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Research article

THE TRANSCRIPTIONAL REGULATION OF PODOCIN (NPHS2) BY Lmx1b AND A PROMOTER SINGLE NUCLEOTIDE POLYMORPHISM

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Abstract: Podocin (NPHS2) is a component of the glomerular slit membrane with major regulatory functions in the renal permeability of proteins. A loss of podocin and a decrease in its resynthesis can influence the outcome of renal diseases with nephrotic syndrome, such as minimal change glomerulonephritis, focal segmental glomerulosclerosis (FSGS) and membranous nephropathy. The transcriptional regulation of podocin may play a major role in these processes. We defined the transcriptional regulation of the human podocin gene and the influence of single nucleotide polymorphisms (SNPs) within its promoter region in the podocytes using reporter gene constructs and gel shift analysis. In addition, we took genomic DNA from healthy Caucasian blood donors and from biopsies of kidneys with defined renal diseases and screened it for podocin promoter SNPs. Our data shows that the transcription of podocin is mainly regulated by the transcription factor Lmx1b, which binds to a FLAT-F element and displays enhancer function. With the SNP variant -116T, there was a significant reduction in luciferase activity, and nuclear protein binding was observed, while the SNP -670C/T did not display functionality. The allelic distribution of -116C/T in patients with kidney diseases leading to nephrotic syndrome was not significantly different from that in the control group. Our data indicates that among other factors, podocin is specifically regulated by the transcription factor Lmx1b and by the functional polymorphism -116C/T. However, there is no association between -116C/T and susceptibility to minimal change glomerulonephritis, focal segmental glomerulosclerosis or membranous nephropathy.

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Abbreviations used: DNA – deoxyribonucleic acid; EMSA – electrophoretic mobility shift assay; FSGS – focal segmental glomerulosclerosis; INF-γ – interferon-γ; PCR – polymerase chain reaction; SNP – single nucleotide chain reaction

Key words: Lmx1b, Nephrotic syndrome, NPHS2 gene, Podocin promoter, Proteinuria, SNP, Transcription

INTRODUCTION

Podocin is an important protein expressed by the visceral glomerular epithelial cells (podocytes). Along with several other proteins, it acts as scaffolding in the structural organization of the glomerular slit membrane, which regulates the filtration function of the kidney [1]. Altering the glomerular filtration barrier leads to proteinuria due to damage of the glomerular podocytes and their tightly regulated slit membrane proteins [1]. Several renal diseases, including minimal change glomerulonephritis, focal segmental sclerosis, and membranous glomerulonephritis, are associated with high-level proteinuria resulting in nephrotic syndrome. The role of podocin in nephrotic kidney diseases has also been experimentally demonstrated in an animal model of podocin-deficient mice, which develop massive proteinuria within a few days of birth [2]. Furthermore, the discovery of mutations of the podocin-encoding gene (NPHS2) in familial nephrotic syndrome was a major breakthrough in research into the mechanisms of nephrotic kidney diseases [3]. Several screening studies on NPHS2 mutations involving familial cases and sporadic nephrotic syndrome were published in the years following this discovery [4-6].

Podocin expression in proteinuric renal diseases is a dynamic process that results in a decrease in its protein expression, although discrepancies remain in the data about the up- or down-regulation of podocin mRNA in renal diseases [7-9]. However, according to structural models, abnormal podocin expression and distribution interferes with slit-diaphragm architecture and function, and is associated with proteinuria [10, 11]. In studies of patients with nephrotic syndrome, it was discovered that the podocin promoter has several rare functional variants which give rise to a decrease in podocin mRNA expression [12]. In addition, a specific haplotype of the 5' regulatory region of the human podocin gene with wild-type variants in positions -51, -116, and -535 was found to be associated with a better outcome in terms of proteinuria and creatinine levels in patients with IgA nephropathy [13]. Furthermore, the transcription factor Lmx1b, which is primarily expressed in the podocytes in the kidneys, was found to bind a sequence within the human podocin promoter [14, 15]. Mutations within the LMX1B gene were reported to lead to nail-patella syndrome [16], a disease which is associated with proteinuria, and $Lmx1b^{-/-}$ mice show a reduced number of podocyte foot processes and a lack of podocin expression in immunohistological analyses [14].

The aim of our study was to further decipher the elements involved in the transcriptional regulation of the podocin gene, with a special focus on Lmx1b and the SNPs described above. In addition, we sought to evaluate whether the C/T polymorphism in position -116 in the regulatory region of the podocin gene

is related to the incidence of various kidney diseases associated with nephrotic syndrome.

MATERIALS AND METHODS

Cell culture

Conditionally immortalized murine podocytes were donated by Karlhans Endlich, Heidelberg, Germany. For propagation, the cells were maintained at 33ºC in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum, 1% sodium pyruvate, 100 µg/ml streptomycin, 100 units/ml penicillin and 10 U/ml mouse recombinant INF-γ (Life Technologies). To induce differentiation, the podocytes were maintained at 38ºC without INF-γ for at least ten days before they were used for the experiments.

Human podocin promoter constructs

Reporter constructs of the human podocin promoter were prepared via PCR, and directionally subcloned into the promoterless luciferase expression vector pGL3-Basic (Promega) using the KpnI-BglII sites. The resulting constructs were terminated at bp -400, -800, -820 and -870, relative to the translational start site, and were respectively denoted pT4-Luc400, pT4-Luc800, pT4-Luc820 and pT4-Luc870. To test the functional significance of the -116 C/T and -670 C/T polymorphisms, site-specific mutagenesis was performed on wild-type genomic templates. These constructs were denominated pT4-Luc $400C_{116}$, pT4-Luc$400T_{116}$, pT4-Luc$800C_{670}C_{116}$, and pT4-Luc$800T_{670}C_{116}$.

Transient transfection and luciferase activity

Transient transfections of differentiated podocytes were performed with polyethyleneimine according to Boussif *et al.* [17]. The respective pT4-Luc expression plasmids and a normalizing pCMV-β-galactosidase plasmid were used in concentrations of 2 µg/well. The total incubation time after transfection was 24 to 48 h. All the experiments were carried out in triplicate and performed independently at least three times. Luciferase and β-galactosidase assays of the cell lysates were performed as described previously [18, 19]. The results are expressed as the ratio of luciferase activity to β-galactosidase activity. For the cotransfection experiments, 1 µg of the Lmx1b expression plasmid pcDNA3.1-LMX1B or the empty vector control pcDNA3.1 (donated by Brendan Lee, Houston, USA) was included in the transfection mixture.

Preparation of nuclear extracts

Nuclear extracts from differentiated podocytes grown to 90% confluence in 150-mm tissue culture dishes were prepared according to Dignam [20]. A 1 M potassium chloride-containing buffer was used for the extraction, and the resultant proteins were dialyzed overnight in a solution of 20 mM Hepes, pH 7.9, 20% glycerol, 100 mM potassium chloride, 0.2 mM EDTA, 0.5 mM dithiothreitol and 0.2 mM

phenylmethylsulfonyl fluoride. The protein concentrations were determined via the BCA protein assay (Pierce) using bovine serum albumin as the standard.

Electrophoretic mobility shift assay (EMSA)

Synthetic oligonucleotides were annealed and end-labelled with polynucleotide kinase and [γ-^{32}P]dATP according to the standard methodology. 5 µg of nuclear protein was incubated in a binding buffer (18 mM HEPES (pH 7.9), 200 nM EDTA, 100 nM EGTA, 40 nM NaCl, 200 nM DTT and 100 nM PMSF) with 2 µg of poly(dI-dC) for 30 min at room temperature with 1 µl of the radiolabelled oligonucleotide (100,000 cpm/µl). The samples were electrophoresed on 4% polyacrylamide and 30% w/v glycerol gels in a buffer containing 1 x Tris borate/EDTA followed by autoradiography. Supershift experiments were performed by first incubating the ready reaction mixture overnight at 4ºC with 4 µg/reaction of polyclonal goat anti-Lmx1b antibody (Santa Cruze Biotechnology) or the control rabbit IgG, and then adding the labelled oligonucleotide and performing electrophoresis.

Isolation of genomic DNA

Blood was collected form 151 healthy Caucasian blood donors from northern Germany. Genomic DNA was extracted using the QIAamp DNA Minikit (Qiagen). Kidney biopsy samples from 135 patients with minimal change glomerulonephritis, 148 patients with focal segmental sclerosis, and 139 patients with membranous nephropathy were collected and provided by Udo Helmchen, Nierenstiftung am Universitätsklinikum Hamburg-Eppendorf. This study was approved by the ethics committee of the physicians' board of the city of Hamburg, and appropriate informed consent was obtained from the human subjects involved. Genomic DNA was obtained from 30-µm slices of the paraffin-embedded kidney biopsy using the DNeasy Blood & Tissue kit (Macherey and Nagel).

Determining the genotypes for the SNPs -116 C/T

To determine the genotype for the SNP of interest, a genomic fragment of the human podocin 5' untranslated region was amplified by PCR using the following flanking primer pair: $^{5'}$CTCTAGCATGACATTAGGAA$^{3'}$ and $^{5'}$CAGCAGCGCGGGAGCGCTAG$^{3'}$. They generate a 350-bp fragment spanning bp -26 to -375. When the -116T allele is present, a unique HpyCH4III restriction site is eliminated from the wild-type -116C allele. This permitted the restriction enzyme analysis for the PCR products for the genotyping of individuals with HpyCH4III for the -116C/T polymorphism.

Statistical analysis

Statistical significances were determined for paired comparisons using Student's t test or by an analysis of variance for multiple comparisons where appropriate. Statistical levels of significance for deviation from the expected Hardy-Weinberg distribution were determined by χ^2 analysis.

RESULTS

Transcriptional regulation of podocin by Lmx1b in podocytes

Transcription experiments with a set of 5' deletion constructs revealed basal promoter activity up to bp -820 5' of the translational start site (Fig. 1) even though the construct pT4-Luc 820 contains a FLAT-E element (5'-TAATTA-3'), which can be activated by the transcription factor Lmx1b, and is fully sufficient for mini-enhancer function [21]. Construct pT4-Luc 870 expressed a 10.5-fold increase in luciferase activity compared with construct pT4-Luc 820. The region between bp -820 and bp -870 includes a FLAT-F element (5'-TTAATAA-3'), which can also bind Lmx1b [22].

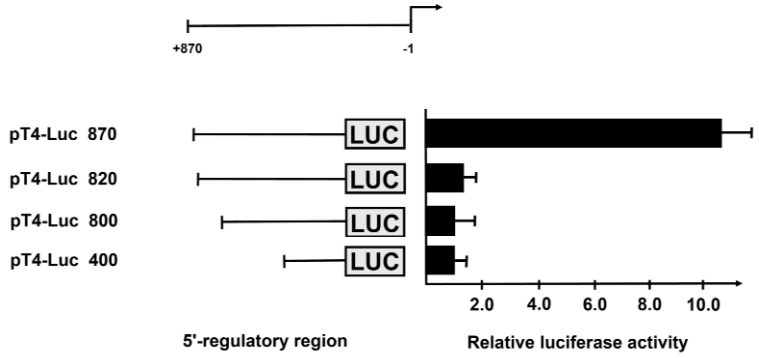

Fig. 1. A transcriptional analysis of 5' deletion constructs of the human podocin promoter. The nucleotide positions with respect to translational start site are given in the name of the constructs. The bars are the ratios of luciferase versus β-galactosidase activities, arbitrarily set equal to 1 for pT4-Luc 400. The values are the means (± SEM) of at least six independent experiments.

Transfecting podocytes with construct pT4-Luc 870 plus the Lmx1b expression vector pcDNA3.1-LMX1B resulted in a further 8-fold increase in luciferase activity, whereas using the empty vector pcDNA3.1 did not lead to any difference in luciferase expression (Fig. 2).

Since it had been demonstrated that an oligonucleotide from the 5' untranslated region of podocin containing the FLAT-E and the FLAT-F element would bind to *in vitro* translated Lmx1b [16], but our transfection data revealed that the FLAT-F element alone seemed to be sufficient for transcriptional upregulation, we performed EMSA experiments with an oligonucleotide that only includes the FLAT-F element (Fig. 3). In the presence of nuclear extracts from the podocytes, the oligonucleotide containing the FLAT-F element shows significant retardation and reveals a major DNA-protein complex (Fig. 3, lane 2). This DNA-protein complex showed a supershift when the probe and nuclear extracts were incubated with an anti-Lmx1b antibody, strongly suggesting the presence of the Lmx1b protein in the DNA-protein complex (Fig. 3, lane 3). No change in

the intensity of DNA-protein complex formation occurred when rabbit IgG alone was added to the oligonucleotide/protein mix (Fig. 3, lane 4).

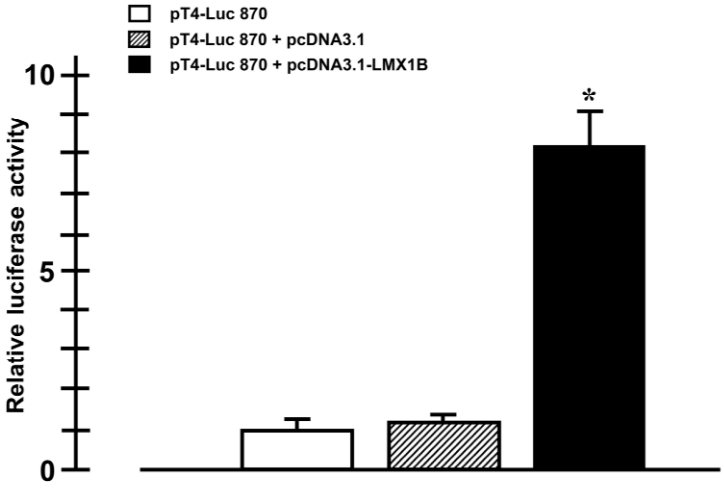

Fig. 2. The luciferase expression of the construct pT4-Luc 870 in the podocytes after cotransfection with the Lmx1b expression vector pcDNA3.1-LMX1B. The vector pcDNA3.1 serves as an empty control. The data is given as the ratios of luciferase to β-galactosidase activities with construct pT4-Luc 870 assigned a value of 1. The results are the means of three independent experiments. *A significant increase in luciferase activity ($p < 0.05$).

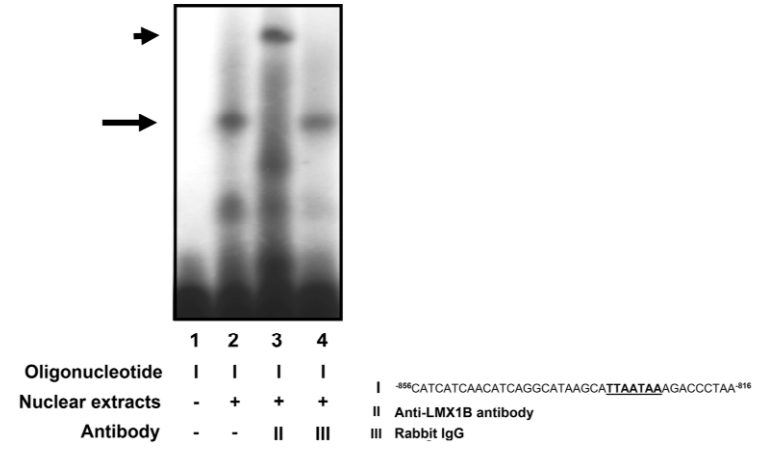

Fig. 3. A supershift EMSA with an antibody against Lmx1b. EMSA was carried out with the radiolabelled oligonucleotide I containing the FLAT-F element. The long arrow marks the shifted DNA/protein complex with nuclear extracts from podocytes (lane 2). The short arrow marks the supershifted band when the Lmx1b antibody is present (lane 3), while no supershift is visible with a non-specific antibody (lane 4).

Transcriptional regulation of podocin by the functional promoter SNP -116C/T

Within the sequence containing basal promoter activity, two common promoter SNPs were identified: -116C/T and -670C/T. Constructs including either the wild-type or mutant variant in position -116 (Fig. 4A) or in position -670 (Fig. 4B) were transfected into the podocytes.

Luciferase expression was significantly reduced with construct pT4-Luc400T_{116} (the mutant variant in position -116), but constructs with the wild-type or mutant variant in position -670 showed no difference in luciferase activity (Fig. 5).

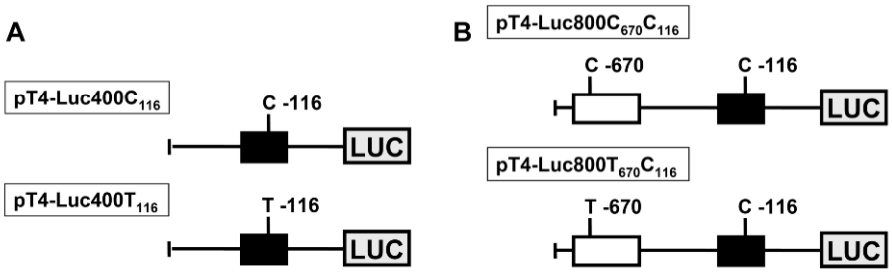

Fig. 4. The design of constructs with two different SNPs. A – pT4-Luc400 with either C or T in position -116. B – pT4-Luc800 with the wild-type C in position -116 and either C or T in position -670.

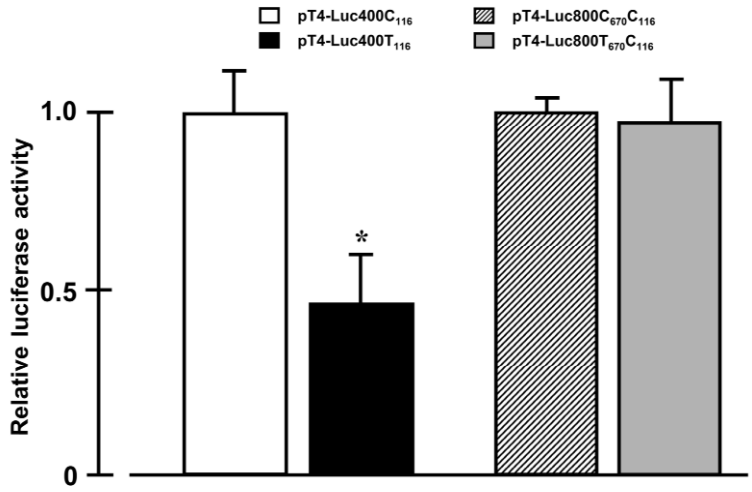

Fig. 5. Transient transfection of podocytes with constructs from Fig. 4. The mutant construct pT4-Luc400T_{116} leads to a significant (*$p < 0.05$) reduction in luciferase expression, while no difference in luciferase activity was observed with constructs pT4-Luc800$C_{670}C_{116}$ and pT4-Luc800$T_{670}C_{116}$. The data is given as the ratios of luciferase to β-galactosidase activities, arbitrarily set equal to 1 for pT4-Luc400C_{116} and pT4-Luc800$C_{670}C_{116}$, respectively. The values are the means (± SEM) of at least three independent experiments.

A gel shift analysis with oligonucleotides including either the -116C or the -116T variant showed a shifted DNA/protein complex for the wild-type oligonucleotide (Fig. 6, lane 2) and decreased protein binding to the mutant oligonucleotide (Fig. 6, lane 4). Hence, reduced activity demonstrated by the luciferase data could be due to decreased protein binding to this site when the mutant variant -116T is present. No DNA/protein complexes were found in EMSA experiments with oligonucleotides containing the wild-type or mutant in position -670 (data not shown).

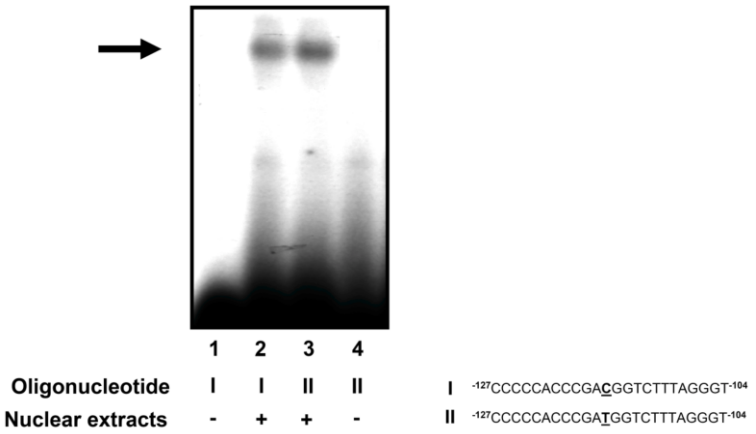

Fig. 6. EMSA with oligonucleotides containing C (I) or T (II) in position -116. The arrow marks a shifted DNA/protein complex with oligonucleotide I and nuclear extracts from podocytes (lane 2). A decrease in nuclear protein binding can be seen in lane 4 when oligonucleotide II with the T in position -116 is present.

Screening of patients with nephritic syndrome for the SNP -116C/T

Since podocin mutations have been shown to play a role in certain renal diseases [12, 13], we screened healthy blood donors and patients with biopsy-proven renal diseases with nephrotic syndrome due to minimal change glomerulonephritis, focal segmental sclerosis or membranous nephropathy for the functional promoter SNP in position -116. The podocin -116 genotype was identified via PCR, followed by restriction analysis with HpyCH4III (Fig. 7).

No differences in allele frequency or genotype frequency were discovered between the four groups (Tab. 1). The distribution of the genotypes was according to the Hardy-Weinberg equilibrium in all the groups, and no association of the -116C/T polymorphism with the occurrence of a specific disease with nephrotic syndrome was detected.

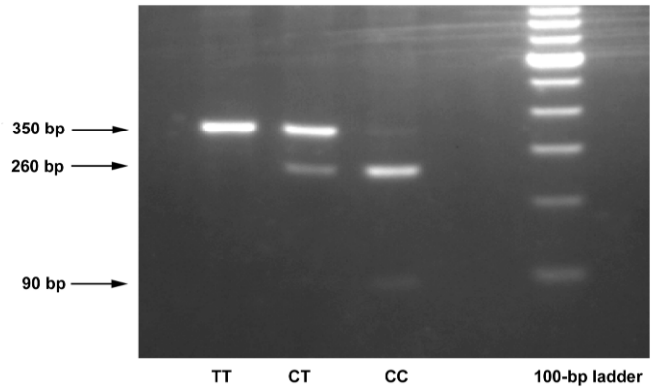

Fig. 7. An example of genotyping for podocin -116C/T promoter polymorphism. HpyCH4III digestion after PCR amplification of genomic DNA yields different fragments according to genotype: TT, 350 bp; TC, 350, 260, and 90 bp; CC, 260 and 90 bp.

Tab. 1. Genotype and allelic frequencies of the -116C/T polymorphism in the 5' regulatory region of the human podocin gene. The data was derived from healthy northern German Caucasian blood donors and from kidney biopsies from patients with different renal diseases presenting with nephrotic syndrome: minimal change glomerulonephritis (Minimal change), focal segmental glomerulosclerosis (FSGS), and Membranous nephropathy.

Genotype -116	#	Genotype frequency	Allele	Allele frequency
Controls	151		C	.665
			T	.335
CC	69	.457		
CT	63	.417		
TT	19	.126		
Minimal change	135		C	.695
			T	.305
CC	63	.466		
CT	62	.459		
TT	11	.081		
FSGS	148		C	.618
			T	.382
CC	58	.392		
CT	67	.453		
TT	23	.155		
Membranous nephropathy	139		C	.650
			T	.350
CC	59	.424		
CT	63	.453		
TT	17	.123		

DISCUSSION

Podocin has a central role in maintaining slit membrane homeostasis. Accordingly, podocin knockout is linked to proteinuria and renal failure in mice [23], and the same occurs in patients with mutations of the NPHS2 gene [3, 13]. During proteinuria, podocin is lost from the podocyte body [8], and should be restored by resynthesis. Knowledge about the transcriptional regulation of the podocin gene might provide key insight into the development and treatment of proteinuric renal diseases. Earlier studies showed that the transcription factor Lmx1b binds a putative FLAT-E site in the podocin 5' regulatory region, and when this site was subcloned into a promoter containing an expression vector, transfected into NIH 3T3 cells, and cotransfected with an Lmx1b expression vector, it revealed enhancer activity [14]. Another study also revealed Lmx1b-binding activity but failed to demonstrate reporter gene activation by the podocin promoter in COS-7 and HeLa cells, suggesting the role of additional transcription factors in this context [15]. Our data shows for the first time an enhancing activity between bp -820 and -870 of the podocin promoter within its own genetic context in the podocytes. This enhancing activity was further increased by cotransfection with an Lmx1b expression vector, and EMSA supershift experiments revealed binding activity to a FLAT-F element localized in this region. In addition to activation by the FLAT-E element [14], binding to the FLAT-F element in the podocin promoter by Lmx1b seems to be important for podocin up-regulation, and might play a role in the restoration of the slit membrane in proteinuric disease processes. So far a pathogenetic role for Lmx1b in renal diseases involving podocin dysregulation has only been defined for knee patella syndrome [24].

Our further studies involved the common promoter SNPs -116C/T and -670C/T in the 5' regulatory region of the podocin gene. While the variant -116T led to a 50% reduction of transcriptional activity, no difference in transcription was seen between -670C and -670T. EMSA experiments revealed a decreased binding activity of nuclear proteins from podocytes to the mutant -116T variant, suggesting an enhancing activity for the protein that binds to this region. The -116C wild-type variant has been shown to belong to a podocin gene haplotype which is associated with a better clinical outcome and reduced proteinuria in patients with IgA nephropathy, but not in patients with renal diseases leading to nephrotic syndrome. However, the patient numbers were small [13]. The decreased activity for the mutant -116T variant in our study with reduced podocin mRNA formation might lead to less podocin protein formation and less reconstruction of the slit membrane. This hypothesis would confirm the *in vivo* data of Di Duca *et al.* [13]. Since the -116C/T SNP does not belong to any defined nuclear protein-binding site, further studies are needed to characterize the binding protein.

As the -116T variant is involved in the reduced activity of the podocin gene and the -116C variant showed improved outcome of IgA nephropathy [13], we

studied the allelic distribution of the -116C/T SNP in patients with renal diseases associated with nephrotic syndrome compared with healthy controls. For none of the three studied diseases (minimal change glomerulonephritis, focal segmental sclerosis and membranous nephropathy) did we find a genotypic frequency that was significantly different from the genotypes of healthy blood donors. These findings strongly argue against a role of the podocin -116C/T polymorphism in susceptibility to any of the common renal diseases associated with nephrotic syndrome. However, since reduced activity was demonstrated for the -116T variant, and the presence of this variant leads to a different outcome in IgA nephropathy [13], it cannot be excluded that changes in the expression of the as yet unknown binding protein might lead to differences in the occurrence or progression of different renal diseases associated with nephrotic syndrome. Mutations in the podocin regulating transcription factor Lmx1b have been shown to lead to such different patterns in renal disease [16, 25].

In summary, our data suggests that the transcription factor Lmx1b is a relevant enhancer for the regulation of the podocin gene in podocytes. The occurrence of the SNP variant -116T leads to a significant reduction in the transcriptional activity of podocin, but is not associated with the incidence of minimal change glomerulonephritis, focal segmental sclerosis or membranous nephropathy in a Caucasian population.

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