Nephrology and Renal Diseases

Original Research Article

Knockdown of Glypican-1 and 5 isoforms causes proteinuria and glomerular injury in zebrafish (*Danio rerio*)

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Abstract

Components of the glycocalyx are being intensively studied for their involvement in a vast array of glomerular diseases. Among the most abundant proteoglycans in the glomerular cell glycocalyx are the heparan sulfate proteoglycans *Glypican-1* and *5*, expressed predominantly in glomerular endothelial and epithelial cells, respectively.

We used a previously characterized zebrafish model to define the role of all different in zebrafish existing isoforms of *Glypican-1* (*a* and *b*) and *5* (*a,b* and *c*) in vascular and renal pathology and tested the hypothesis that *Glypican* expression is important for the integrity of the glomerular barrier. *Glypican* morpholino knockdown in *l-fabp:DBP-eGFP* fish showed edematous phenotypes and a significant reduction of eye fluorescence for *Glypican-1a*, *5a* und *5c*, but not for *Glypican-1b* and *5b* isoforms. This indicates a significant loss of plasma proteins following knockdown of *Glypican-1a*, *5a* and *5c* in contrast to a conserved filtration barrier following knockdown of *Glypican-1b* and *5b* isoforms. Knockdown of all different examined *Glypican* isoforms did not interfere with early nephron development as shown in the *WT1b* fish strain. Using electron microscopy, we could demonstrate that *Glypican-1a* knockdown leads to a predominantly endothelial cell injury with preservation of podocyte foot processes, while knockdown *of Glypicans- 5a* and *5c* shows focal podocyte effacement with conserved endothelial structures. Ultrastructural analysis of glomeruli following knockdown of *Glypicans-1b* and *5b* reveals normal glomerular structure.

In this brief study we demonstrate in an in-vivo model that the *Glypican* proteoglycan family is critical in vascular and glomerular integrity. Our observations suggest a role for *Glypican- 1a* in endothelial cell and *Glypican-5a* and *5b* in podocyte cell injury leading to subsequent loss of glomerular filtration barrier function and proteinuria.

Abbreviations: hpf: hours post fertilization

Introduction

Quantitative changes in heparan sulfate proteoglycans have been associated with a wide range of inflammatory and proteinuric nephropathies, including diabetic nephropathy, minimal change disease, membranous nephropathy, systemic lupus associated nephropathy and various rodent models of nephrotic syndrome [1,2]. Of the diverse Glypican proteoglycan family *Glypicans-1, 3, 4* and *5* are found in the human kidney. [3-5]. Recently, Okamoto et. al. identified through a genome wide association study and replication analysis a certain variant of *Glypican-5* as a new susceptibility gene for acquired nephrotic syndrome [6]. *Glypican-1* is one of the most important cell surface associated glomerular heparan sulfate proteoglycans and is expressed in both glomerular epithelial as well as glomerular endothelial cells 1. However, not much is known until now about a possible association of *Glypican-1* with kidney disease. While in humans only six different Gypican proteins exist [7], in zebrafish ten different Glypican proteins have been isolated [8]. In contrast to humans, in zebrafish two different isoforms of *Glypican-1*, named *Glypican-1a* and *1b*, and three different isoforms of *Glypican 5*, named *Glypican-5a*, *5b* and *5c*, have been identified 8.

In this present study we examined the role of the *Glypican-1 (a and b) and Glypican-5 (a,b and c)* genes in glomerular and vascular physiology employing a zebrafish model. We present evidence for an important role of this protein in the development of proteinuria as well as vascular permeability and describe for the first time a model of glomerular pathology of *Glypican-1* and *Glypican-5* in an *in-vivo* setting.

Materials and methods

All methods applied in this study have been extensively described by our laboratory previously 9, and will therefore be described in a short fashion.

Zebrafish stocks and embryos

Zebrafish, Wildtype AB and the Transgenic zebrafish lines *Tg(l-fabp:DBP-eGFP)* 10, *Tg(flk:mcherry/l-fabp:DBP-eGFP)* and *Wt1b-GFP* 11, were grown and mated at 28.5°C. Embryos were kept and handled in standard E3 solution buffered with 2 mM HEPES as previously described previous 12,13. All zebrafish studies were conducted in accordance with the National Institutes of Health Guide

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for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) under approval #14-06.

Zebrafish morpholino injection

As decribed before by our group, *Glypican-1a* and *1b, Glypican 5a, 5b* and *5c* as well as control morpholinos were injected into one to four-cell stage fertilized embryos at a concentration of 100μM. Morpholino sequences were ordered from GeneTools (Philomath, OR) as follows: control sequence - 5'- CCTCTTACCTCAGTTACAATTTATA-3', *Glypican-1a* sequence 5'- AAAAAGAGCTGTTGACTCACTGTCA-3', *Glypican-1b* sequence 5'- CAGCAGGACCGATTCACCTACCGGA-3', *Glypican-5a* sequence 5'- TAACCACACACTCATCCTCTCACCT-3', *Glypican-5b* sequence 5'- AATGTTTTTGGACCTACCTGGTTGA-3' and *Glypican-5c* sequence 5'- ACATGACGGCATTTCAGTGGAGAAA -3'. The *Glypican-1a/1b/5a/5b* morpholino constructs act as a splice donor, initiating wrong pre-mRNA splicing in the region between exon2-intron2/exon1-intron1/exon1-intron1/exon7-intron7, respectively. The *Glypican-5c* morpholino construct works as a translation blocker by binding close to the translational start site of the′complementary mRNA in the 5 -untranslated region.

Fluorescence proteinuria measurements –fabp eye assay

72 hours post fertilization (hpf) dechorionated Tg(l- fabp:DBP-eGFP) embryos were anesthetized with a 1:20 to 1:100 dilution of 4 mg/ml Tricaine, placed in 200 μl E3 in single wells of 96 well plates and allowed to recover. Sequential images of live fish were then generated 96 and 120hpf using the Axiovert 200 microscope connected to an AxioCam charge-coupled device camera, and images were taken using the Axio Vision release 4.5 SP1 software package. The maximum fluorescence intensities of grayscale images of the pupil of the fish were measured using NIH's ImageJ application and reported in relative units of brightness as previously described by our group.

Assay for glomerular fusion – WT1b fish

Normal development and the fusion of the zebrafish pronephros was documented in the *WT1b:eGFP* fish line 72hpf for all Glypican isoforms at a morpholino concentration of 100 μM.

Histology and transmission electron microscopy (TEM)

Morphant larval zebrafish were sampled at 120 hpf and fixed, washed and embedded with epon according to manufacturer's protocol (Hard Plus Resin 812, Electronmicroscopy Sciences, Hatfield, PA). Ultrathin sections of 70-90 nm were imaged using a JEOL-1230 or a Morgagni (FEI, Eindhoven) transmission electron microscope, operated at 80 kV.

Statistics

We used mean ± SEM throughout this study. For comparing two different groups of eye fluorescencé T results, Student test was applied. p-values (2-sided) were considered significant at $p<0.05$. Statistical analysis was performed using GraphPad Prism Software (San Diego, California).

Results

Knockdown of *Glypican-1a, 5a and 5c* in zebrafish induces a renal phenotype

Following injection of 100 μM of *Glypican-1a* and *b* as well as *Glypican 5a, b* and *c* specific antisense morpholinos, we could detect the development of an edematous phenotype in *Glypican-1a, 5a* and *5c* knockdown fish, but not in *Glypican-1b* and *5b* knockdown fish. This phenotype was characterized by general body edema, including pericardial effusion, yolk sac edema and a severely arched back. Those fish displayed reduced activity and had high rates of mortality at 96 and 120 hpf. The phenotype was worst in *Glypican-5c* knockdown fish, and about equal severe in *Glypican-1a* and *5a* knockdown fish. The heartbeat and blood flow of these fish was normal pointing towards normal cardiac development. Edema developed already at 72hpf and was progressing in severity by 96 and 120hpf. This phenotype was not seen in WT fish, fish injected with a control morpholino, and in fish injected with a *Glypican-1b* and *5b* knockdown morpholino. Representative phenotype pictures are shown in Figure 1A.

Knockdown of *Glypican-1a, 5a and 5c* leads to proteinuria

We measured the loss of high molecular weight GFP tagged DBP protein as loss of eye vasculature fluorescence employing the Tg(l- fabp:DBP-eGFP) fish line. This revealed a highly significant loss of fluorescence in the *Glypican-1a, 5a* and *5c* knockdown fish compared with control fish for both 96hpf and 120hpf. *Glypican-1b* and *5b* knockdown fish showed no significant loss of eye fluorescence, neither at earlier nor at later time points. Results are shown in Figure 1B and where as follows: 96hpf: control = 48.1 ± 1.6, *Glypican-1a* = 23.5 ± 1.9, *1b* = 43.2 ± 2.5, *5a* = 23.2 ± 3.0, *5b* = 47.7 ± 2.0, *5c* = 13 ± 0.8 and 120hpf: control = 66.7 ± 3.2, *Gypican-1a* = 43.9 ± 7.1, *1b* = 68.7 ± 5.3, *5a* = 15.9 ± 4.6, *5b* = 57.9 ± 3.9, *5c* = 0.7 ± 0,1). Loss of fluorescence was most pronounced with knockdown of *Glypican-5c*, corresponding well with the highest severity of edematous phenotype seen with this knockdown. Knockdown of *Glypican-1a* and *5a* were about equal at 96hpf and more pronounced with *Glypican-5c* at the later time point of 120hpf.

Knockdown of *Glypicans* does not interfere with early zebrafish nephron development.

The zebrafish embryonic pronephros consists of two nephrons with glomeruli fused at the embryo midline and two pronephric tubules that connect the glomerulus to the pronephric ducts, that fuse just before the cloaca [9,14]. As seen in Figure 2 we could demonstrate in the WT1b fish strain that knockdown of all different Glypican isoforms studied does not interfere with early nephron development. Two glomeruli give rise to two seperate tubular structures. Fusion of the two pronephros takes place about 72hpf.

Knockdown of *Glypican-1a* disrupts glomerular filter structure by causing endothelial cell injury, while knockdown of *Glypican-5a* and *5c* leads to podocyte foot process effacement

To directly visualize the effect of *Glypican* knockdown on glomerular slit diaphragm architecture, we analyzed the glomeruli of 120hpf embryos by transmission electron microscopy. Diffuse endothelial cell swelling was seen in the glomeruli of *Glypican-1a* knockdown fish, whereas the endothelium appeared healthy in control injected fish and wildtype fish as shown in Figure 3. However, regular podocyte foot processes and basement membrane structures were observed in *Glypican-1a* morpholino injected fish. When electron microscopy pictures of glomeruli following knockdown of *Glypican-5a* and *5c* were analyzed, focal podocyte foot process effacement could be recognized in both isoforms. In contrast to *Glypican-1a* knockdown fish endothelial cell morphology and endothelial

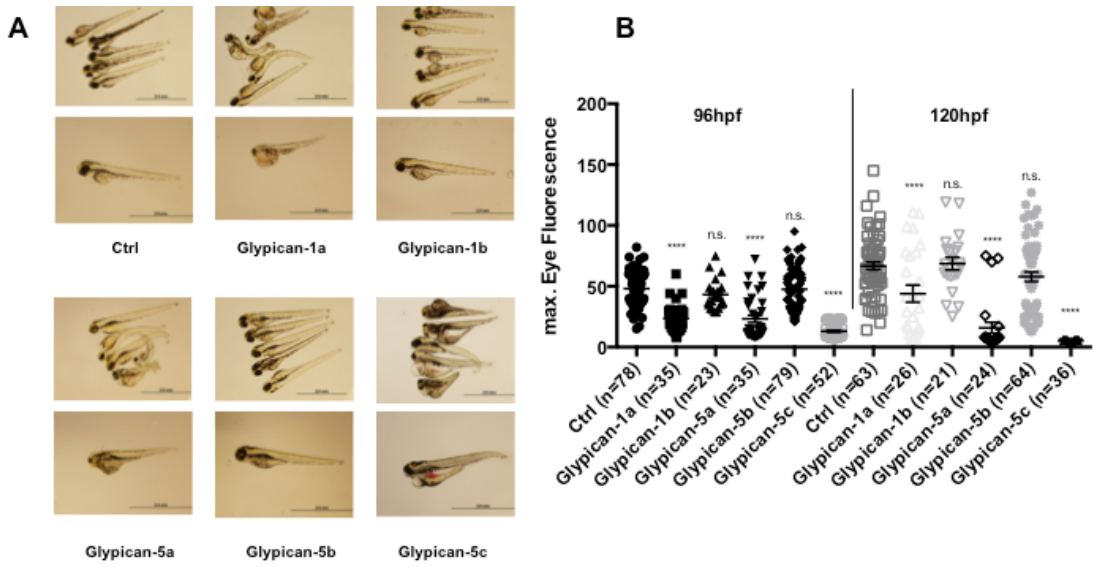

Figure 1: Knockdown of *Glypican-1a, Glypican-5a* and *Glypican-5c* in zebrafish induces an edematous phenotype and proteinuria.
Edematous phenotype of *Glypican* knockdown (A). Compared are *Glypican* knockdown and control fish at 120hpf, morpholino concentration is 100µM. *Glypican-1a, Glypican-5a* and *Glypican-5c* knockdown fish exhibit yolk sac and pericardial edema with severely curved arches. In contrast, control fish, *Glypican-1b* and *5b* knockdown fish retain their linear shape and show no edema.
Knockdown of *Glypican-1a, Glypican-5a* and *Glypican-5c* leads to proteinuria – Quantitative measurement of eye fluorescence (B). Compared are results for *Glypican* knockdown and control fish at a morpholino concentration of 100uM Eye fluorescence of *Glypican-1a, Glypican-5a* and *Glypican-5c* knockdown fish is highly significantly reduced compared to control as well as *Glypican-1b* and *5b* knockdown fish at both 96 and 120hpf.
Values are means ± SEM. ns = non significant, *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.001.

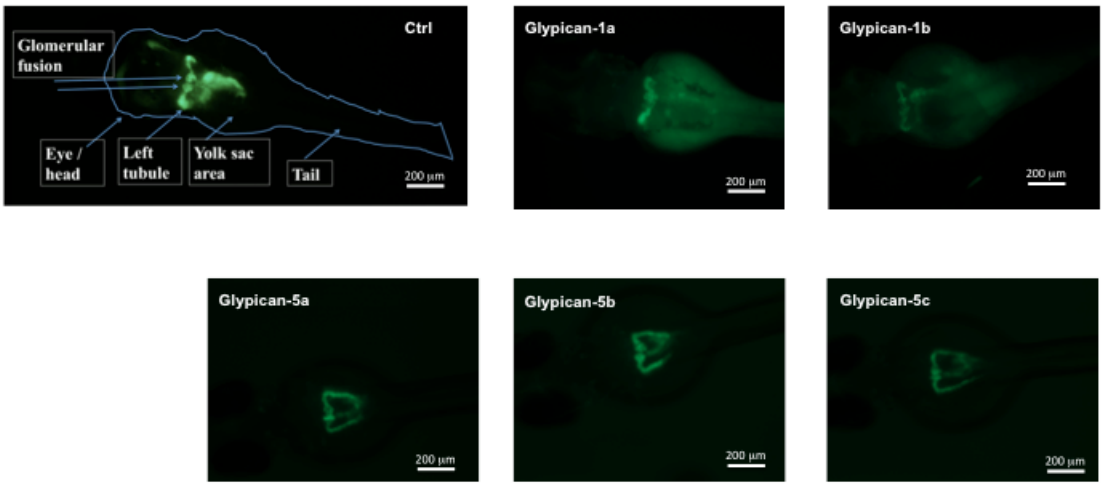

Figure 2: Knockdown of *Glypicans* in zebrafish does not interfere with early zebrafish nephron development .
Fluorescence microscopy showing pronephron structures of WT, control morpholino and *Glypican* knockdown fish at 72hpf. In all fish two separate glomerular tufts and tubular structures and fusion of the two pronephrons are seen. There is no defect of development or disruption of fusion of the pronephrons in *Glypican* knockdown fish. Anatomical structures are labeled accordingly in the left picture.

fenestrae appeared to be normal. For all *Glypican* isoforms a normal glomerular basement membrane structured could be recognized. In marked contrast, *Glypican-1a* and *5b* knockdown fish showed normal ultrastructural glomerular anatomy comparable with control and wild type fish.

Discussion

In this manuscript, we present evidence for an important role of the *Glypican* protein family in the development of proteinuria as well as vascular permeability in a zebrafish in-vivo seeting. Evidence for this conclusion from the present experiments is as follows:

1). *Glypican-1a, 5a* and *5c* knockdown fish develop a severely disturbed edematous phenotype that is not seen in control morpholino injected fish nor in *Glypican-1b* and *5b* knockdown fish;

2). Employing a transgenic fish line expressing a GFP tagged DBP protein, *Glypican-1a, 5a* and *5c* knockdown fish show a highly significant loss of eye fluorescence which corresponds to significant loss of high molecular weight protein via the urine, whereas *Glypican-1b* and *5b* knockdown fish exhibit conserved vascular fluorescence

3). Proteinuria appears to correspond with the severity of the edematous phenotype, is worst in *Glypican-5c* knockdown fish and about equal in *Glypican-1a* and *5a* knockdown fish

4). Renal pathology is not due to gross renal developmental defects as seen in the WT1b fish strain.

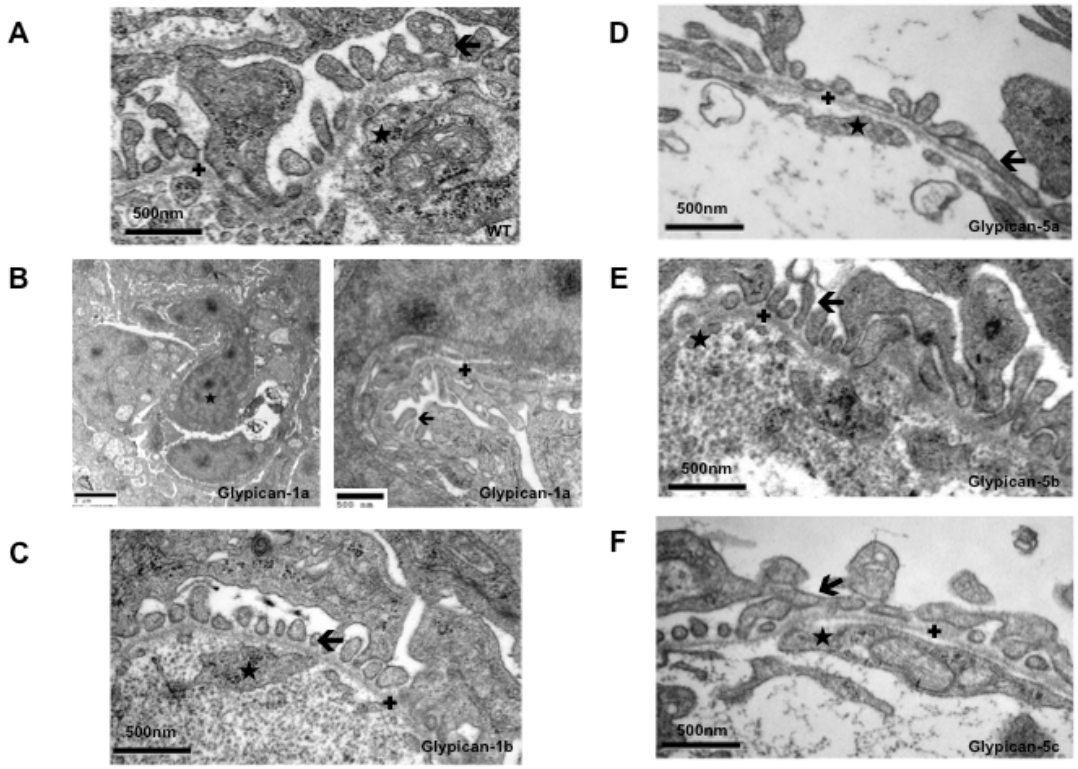

Figure 3: Knockdown of *Glypicans* disrupts glomerular filter structure by causing endothelial cell and podocyte injury – Transmission electron microscopy analysis of glomeruli at 120hpf. WT fish (A) as well as *Glypican-1b* (C) and *5b* (E) knockdown fish show regular endothelial cell (★) and podocyte foot process morphology (·) as well as a normal basement membrane structure (✚).

Glypican-1a (B) knockdown fish glomeruli exhibit diffuse endothelial cell swelling (★), but regular podocyte foot processes (·) and basement membrane (✚) structures.14In contrast, glomeruli of *Glypican-5a* (D) and *5c* (F) knockdown fish show podocyte foot process effacement (·), which has a focal character for both isoforms. Normal endothelial cell morphology (★) and glomerular basement membrane (✚) structures are seen in both isoforms.

Proteinuria is considered to be a hallmark of glomerular pathology [15].

Given the fact that *Glypican-1*is expressed in both glomerular endothelial cells and in podocytes [3,16], we proceeded to investigate which cell type might be primary responsible for the disruption of glomerular filter integrity following *Glypican* knockdown in the zebrafish model. Our experiments provide further evidence that *Glypican-1* gene knockdown leads to a predominant endothelial injury, while *Glypican-5a* and *5c* results in a podocyte phenotype. Transmission Electron Microscopy pictures of *Glypican-1a*knockdown fish glomeruli showed substantial endothelial cell swelling whereas no significant podocyte effacement was seen and the basement membrane architecture principally remained intact. The observed tubular damage is most likely secondary to the predominant endothelial injury that is taking place in the glomerulus. In contrast, *Glypican-5a* and *5c* knockdown fish showed focal podocyte effacement but conserved endothelial cell structures when glomerular structures were analyzed on an ultrastructural level using transmission electron microscopy. These electron microscopy findings provide direct evidence that the nephrotic phenotype observed in *Glypican-1a* knockdown fish is likely due to primary endothelial cell injury while *Glypican-5a* and *5c* is associated with podocytic cell injury that both ultimately lead to disintegrity of the glomerular filter function and secondary tubular cell injury.

It is not clear why knockdown of *Glypican-1a, 5a* and *5c* lead to glomerular injury, whereas *Glypican-1b* and *5b* knockdown does not. Based on comparable expression patterns and partial synteny a previous investigation has concluded that *Glypican-1a* is more likely to be the true ortholog of mammalian *Glypican-1 8*. Of all three *Glypican-5* isoforms, *Glypican-5a* has the highest amino acid sequence identity/similarity compared to mammalian *Glypican-5*, followed by *Glypican-5c* and then *5b*.

In endothelial cells, *Glypican-1* is also involved in flow sensation: Exposure to an increase in flow leads to mobilisation of *Glypican-1* and caveolin 1 to the plasma membrane and the activation of endothelial nitric oxide synthase [17,18]. One could suspect, that *Glypican-1* knockdown leads to structural disintegrity and maladaption of endothelial cell response to shear stress and thus to endothelial injury, glomerular filtration barrier dysfuntion and proteinuria. Among the different glypicans *Glypican-1* is the only member expressed in the vascular system [19,20]. Both angiogenic and anti-angiogenic proteins, endostatin and VEGF-165, were shown previously to require interaction with *Glypican-1* for the binding to their corresponding receptors in endothelial cells [16,21]. Glypican`s heparin sulfate proteoglycans are essential to chaperone both endostatin and VEGF binding. Endostatin appears to antagonize proangiogenic actions of VEGF not by cross-competition for receptor binding but rather via antagonizing intracellular signaling [21]. VEGF is able to cause endothelial dysfunction in patients with diabetic nephropathy and chronic kidney disease, whereas it appears to have a protective role in preeclampsia associated kidney disease [22]. It is conceivable that knockdown of *Glypican-1* causes a dysbalance of VEGF and endostatin

and therefore leads to endothelial dysfunction that we observe in the zebrafish model.

Recent studies focusing on the role of *Glypican-5* in glomerular pathology, using cell culture experiments in rat glomerular epithelial cells and employing a podocyte specific *Glypican-5* knockdown mouse model, have been suggested a close association of *Glypican-5* with FGF2 signaling and podocyte injury 6. A potential harmful role of FGF2 signaling has been suspected in several different etiologies of nephrotic syndrome [23-25]. It is feasible, given the same podocyte phenotype we observed comparing Glypican-5a and 5c knockdown fish, that this is the predominant injury mechanism also for *Glypican-5c* knockdown associated podocyte injury.

In this present study we investigated in a zebrafish vivo model the role of the Glypican proteoglycan family in glomerular physiology and conclude that *Glypican-1a, 5a* and *5c* play an important role in the integrity of the glomerular filtration barrier and reduced *Glypican-1a/5a and 5c* expression may contribute to injury to glomerular endothelial cells and podocytes, respectively, the loss of glomerular filtration barrier function and the onset of proteinuria.

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Conflicts of interest statement

None declared.

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