 bp, respectively. The band intensities of podocin and nephrin were evidently lower in the interference group than in the control groups, whereas that of α-actinin and GAPDH showed no appreciable change between the interference group and the control groups. In relation to GAPDH, the mRNA levels of podocin and nephrin decreased by about 65% and 70% in the interference group, respectively (Fig. 4A).

Western Blotting. The specific protein bands of podocin, nephrin, α-actinin, and β-actin were detected with the sizes of 45 kDa, 180 kDa, 100 kDa, and 43 kDa, respectively. The band of podocin protein was too low to be seen in the interference group. In relation to β-actin, the band intensity of nephrin decreased remarkably by about 78% in the interference group. The expression of α-actinin showed no appreciable difference between the interference group and the control groups (Fig. 4B).

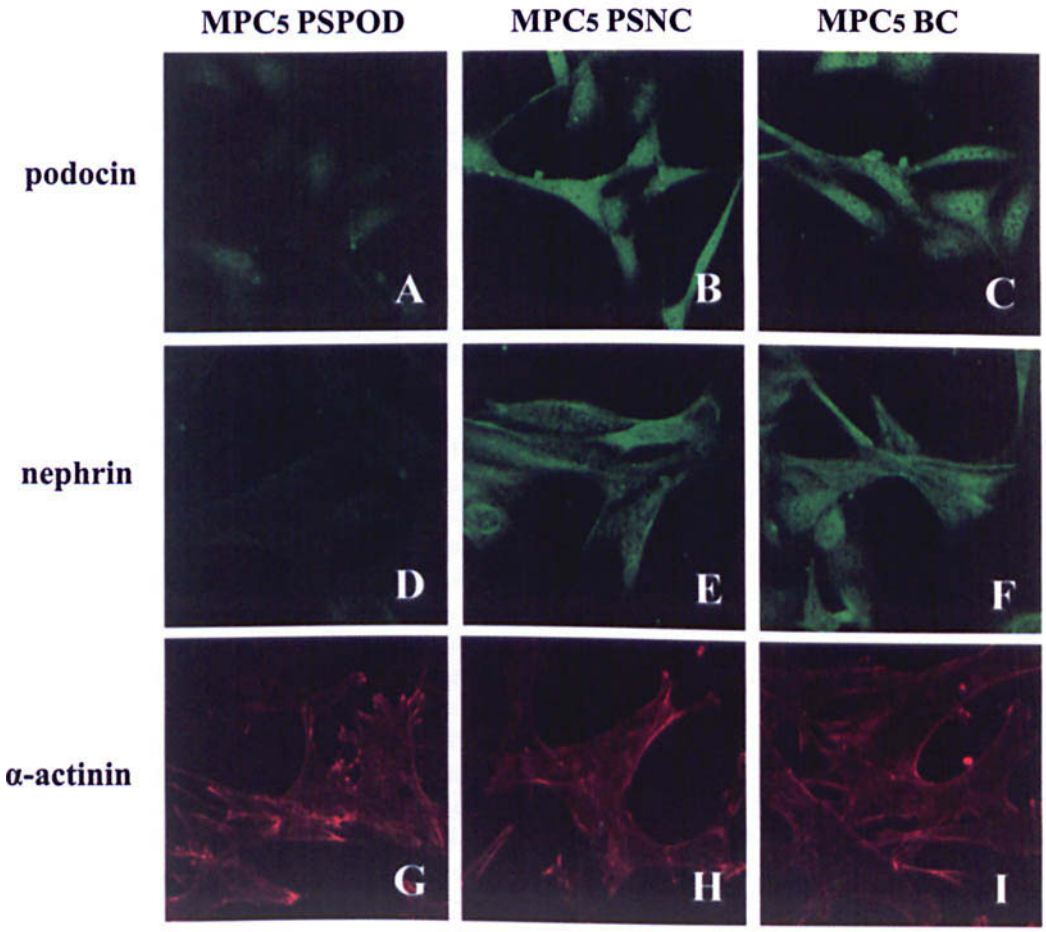

Figure 2. Immunofluorescence staining for podocin, nephrin, and α-actinin in cultured mouse podocytes (Magnification: ×700). In the interference group (MPC5 PSPOD), the fluorescence intensities of podocin and nephrin were lower than in the control groups (MPC5 PSNC and MPC5 BC); the staining for α-actinin showed no appreciable difference between the interference group and the control groups.

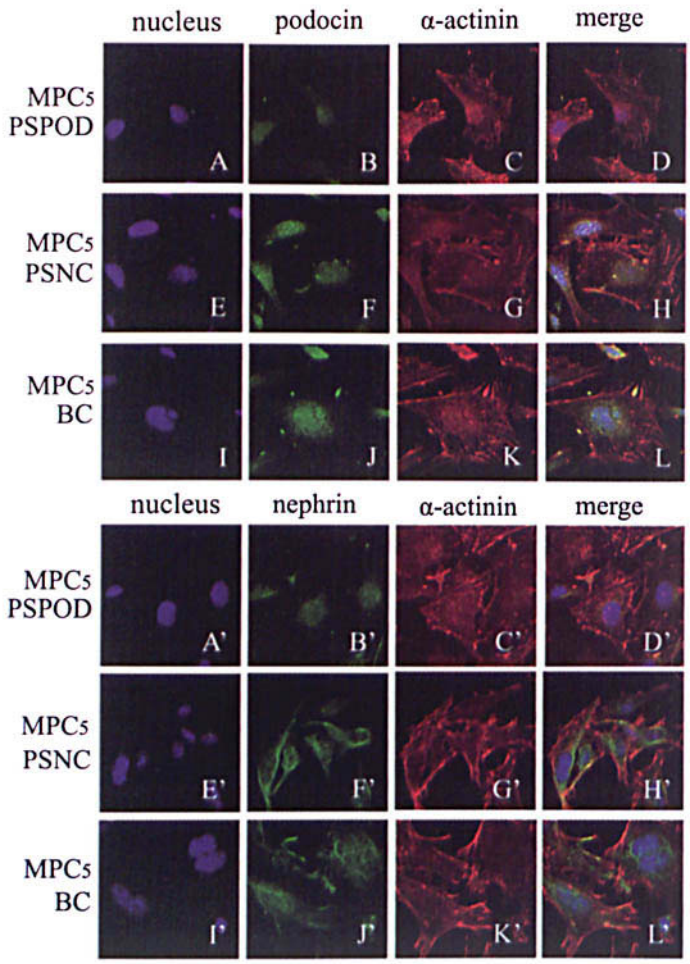

Figure 3. Immunofluorescence double staining for podocin and α-actinin, and nephrin and α-actinin in cultured mouse podocytes (Magnification: ×700). (D), (H), and (L). Podocin and α-actinin double immunolabeling. (D'), (H'), and (L'). Nephrin and α-actinin double immunolabeling. Podocin and nephrin were stained in green with FITC, α-actinin was stained in red with TRITC, and the nuclei were stained in blue with Hoechst. The overlaps of podocin and α-actinin, and nephrin and α-actinin were indicated by the orange color. In the control groups (MPC5 PSNC and MPC5 BC), the overlaps of podocin and α-actinin, and nephrin and α-actinin localized around nuclei and mainly on the cell membrane surface as indicated by the orange color; their overlaps in the interference group (MPC5 PSPOD) localized predominantly around the nuclei. The distribution of α-actinin localized mainly in the cytoplasm and also extended to the processes in the interference group and the control groups.

Discussion

Nephrin, the first molecule of the slit diaphragm, was identified in congenital nephrotic syndrome of the Finnish type in 1998 (3). It is very important to recognize the podocyte as the key layer of the glomerular filtration barrier from the molecular sight. Shortly after this, another novel podocyte protein, podocin, was identified in autosomal recessive steroid-resistant nephrotic syndrome (4). At the same time, mutations on *ACTN4* encoding α-actinin-4 were detected in some kindreds with autosomal dominant familiar focal segmental glomerulosclerosis (FSGS; Ref. 5). These unrelated congenital and familiar nephrotic syndromes, which might be taken as a kind of gene knockout model, enlightened us on the understanding of the molecular mechanism of proteinuria. Further studies demonstrated that nephrin, podocin, and α-actinin-4, as well as some other molecules, also showed abnormality in some acquired nephrotic syndromes (11–14) and some types of experimental proteinuria (15–18). Thus, it is suggested that more than one podocyte molecule might be involved in the occurrence and development of proteinuria. In other words, the integrity of the slit diaphragm and the normal function of the selective filtration barrier may be maintained by more than one podocyte protein. However, the molecular interactions among these proteinuria-associated proteins in the maintenance of the normal function of filtration barrier or in the development of proteinuria have not been elucidated clearly. It is possible that one molecule would play as a trigger to induce or influence the behavior changes of the rest of the podocyte molecules, whereas it is not impossible that each molecule would react to injury factors individually or respectively, and that the involvement of these molecules in the normal function of the filtration barrier and the development of proteinuria would not rely on the molecular interactions. Therefore, we designed this

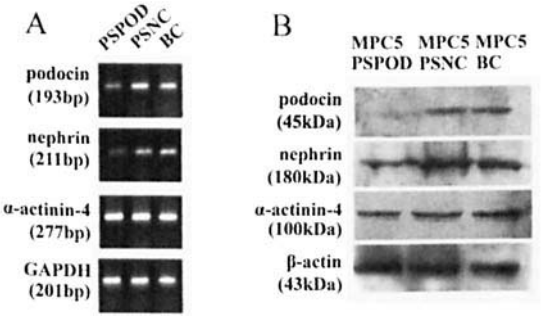

Figure 4. Expression of nephrin, podocin, α-actinin, and GAPDH/β-actin at the mRNA (A) and protein (B) level in cultured mouse podocytes by using RT-PCR and Western blotting assay. (A). In relation to GAPDH, the mRNA levels of podocin and nephrin decreased evidently in the interference group (MPC5 PSPOD). The band intensity of α-actinin showed no appreciable change between the interference group and the control groups (MPC5 PSNC and MPC5 BC). (B). The expression of podocin was too low to be detected in the interference group. In relation to β-actin, the protein level of nephrin decreased remarkably compared with the control groups; the expression of α-actinin showed no appreciable difference between the interference group and the control groups.

experiment in order to explore the molecular interactions among podocin, nephrin, and α-actinin-4 by using the novel gene block technique, RNAi. Compared with gene knockout, RNAi is also termed as gene knockdown, which can specifically lead to gene silencing by generating an active RNA-induced silencing complex (RISC) in a sequence-specific manner (19). Studies from other groups have shown that the RNAi technique can be used to study the gene function and to identify interacting pairs of genes in a rapid and inexpensive way (19, 22, 23). Recently, it has been demonstrated that 21-nucleotide hairpin siRNA duplexes can efficiently and specifically inhibit the expression of cognate genes in mammalian cells (24–27).

In this study, after the specific knockdown of podocin mRNA, we detected an obvious reduction of nephrin at mRNA and protein levels in podocyte. Namely, the expression of nephrin protein decreased evidently with the knockdown of podocin mRNA. Our results indicated that there might be a direct molecular interaction between podocin and nephrin, and that the existence of podocin might be very necessary for the expression of nephrin. Studies from Huber's group showed that nephrin might be a signaling molecule, and that nephrin-induced signaling could be greatly enhanced by podocin in HEK293 (28). Schwarz *et al.* (29) revealed that podocin might recruit nephrin at the cytoplasmic side of the slit diaphragm, and react via its COOH-terminal domain to CD2AP, a cytoplasmic binding partner of nephrin. Although these experiments implied the possible correlation between podocin and nephrin, our studies provided the first direct evidence that podocin could interact with nephrin in podocyte.

With the knockdown of podocin mRNA, we detected not only the decreased expression of nephrin, but also the obvious redistribution of podocin and nephrin in podocyte, which shifted from the cell membrane surface to the nucleus circumference of the podocyte. This distribution change suggested that there could be a possible mechanism whereby podocin might functionally link to nephrin at the cell membrane surface, and that the loss of surface expression of podocin might subsequently result in the redistribution of nephrin because of the loss of molecular reaction between them in the membrane surface of podocyte. Interestingly, such mRNA reduction and redistribution of nephrin was reported by Roselli *et al.* (30) in podocin-deficient mice; it shifted from a linear to a granular pattern, and nephrin was localized at some distance from the GBM. Studies from our group also showed that the decreased expression of podocin was detected first, followed by the reduction of nephrin in the development of proteinuria in puromycin aminonucleoside nephrosis rat (18). In contrast, Hamano *et al.* (31) revealed that nephrin deficiency did not lead to alterations in the expression and distribution of podocyte molecules such as podocin, α-actinin-4, CD2AP, and synaptopodin in the nephrin knockout mice. The study by Hamano *et al.* led to a strong suggestion that nephrin is a pivotal protein for the maintenance of normal podocyte slit diaphragm structure and the proper function of the glomerular filtration barrier. Our results of podocin knockdown and the studies by Roselli *et al.* of podocin knockout all showed that the decreased expression of podocin led to the reduction of nephrin expression and the redistribution in podocyte, which suggested that there is an interaction between podocin and nephrin. On the other hand, podocin might act as an organizer to assemble the normal slit diaphragm structure with nephrin as well as other molecules. Simultaneously, we supposed that there might be a close correlation between the expression and the intracellular distribution of podocin and nephrin. In other words, the expression reduction of podocin and nephrin might cause their redistribution in podocyte. Studies by Saleem *et al.* (32) showed that podocin and nephrin localized in the cytoplasm at the early time of synthesis, then transported to the cell membrane along the cytoskeleton, and finally localized to the slit diaphragm with the increased expression level. Because their synthesis and distribution positions are different, the decline in the synthesis of podocin and nephrin might affect their distribution pattern in podocyte. Therefore, the distribution changes of podocin and nephrin revealed in our study might be attributed to the decrease of the molecular expression caused by the knockdown of podocin mRNA. Accordingly, we supposed that the normal localization of molecules would depend on their normal expression quantity, and both the localization and the quantity of molecules might be very important for maintaining their behavior in cells.

In this study, the distribution, the mRNA, and the protein expression of α-actinin showed no appreciable difference between the interference group and the control groups. Namely, the knockdown of podocin mRNA did not

influence the distribution and expression of α-actinin. So, we postulate that podocin might not directly interact with α-actinin.

In summary, we detected both the significant decreased expression of nephrin and the obvious redistribution of podocin and nephrin in podocyte with the knockdown of podocin mRNA. These results demonstrate that there is a direct molecular interaction between podocin and nephrin, which can maintain their normal distribution in podocyte. Although the redistribution of podocin and nephrin might reveal that the molecular expression quantity will affect their localization and distribution in cells, the internal mechanisms involved in this process need to be further studied. Our study demonstrates that the expression and distribution of α-actinin were not influenced by the knockdown of podocin mRNA.

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