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Heparanase: an essential factor for the development of proteinuria

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Chapter 1

General introduction and outline of this thesis

The kidney is a bean-shaped organ that plays a vital role in maintaining body homeostasis. The kidney filters approximately 180 liters of primary urine a day, thereby excreting waste products such as urea, minerals and toxic substances. In addition, the kidney is important for the regulation of blood pressure and the release of hormones, such as renin, erythropoietin, prostaglandins and vitamin D. Each kidney consists of about 1 million nephrons, which are the functional units of the kidney. A nephron is composed of a glomerulus, where the filtration of the blood takes place, and a tubule, which is responsible for the reabsorption and secretion of ions and water. The glomerulus is a network of capillary loops that is enclosed by the Bowman's capsule. Glomeruli receive their blood supply from an afferent arteriole. Unlike most other capillary beds, blood is drained away from the glomerulus into an efferent arteriole rather than a venule. This creates resistance to blood flow and produces high pressure on glomerular capillaries, aiding the process of glomerular ultrafiltration. Glomerular capillaries consist of three layers: glomerular endothelial cells, covered with a glycocalyx, at the inner side of the capillary wall, the glomerular basement membrane (GBM), and podocytes with interdigitating foot process covering the outer side of the capillaries (Figure 1). Together these three layers form the glomerular filtration barrier (GFB), which is responsible for the size- and charge-selective filtration of the blood. Small and positively charged proteins can pass the GFB freely, whereas the passage of large and negatively charged proteins is restricted. Damage to any of the 3 layers of the GFB will facilitate passage of blood-derived proteins, thereby resulting in the development of proteinuria [1].

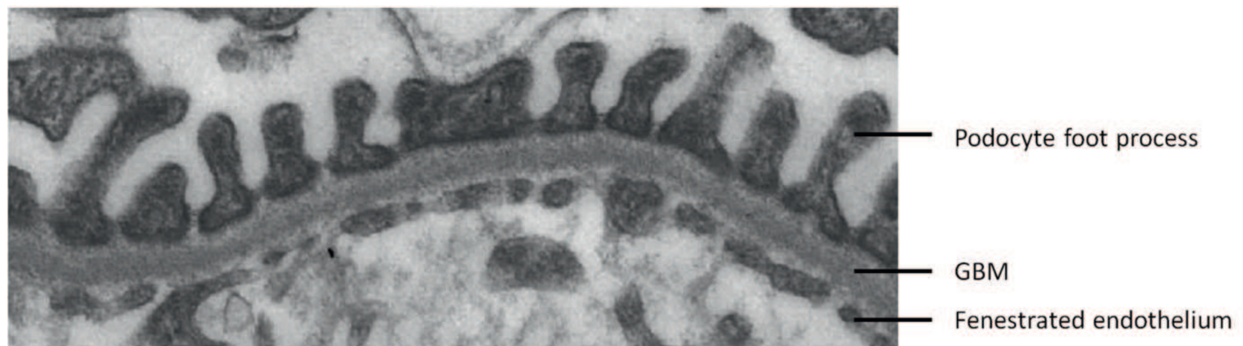

Figure 1: Transmission electron microscopic picture of the glomerular filtration barrier. The glomerular filtration barrier is composed of fenestrated glomerular endothelial cells, the glomerular basement membrane (GBM) and podocytes with interdigitating foot processes. Courtesy of H. Dijkman and B. Willemsen.

Proteinuria

Proteinuria is characterized by the presence of an excess amount of proteins in the urine. Proteinuria is an early sign of many renal diseases and an independent risk factor for the progression to renal failure, all-cause mortality, and cardiovascular mortality [2, 3]. Proteinuria can be caused by damage to the GFB, increased quantity of proteins in the

serum (overflow proteinuria), or a reduced reabsorption of proteins in the tubuli [4]. Previous studies showed that damage to any layer of the GFB can eventually result in the development of proteinuria [1]. It is therefore important that all layers of the GFB remain intact to maintain a normal filtration barrier function.

The role of the endothelial glycocalyx in proteinuric diseases

Glomerular endothelial cells and podocytes are covered with a carbohydrate-rich layer, called the glycocalyx. The glycocalyx is connected to the endothelium and podocytes through cell membrane-bound proteoglycans, which, together with their glycosaminoglycan (GAG) chains, form the structural and functional backbone of the glycocalyx [5]. Soluble molecules like albumin, orosomucoid and lamican are incorporated in the glycocalyx and are connected either directly or via soluble proteoglycans and/or GAG chains [5, 6]. The presence of the endothelial glycocalyx is essential for controlling vascular permeability, signaling, vascular protection, mechanotransduction and attenuating blood cell-vessel wall interactions [5, 7-10]. Not much is known about the physiological role of the podocyte glycocalyx, but it has been suggested to play a role in maintaining foot process structure and keeping a certain distance between podocytes and parietal epithelial cells lining Bowman's capsule [11].

Several studies showed that the endothelial glycocalyx is degraded during renal failure. Patients with end-stage renal disease have a reduced glycocalyx thickness and elevated levels of circulating syndecan-1, which are both normalized after kidney transplantation [12]. Glycocalyx thickness was also reduced in patients with type 1 diabetes and correlated with the development of proteinuria, suggesting that the endothelial glycocalyx plays an important role in vascular permeability and the development of proteinuria [13]. This is further supported by several animal studies, where a reduced glycocalyx thickness was associated with the development of proteinuria [7, 14-16]. A recent study showed that infusion of mice with hyaluronidase, an enzyme that degrades hyaluronan, one of the constituents of the glycocalyx, reduced glycocalyx thickness and increased the passage of albumin across the endothelium [16]. Together, these studies suggest that an intact endothelial glycocalyx is essential to maintain a normal filtration barrier.

Heparan sulfate proteoglycans

Important constituents of the endothelial glycocalyx are heparan sulfate proteoglycans (HSPGs). HSPGs are negatively charged polysaccharides consisting of a core protein to which heparan sulfate (HS) side chains are covalently attached. HSPGs are located in extracellular matrices and at the cell surface of many cell types, including endothelial cells and podocytes. HSPGs play an important role in the interaction between cells and between cells and the extracellular matrix, and function as a receptor or co-receptor for the binding of

chemokines, cytokines, enzymes, growth factors or other bioactive molecules [17, 18]. The linear HS side chain consists of up to 150 repeating N-acetyl-glucosamine and glucuronic acid residues. HS can be modified extensively by several modification steps, including N-deacetylation/N-sulfation of N-acetyl-glucosamine, C-5 epimerization of glucuronic acid to iduronic acid and O-sulfation at various positions (C-2, C-3, and C-6). In addition, the structure and length of the HS chain can be modified by the HS-modifying enzymes 6-O-endosulfatase and heparanase [19-21]. By the combination of all aforementioned modifications, the structure of HS is extremely diverse, thereby enabling the specific binding of several soluble ligands, such as cytokines, chemokines and growth factors [18].

Previous studies showed that the expression of HS in the GBM is reduced in many human and experimental glomerular diseases [22-28]. By using GAG-degrading enzymes or anti-HS antibodies to reduce HS expression in the GBM, it was shown that loss of HS in the GBM is associated with the development of proteinuria [29-31]. However, the primary role of HS in charge-selective filtration was questioned when animal models lacking HS in the GBM did not display overt proteinuria, despite the fact that they lacked the majority of anionic sites in the GBM [32-35]. However, in these aforementioned studies HS expression was mainly targeted in the GBM and not in the glomerular endothelium. Therefore, an important role for HS in the endothelial glycocalyx in charge selective filtration is still highly plausible.

Heparanase

Heparanase is the only known mammalian enzyme that can cleave HS, thereby yielding small HS fragments. Heparanase is synthesized as a 68 kDa pre-proheparanase and processed into a 65 kDa inactive proheparanase in the endoplasmatic reticulum by cleaving off the signal peptide. After transport to the Golgi apparatus, proheparanase is packaged into vesicles and secreted. Once secreted, heparanase is re-internalized through binding to cell-associated HSPGs and other receptors such as low density lipoprotein receptor-related proteins and mannose-6-phosphate receptors [36]. After re-internalization, proheparanase is transported to the lysosomes, where it is processed and activated by cathepsin L (Figure 2) [37].

Heparanase is active at a pH between 5.0 and 6.0, thereby limiting its activity to acidic microenvironments such as the glycocalyx, inflammatory sites, tumor cores, and the GBM facing sites of podocytes [24]. Heparanase is not enzymatically active at neutral pH, but through its binding to HS it can serve as a proadhesive molecule [38]. Heparanase plays an important role in several physiological processes, such as HS turnover, embryo development, hair growth and wound healing. In addition, heparanase is involved in various pathological processes, such as tumor growth, angiogenesis, metastasis, inflammation and glomerular diseases [39, 40].

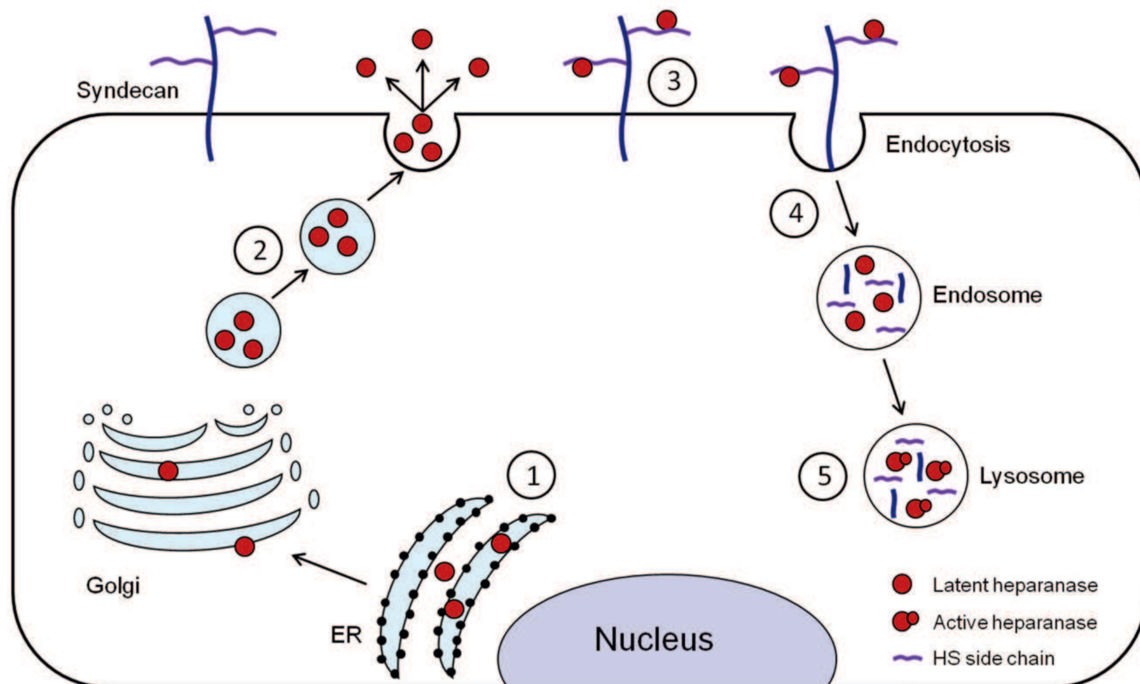

Figure 2: Schematic representation of heparanase biosynthesis and trafficking. (1) Heparanase is synthesized as a pre-proheparanase in the nucleus. Pre-proheparanase is processed into a latent proheparanase in the endoplasmatic reticulum (ER) by cleaving off the signal peptide. (2) After transport to the Golgi apparatus, proheparanase is packaged into vesicles and secreted. (3) Once secreted, proheparanase binds to cell-associated HSPGs (in particular syndecan). (4) Binding of proheparanase to HSPGs is followed by endocytosis of the proheparanase-HSPG complex. (5) Endosomes are converted to lysosomes, where proheparanase is processed into an active heparanase heterodimer by cathepsin L after cleaving out an internal linker domain. HSPGs, heparan sulfate proteoglycans.

Regulation of heparanase expression

An increased heparanase expression plays an important role in several pathological processes. Heparanase mRNA expression is positively regulated by various transcription factors, including early growth response 1 (EGR1), Ets1 and Ets2, and Sp1 [40-42]. On the other hand, heparanase expression is negatively regulated by heparanase promoter methylation and by the tumor suppressor p53 [43, 44]. A previous study showed that heparanase expression is induced by estrogen in breast cancer, thereby contributing to breast tumor growth and neovascularization [45]. Several other studies showed that heparanase expression is also induced by high glucose, angiontensin II, aldosterone, reactive oxygen species (ROS), and proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-1β [26, 46-51]. All aforementioned factors can be involved in the pathogenesis of glomerular diseases.

Heparanase expression in proteinuric diseases

Previous studies showed that the expression of heparanase is increased in several human and experimental glomerular diseases, such as diabetic nephropathy, IgA nephropathy, minimal change disease, dense deposit disease, membranous glomerulopathy and adriamycin-induced nephropathy (AN) [23-26]. This increased glomerular heparanase expression inversely correlated with a reduced glomerular HS expression [24, 25, 52]. Several studies also showed an increased heparanase activity in the serum and urine of patients with type 1 and type 2 diabetes [53-55]. Inhibition of heparanase activity by an anti-heparanase antibody or the heparanase inhibitor PI-88 reduced proteinuria in respectively rats with passive Heymann nephritis, which is a model for membraneous glomerulopathy, and rats with accelerated anti-GBM disease [27, 28, 56]. Moreover, a recent study showed that inhibition of heparanase activity by heparin reduced renal dysfunction after induction of severe systemic sepsis by cecal ligation and puncture in mice [57]. Together, these studies suggest that heparanase plays an important role in the development of proteinuria and renal damage.

Established and hypothetical factors regulating heparanase expression and activity

Cathepsin L

Cathepsin L is a lysosomal cysteine protease that plays an important role in the degradation of proteins and the activation of enzymes. The expression of cathepsin L is increased in several glomerular diseases, including diabetic nephropathy, membraneous glomerulopathy, minimal change disease and focal segmental glomerulosclerosis (FSGS) [58]. Inhibition of cathepsin L activity results in a reduction of proteinuria in rats with accelerated anti-GBM disease, suggesting that cathepsin L plays an important role in the development of proteinuria [59]. More recent studies showed that induction of cathepsin L activity in podocytes results in a migratory event in podocyte foot processes, which subsequently results in the onset of proteinuria [60]. The CD2-associated protein (CD2AP), synaptopodin and dynamin are three known substrates of cathepsin L in the podocyte, and their presence is crucial for maintaining a normal architecture of the podocyte cytoskeleton [58, 61-63]. Degradation of either of these proteins resulted in the development of foot process effacement, proteinuria and renal failure [58, 62, 64-67]. In addition to the degradation of CD2AP, synaptopodin and dynamin, the presence of cathepsin L is essential for the activation of heparanase [37], indicating that the role of cathepsin L in the development of proteinuria may also be by the activation of heparanase.

Endothelin-1

Endothelin-1 is a powerful vasoconstrictor that is released upon activation of endothelial cells. Endothelin-1 signals through two G-protein coupled receptors, the endothelin receptor type A and the endothelin receptor type B, which are both present in the kidney [68-70]. Endothelin-1 induces several intracellular signaling cascades, resulting in vascoconstriction, proliferation, inflammation, extracellular matrix production and fibrosis [71-74]. The expression of endothelin-1 is increased in several human and experimental glomerular diseases, including diabetic nephropathy, FSGS and glomerulonephritis [75-77]. Treatment with endothelin receptor antagonists reduced proteinuria and improved renal function in several human and experimental glomerular diseases [78-84], indicating that endothelin-1 plays an important role in the development of proteinuria and renal damage in several glomerular diseases. Whether heparanase is contributing to the molecular mechanisms underlying the renoprotective effect of endothelin receptor antagonists, however, remains an open question.

Endothelial nitric oxide synthase

Endothelial nitric oxide synthase (eNOS) is an enzyme present in endothelial cells that is responsible for the generation of nitric oxide (NO). Previous studies showed that the presence of NO is important to maintain a healthy endothelium and that a reduced NO production contributes to endothelial dysfunction [85, 86]. The availability of eNOS is regulated by asymmetric dimethylarginine (ADMA), ROS, angiotensin II, protein kinase C, advanced glycation end products (AGEs), TNF-α, and vitamin D [87, 88]. Several studies showed that eNOS deficiency exacerbates renal injury in experimental FSGS, accelerated anti-GBM glomerulonephritis, and diabetic nephropathy [89-94]. Moreover, eNOS gene delivery reduced proteinuria and renal failure in a rat model for FSGS [95]. Whether heparanase is contributing to the molecular mechanisms underlying the renoprotective effect of eNOS, remains to be established.

Vitamin D

Vitamin D is a steroid hormone that plays an important role in the regulation of calcium and phosphate balance. Vitamin D_3 is taken up from the diet and produced in the skin in response to ultraviolet light. Vitamin D_3 is converted to 25-hydroxyvitamin D_3 in the liver, and to the active form of vitamin D_3, 1,25-dihydroxyvitamin D_3, in the kidney. The availability of 1α-hydroxylase, the enzyme responsible for the activation of vitamin D_3 in the kidney, is reduced in patients with chronic kidney disease (CKD) as a result of the reduced renal mass [96]. As a consequence, many CKD patients become 1,25-dihydroxyvitamin D_3-deficient [97]. Several clinical studies showed that 1,25-dihydroxyvitamin D_3 supplementation reduced

proteinuria in CKD patients, in addition to standard anti-proteinuric therapy [98-104]. Moreover, proteinuria and podocyte loss were also reduced in several experimental models for FSGS [105-107]. Vitamin D may reduce proteinuria by reducing the expression of the transient receptor potential cation channel 6 (TRPC6), renin, or TNF-α [107-110]. Whether heparanase is also regulated by vitamin D remains to be elucidated.

Scope and outline of this thesis

The major aim of this thesis is to evaluate the role of heparanase in the development of proteinuria. The first part of the research focuses on the identification of the exact role of heparanase in the development of proteinuria in experimental diabetic nephropathy and glomerulonephritis. In addition, possible mechanisms involved in the regulation of glomerular heparanase expression are evaluated *in vivo* and *in vitro* in the second part of this thesis.

Heparanase expression in experimental diabetic nephropathy and glomerulonephritis

In the first part of this thesis the role of heparanase in the development of proteinuria in experimental diabetic nephropathy and glomerulonephritis is examined. The role of heparanase in experimental diabetic nephropathy is evaluated by induction of diabetes with streptozotocin in wild type and heparanase-deficient mice (**chapter 2**). Albuminuria, heparanase and HS expression, and renal histology are analyzed. Furthermore, the molecular mechanism underlying the induction of heparanase expression in DN is evaluated. Finally, the effect of heparanase inhibition on the development of albuminuria and renal damage is evaluated in experimental DN.

Cathepsin L is a lysosomal protease that can be involved in the development of proteinuria by the activation of heparanase or the degradation of the CD2-associated protein, synaptopodin and dynamin, three proteins that are important for normal podocyte architecture and function. In **chapter 3** the exact role of cathepsin L in streptozotocin-induced diabetes in wild type and cathepsin L-deficient mice is studied. Albuminuria, blood urea nitrogen levels and renal histology are measured. In addition, heparanase activity, HS expression and synaptopodin expression are determined.

Since heparanase expression is increased in other glomerular diseases in addition to DN, in **chapter 4** the role of heparanase is evaluated in two models of experimental glomerulonephritis, being anti-GBM and lipopolysaccharide (LPS)-induced glomerulonephritis, in wild type and heparanase-deficient mice. Albuminuria, blood urea nitrogen levels, renal histology, heparanase activity, glomerular HS expression and the influx of inflammatory cells are determined. In addition, the ability of tumor necrosis factor (TNF)-α and LPS to induce heparanase expression in cultured glomerular endothelial cells and podocytes is studied.

The literature on the role of heparanase and the endothelial glycocalyx in the development of proteinuria is reviewed in **chapter 5**.

Regulation of heparanase expression

In the second part of this thesis, mechanisms that could play a role in the regulation of heparanase expression are addressed. Treatment with endothelin receptor antagonists (ERAs) has a beneficial effect on proteinuria in several renal diseases. In **chapter 6** the mechanism underlying the renoprotective effect of ERAs are evaluated. The effect of endothelin-1 on heparanase expression in cultured mouse glomerular endothelial cells and mouse podocytes is determined. Moreover, the effect of endothelin-1 on transendothelial albumin passage *in vitro* is studied. Additionally, podocyte-specific endothelin receptor knockout mice are used to evaluate the effect of endothelin-1 signaling *in vivo* on glomerular heparanase and HS expression and the thickness of the glomerular endothelial and podocyte glycocalyx.

Endothelial nitric oxide synthase (eNOS) deficiency exacerbates proteinuria in several glomerular diseases, but the underlying mechanism is not completely understood. Previous studies showed that the glomerular endothelial glycocalyx is impaired in diabetic eNOS-deficient mice and that heparanase is involved in the impairment of the glycocalyx [8, 94]. In **chapter 7** the effect of eNOS deficiency on glomerular heparanase expression is studied. AN is induced in C57BL/6 wild type mice, an AN resistant strain, and C57BL/6 eNOS-deficient mice, and glomerular heparanase and HS expression are evaluated. *In vitro*, mouse glomerular endothelial cells are treated with an eNOS inhibitor and heparanase expression and transendothelial albumin passage are determined.

Another novel therapeutic agent for the treatment of proteinuria is vitamin D. Vitamin D supplementation reduces proteinuria in many experimental and human glomerular diseases. In **chapter 8** the effect of vitamin D on heparanase expression and promoter activity was studied. AN rats and 1,25-D_3-deficient 25-hydroxy-1α-hydroxylase knockout mice, which are not able to produce the active form of vitamin D, are treated daily with active vitamin D and glomerular heparanase and HS expression are determined. *In vitro*, the effects of vitamin D on heparanase expression, HS expression and transendothelial albumin passage are evaluated in mouse glomerular endothelial cells and mouse podocytes. Finally, the effect of vitamin D on heparanase promoter activity and the direct binding of the vitamin D receptor to the heparanase promoter are evaluated.

Interpretation

In **chapter 9** and **chapter 10** the findings of the studies presented in this thesis are summarized and integrated, translational aspects are outlined and possible future directions of research are proposed.

References

1. Patrakka, J. and K. Tryggvason, *Molecular make-up of the glomerular filtration barrier.* Biochem Biophys Res Commun, 2010. **396**(1): p. 164-9.
2. Tryggvason, K. and E. Pettersson, *Causes and consequences of proteinuria: the kidney filtration barrier and progressive renal failure.* J Intern Med, 2003. **254**(3): p. 216-24.
3. Matsushita, K., et al., *Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis.* Lancet, 2010. **375**(9731): p. 2073-81.
4. Carroll, M.F. and J.L. Temte, *Proteinuria in adults: a diagnostic approach.* Am Fam Physician, 2000. **62**(6): p. 1333-40.
5. Reitsma, S., et al., *The endothelial glycocalyx: composition, functions, and visualization.* Pflugers Arch, 2007. **454**(3): p. 345-59.
6. Friden, V., et al., *The glomerular endothelial cell coat is essential for glomerular filtration.* Kidney Int, 2011. **79**(12): p. 1322-30.
7. Jeansson, M., et al., *Adriamycin alters glomerular endothelium to induce proteinuria.* J Am Soc Nephrol, 2009. **20**(1): p. 114-22.
8. Singh, A., et al., *Glomerular endothelial glycocalyx constitutes a barrier to protein permeability.* J Am Soc Nephrol, 2007. **18**(11): p. 2885-93.
9. Tarbell, J.M. and M.Y. Pahakis, *Mechanotransduction and the glycocalyx.* J Intern Med, 2006. **259**(4): p. 339-50.
10. Vink, H. and B.R. Duling, *Identification of distinct luminal domains for macromolecules, erythrocytes, and leukocytes within mammalian capillaries.* Circ Res, 1996. **79**(3): p. 581-9.
11. Doyonnas, R., et al., *Anuria, omphalocele, and perinatal lethality in mice lacking the CD34-related protein podocalyxin.* J Exp Med, 2001. **194**(1): p. 13-27.
12. Dane, M.J., et al., *Association of kidney function with changes in the endothelial surface layer.* Clin J Am Soc Nephrol, 2014. **9**(4): p. 698-704.
13. Nieuwdorp, M., et al., *Endothelial glycocalyx damage coincides with microalbuminuria in type 1 diabetes.* Diabetes, 2006. **55**(4): p. 1127-32.
14. Kuwabara, A., et al., *Deterioration of glomerular endothelial surface layer induced by oxidative stress is implicated in altered permeability of macromolecules in Zucker fatty rats.* Diabetologia, 2010. **53**(9): p. 2056-65.
15. Salmon, A.H., et al., *Loss of the endothelial glycocalyx links albuminuria and vascular dysfunction.* J Am Soc Nephrol, 2012. **23**(8): p. 1339-50.
16. Dane, M.J., et al., *Glomerular endothelial surface layer acts as a barrier against albumin filtration.* Am J Pathol, 2013. **182**(5): p. 1532-40.
17. Bernfield, M., et al., *Functions of cell surface heparan sulfate proteoglycans.* Annu Rev Biochem, 1999. **68**: p. 729-77.
18. Rops, A.L., et al., *Heparan sulfate proteoglycans in glomerular inflammation.* Kidney Int, 2004. **65**(3): p. 768-85.
19. Jackson, R.L., S.J. Busch, and A.D. Cardin, *Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes.* Physiol Rev, 1991. **71**(2): p. 481-539.
20. Gallagher, J.T., *Structure-activity relationship of heparan sulphate.* Biochem Soc Trans, 1997. **25**(4): p. 1206-9.
21. Esko, J.D. and S.B. Selleck, *Order out of chaos: assembly of ligand binding sites in heparan sulfate.* Annu Rev Biochem, 2002. **71**: p. 435-71.
22. van den Born, J., et al., *Distribution of GBM heparan sulfate proteoglycan core protein and side chains in human glomerular diseases.* Kidney Int, 1993. **43**(2): p. 454-63.
23. Smith, R.J., et al., *New approaches to the treatment of dense deposit disease.* J Am Soc Nephrol, 2007. **18**(9): p. 2447-56.

24. van den Hoven, M.J., et al., *Heparanase in glomerular diseases.* Kidney Int, 2007. **72**(5): p. 543-8.
25. van den Hoven, M.J., et al., *Increased expression of heparanase in overt diabetic nephropathy.* Kidney Int, 2006. **70**(12): p. 2100-8.
26. Kramer, A., et al., *Induction of glomerular heparanase expression in rats with adriamycin nephropathy is regulated by reactive oxygen species and the renin-angiotensin system.* J Am Soc Nephrol, 2006. **17**(9): p. 2513-20.
27. Levidiotis, V., et al., *A synthetic heparanase inhibitor reduces proteinuria in passive Heymann nephritis.* J Am Soc Nephrol, 2004. **15**(11): p. 2882-92.
28. Levidiotis, V., et al., *Heparanase is involved in the pathogenesis of proteinuria as a result of glomerulonephritis.* J Am Soc Nephrol, 2004. **15**(1): p. 68-78.
29. Kanwar, Y.S., A. Linker, and M.G. Farquhar, *Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion.* J Cell Biol, 1980. **86**(2): p. 688-93.
30. Rosenzweig, L.J. and Y.S. Kanwar, *Removal of sulfated (heparan sulfate) or nonsulfated (hyaluronic acid) glycosaminoglycans results in increased permeability of the glomerular basement membrane to 125I-bovine serum albumin.* Lab Invest, 1982. **47**(2): p. 177-84.
31. van den Born, J., et al., *A monoclonal antibody against GBM heparan sulfate induces an acute selective proteinuria in rats.* Kidney Int, 1992. **41**(1): p. 115-23.
32. van den Hoven, M.J., et al., *Reduction of anionic sites in the glomerular basement membrane by heparanase does not lead to proteinuria.* Kidney Int, 2008. **73**(3): p. 278-87.
33. Harvey, S.J., et al., *Disruption of glomerular basement membrane charge through podocyte-specific mutation of agrin does not alter glomerular permselectivity.* Am J Pathol, 2007. **171**(1): p. 139-52.
34. Goldberg, S., et al., *Glomerular filtration is normal in the absence of both agrin and perlecan-heparan sulfate from the glomerular basement membrane.* Nephrol Dial Transplant, 2009. **24**(7): p. 2044-51.
35. Chen, S., et al., *Loss of heparan sulfate glycosaminoglycan assembly in podocytes does not lead to proteinuria.* Kidney Int, 2008. **74**(3): p. 289-99.
36. Vreys, V., et al., *Cellular uptake of mammalian heparanase precursor involves low density lipoprotein receptor-related proteins, mannose 6-phosphate receptors, and heparan sulfate proteoglycans.* J Biol Chem, 2005. **280**(39): p. 33141-8.
37. Abboud-Jarrous, G., et al., *Cathepsin L is responsible for processing and activation of proheparanase through multiple cleavages of a linker segment.* J Biol Chem, 2008. **283**(26): p. 18167-76.
38. Gilat, D., et al., *Molecular behavior adapts to context: heparanase functions as an extracellular matrix-degrading enzyme or as a T cell adhesion molecule, depending on the local pH.* J Exp Med, 1995. **181**(5): p. 1929-34.
39. Nasser, N.J., *Heparanase involvement in physiology and disease.* Cell Mol Life Sci, 2008. **65**(11): p. 1706-15.
40. Ilan, N., M. Elkin, and I. Vlodavsky, *Regulation, function and clinical significance of heparanase in cancer metastasis and angiogenesis.* Int J Biochem Cell Biol, 2006. **38**(12): p. 2018-39.
41. Jiang, P., et al., *Cloning and characterization of the human heparanase-1 (HPR1) gene promoter: role of GA-binding protein and Sp1 in regulating HPR1 basal promoter activity.* J Biol Chem, 2002. **277**(11): p. 8989-98.
42. de Mestre, A.M., et al., *Early growth response gene 1 (EGR1) regulates heparanase gene transcription in tumor cells.* J Biol Chem, 2005. **280**(42): p. 35136-47.
43. Ogishima, T., et al., *Increased heparanase expression is caused by promoter hypomethylation and up-regulation of transcriptional factor early growth response-1 in human prostate cancer.* Clin Cancer Res, 2005. **11**(3): p. 1028-36.
44. Baraz, L., et al., *Tumor suppressor p53 regulates heparanase gene expression.* Oncogene, 2006. **25**(28): p. 3939-47.

45. Elkin, M., et al., *Regulation of heparanase gene expression by estrogen in breast cancer.* Cancer Res, 2003. **63**(24): p. 8821-6.
46. Han, J., et al., *Heparanase upregulation in high glucose-treated endothelial cells is prevented by insulin and heparin.* Exp Biol Med (Maywood), 2007. **232**(7): p. 927-34.
47. Maxhimer, J.B., et al., *Heparanase-1 gene expression and regulation by high glucose in renal epithelial cells: a potential role in the pathogenesis of proteinuria in diabetic patients.* Diabetes, 2005. **54**(7): p. 2172-8.
48. van den Hoven, M.J., et al., *Regulation of glomerular heparanase expression by aldosterone, angiotensin II and reactive oxygen species.* Nephrol Dial Transplant, 2009. **24**(9): p. 2637-45.
49. Rops, A.L., et al., *Heparan sulfate domains on cultured activated glomerular endothelial cells mediate leukocyte trafficking.* Kidney Int, 2008. **73**(1): p. 52-62.
50. Chen, G., et al., *Inflammatory cytokines and fatty acids regulate endothelial cell heparanase expression.* Biochemistry, 2004. **43**(17): p. 4971-7.
51. Goldberg, R., et al., *Role of heparanase-driven inflammatory cascade in pathogenesis of diabetic nephropathy.* Diabetes, 2014. **63**(12): p. 4302-13.
52. Wijnhoven, T.J., et al., *Heparanase induces a differential loss of heparan sulphate domains in overt diabetic nephropathy.* Diabetologia, 2008. **51**(2): p. 372-82.
53. Shafat, I., et al., *Heparanase levels are elevated in the urine and plasma of type 2 diabetes patients and associate with blood glucose levels.* PLoS One, 2011. **6**(2): p. e17312.
54. Shafat, I., et al., *Elevated urine heparanase levels are associated with proteinuria and decreased renal allograft function.* PLoS One, 2012. **7**(9): p. e44076.
55. Rops, A.L., et al., *Urinary heparanase activity in patients with Type 1 and Type 2 diabetes.* Nephrol Dial Transplant, 2012. **27**(7): p. 2853-61.
56. Levidiotis, V., et al., *Heparanase inhibition reduces proteinuria in a model of accelerated anti-glomerular basement membrane antibody disease.* Nephrology (Carlton), 2005. **10**(2): p. 167-73.
57. Lygizos, M.I., et al., *Heparanase mediates renal dysfunction during early sepsis in mice.* Physiol Rep, 2013. **1**(6): p. e00153.
58. Sever, S., et al., *Proteolytic processing of dynamin by cytoplasmic cathepsin L is a mechanism for proteinuric kidney disease.* J Clin Invest, 2007. **117**(8): p. 2095-104.
59. Baricos, W.H., et al., *Evidence suggesting a role for cathepsin L in an experimental model of glomerulonephritis.* Arch Biochem Biophys, 1991. **288**(2): p. 468-72.
60. Reiser, J., et al., *Podocyte migration during nephrotic syndrome requires a coordinated interplay between cathepsin L and alpha3 integrin.* J Biol Chem, 2004. **279**(33): p. 34827-32.
61. Faul, C., et al., *The actin cytoskeleton of kidney podocytes is a direct target of the antiproteinuric effect of cyclosporine A.* Nat Med, 2008. **14**(9): p. 931-8.
62. Yaddanapudi, S., et al., *CD2AP in mouse and human podocytes controls a proteolytic program that regulates cytoskeletal structure and cellular survival.* J Clin Invest, 2011. **121**(10): p. 3965-80.
63. Lehtonen, S., F. Zhao, and E. Lehtonen, *CD2-associated protein directly interacts with the actin cytoskeleton.* Am J Physiol Renal Physiol, 2002. **283**(4): p. F734-43.
64. Kim, J.M., et al., *CD2-associated protein haploinsufficiency is linked to glomerular disease susceptibility.* Science, 2003. **300**(5623): p. 1298-300.
65. Huber, T.B., et al., *Bigenic mouse models of focal segmental glomerulosclerosis involving pairwise interaction of CD2AP, Fyn, and synaptopodin.* J Clin Invest, 2006. **116**(5): p. 1337-45.
66. Mundel, P. and J. Reiser, *Proteinuria: an enzymatic disease of the podocyte?* Kidney Int, 2010. **77**(7): p. 571-80.
67. Reiser, J., B. Adair, and T. Reinheckel, *Specialized roles for cysteine cathepsins in health and disease.* J Clin Invest, 2010. **120**(10): p. 3421-31.
68. Lehrke, I., et al., *Renal endothelin-1 and endothelin receptor type B expression in glomerular diseases with proteinuria.* J Am Soc Nephrol, 2001. **12**(11): p. 2321-9.

69. Karet, F.E., R.E. Kuc, and A.P. Davenport, *Novel ligands BQ123 and BQ3020 characterize endothelin receptor subtypes ETA and ETB in human kidney.* Kidney Int, 1993. **44**(1): p. 36-42.
70. Wendel, M., et al., *Distribution of endothelin receptor subtypes ETA and ETB in the rat kidney.* J Histochem Cytochem, 2006. **54**(11): p. 1193-203.
71. Barton, M. and M. Yanagisawa, *Endothelin: 20 years from discovery to therapy.* Can J Physiol Pharmacol, 2008. **86**(8): p. 485-98.
72. Clozel, M. and H. Salloukh, *Role of endothelin in fibrosis and anti-fibrotic potential of bosentan.* Ann Med, 2005. **37**(1): p. 2-12.
73. Shi-Wen, X., et al., *Fibroblast matrix gene expression and connective tissue remodeling: role of endothelin-1.* J Invest Dermatol, 2001. **116**(3): p. 417-25.
74. Neuhofer, W. and D. Pittrow, *Role of endothelin and endothelin receptor antagonists in renal disease.* Eur J Clin Invest, 2006. **36 Suppl 3**: p. 78-88.
75. Fukui, M., et al., *Gene expression for endothelins and their receptors in glomeruli of diabetic rats.* J Lab Clin Med, 1993. **122**(2): p. 149-56.
76. Fligny, C., M. Barton, and P.L. Tharaux, *Endothelin and podocyte injury in chronic kidney disease.* Contrib Nephrol, 2011. **172**: p. 120-38.
77. Yoshimura, A., et al., *Endothelin-1 and endothelin B type receptor are induced in mesangial proliferative nephritis in the rat.* Kidney Int, 1995. **48**(4): p. 1290-7.
78. Barton, M., *Reversal of proteinuric renal disease and the emerging role of endothelin.* Nat Clin Pract Nephrol, 2008. **4**(9): p. 490-501.
79. Mann, J.F., et al., *Avosentan for overt diabetic nephropathy.* J Am Soc Nephrol, 2010. **21**(3): p. 527-35.
80. Schievink, B., et al., *Prediction of the effect of atrasentan on renal and heart failure outcomes based on short-term changes in multiple risk markers.* Eur J Prev Cardiol, 2015.
81. Gagliardini, E., C. Zoja, and A. Benigni, *Et and diabetic nephropathy: preclinical and clinical studies.* Semin Nephrol, 2015. **35**(2): p. 188-96.
82. Kohan, D.E. and M. Barton, *Endothelin and endothelin antagonists in chronic kidney disease.* Kidney Int, 2014. **86**(5): p. 896-904.
83. Ortmann, J., et al., *Role of podocytes for reversal of glomerulosclerosis and proteinuria in the aging kidney after endothelin inhibition.* Hypertension, 2004. **44**(6): p. 974-81.
84. Gomez-Garre, D., et al., *An orally active ETA/ETB receptor antagonist ameliorates proteinuria and glomerular lesions in rats with proliferative nephritis.* Kidney Int, 1996. **50**(3): p. 962-72.
85. Tousoulis, D., et al., *The role of nitric oxide on endothelial function.* Curr Vasc Pharmacol, 2012. **10**(1): p. 4-18.
86. Davignon, J. and P. Ganz, *Role of endothelial dysfunction in atherosclerosis.* Circulation, 2004. **109**(23 Suppl 1): p. III27-32.
87. Takahashi, T. and R.C. Harris, *Role of endothelial nitric oxide synthase in diabetic nephropathy: lessons from diabetic eNOS knockout mice.* J Diabetes Res, 2014. **2014**: p. 590541.
88. Andrukhova, O., et al., *Vitamin D is a regulator of endothelial nitric oxide synthase and arterial stiffness in mice.* Mol Endocrinol, 2014. **28**(1): p. 53-64.
89. Nakayama, T., et al., *Endothelial injury due to eNOS deficiency accelerates the progression of chronic renal disease in the mouse.* Am J Physiol Renal Physiol, 2009. **296**(2): p. F317-27.
90. Sun, Y.B., et al., *Glomerular endothelial cell injury and damage precedes that of podocytes in adriamycin-induced nephropathy.* PLoS One, 2013. **8**(1): p. e55027.
91. Heeringa, P., et al., *Lack of endothelial nitric oxide synthase aggravates murine accelerated anti-glomerular basement membrane glomerulonephritis.* Am J Pathol, 2000. **156**(3): p. 879-88.
92. Zhao, H.J., et al., *Endothelial nitric oxide synthase deficiency produces accelerated nephropathy in diabetic mice.* J Am Soc Nephrol, 2006. **17**(10): p. 2664-9.

93. Nakagawa, T., et al., *Diabetic endothelial nitric oxide synthase knockout mice develop advanced diabetic nephropathy.* J Am Soc Nephrol, 2007. **18**(2): p. 539-50.
94. Kanetsuna, Y., et al., *Deficiency of endothelial nitric-oxide synthase confers susceptibility to diabetic nephropathy in nephropathy-resistant inbred mice.* Am J Pathol, 2007. **170**(5): p. 1473-84.
95. Savard, S., et al., *eNOS gene delivery prevents hypertension and reduces renal failure and injury in rats with reduced renal mass.* Nephrol Dial Transplant, 2012. **27**(6): p. 2182-90.
96. Mawer, E.B., et al., *Failure of formation of 1,25-dihydroxycholecalciferol in chronic renal insufficiency.* Lancet, 1973. **1**(7804): p. 626-8.
97. Levin, A., et al., *Prevalence of abnormal serum vitamin D, PTH, calcium, and phosphorus in patients with chronic kidney disease: results of the study to evaluate early kidney disease.* Kidney Int, 2007. **71**(1): p. 31-8.
98. de Borst, M.H., et al., *Active vitamin D treatment for reduction of residual proteinuria: a systematic review.* J Am Soc Nephrol, 2013. **24**(11): p. 1863-71.
99. Agarwal, R., et al., *Antiproteinuric effect of oral paricalcitol in chronic kidney disease.* Kidney Int, 2005. **68**(6): p. 2823-8.
100. Krairittichai, U., R. Mahannopkul, and S. Bunnag, *An open label, randomized controlled study of oral calcitriol for the treatment of proteinuria in patients with diabetic kidney disease.* J Med Assoc Thai, 2012. **95 Suppl 3**: p. S41-7.
101. de Zeeuw, D., et al., *Selective vitamin D receptor activation with paricalcitol for reduction of albuminuria in patients with type 2 diabetes (VITAL study): a randomised controlled trial.* Lancet, 2010. **376**(9752): p. 1543-51.
102. Fishbane, S., et al., *Oral paricalcitol in the treatment of patients with CKD and proteinuria: a randomized trial.* Am J Kidney Dis, 2009. **54**(4): p. 647-52.
103. Liu, L.J., et al., *Oral calcitriol for reduction of proteinuria in patients with IgA nephropathy: a randomized controlled trial.* Am J Kidney Dis, 2012. **59**(1): p. 67-74.
104. Thadhani, R., et al., *Vitamin D therapy and cardiac structure and function in patients with chronic kidney disease: the PRIMO randomized controlled trial.* JAMA, 2012. **307**(7): p. 674-84.
105. Kuhlmann, A., et al., *1,25-Dihydroxyvitamin D3 decreases podocyte loss and podocyte hypertrophy in the subtotally nephrectomized rat.* Am J Physiol Renal Physiol, 2004. **286**(3): p. F526-33.
106. Zou, M.S., et al., *1, 25-dihydroxyvitamin D3 decreases adriamycin-induced podocyte apoptosis and loss.* Int J Med Sci, 2010. **7**(5): p. 290-9.
107. Sonneveld, R., et al., *Vitamin D down-regulates TRPC6 expression in podocyte injury and proteinuric glomerular disease.* Am J Pathol, 2013. **182**(4): p. 1196-204.
108. Zhou, C., et al., *Calcium-independent and 1,25(OH)2D3-dependent regulation of the renin-angiotensin system in 1alpha-hydroxylase knockout mice.* Kidney Int, 2008. **74**(2): p. 170-9.
109. Kuo, Y.T., et al., *Effects of vitamin D3 on expression of tumor necrosis factor-alpha and chemokines by monocytes.* J Food Sci, 2010. **75**(6): p. H200-4.
110. Tan, X., X. Wen, and Y. Liu, *Paricalcitol inhibits renal inflammation by promoting vitamin D receptor-mediated sequestration of NF-kappaB signaling.* J Am Soc Nephrol, 2008. **19**(9): p. 1741-52.

Chapter 2

Heparanase is essential for the development of diabetic nephropathy in mice

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Abstract

Diabetic nephropathy (DN) is the major life-threatening complication of diabetes. Abnormal permselectivity of the glomerular basement membrane (GBM) plays an important role in DN pathogenesis. Heparanase is the predominant enzyme that degrades heparan sulfate (HS), the main polysaccharide of the GBM. Loss of GBM HS in diabetic kidney was associated with increased glomerular expression of heparanase. However, the causal involvement of heparanase in the pathogenesis of DN has not been demonstrated. We report for the first time the essential involvement of heparanase in DN. With the use of heparanase knockout mice, we found that deletion of the heparanase gene protects diabetic mice from DN. Furthermore, by investigating the molecular mechanism underlying induction of the enzyme in DN, we found that transcription factor early growth response 1 (Egr1) is responsible for activation of heparanase promoter under diabetic conditions. The specific heparanase inhibitor SST0001 markedly decreased the extent of albuminuria and renal damage in mouse models of DN. Our results collectively underscore the crucial role of heparanase in the pathogenesis of DN and its potential as a highly relevant target for therapeutic interventions in patients with DN.

Introduction

The kidneys represent primary targets of diabetes, and diabetic nephropathy (DN) is the leading cause of end stage renal disease in the western world [1-3]. DN is characterized by glomerular hyperfiltration, increased renal albumin permeability, cellular and extracellular changes in the glomerular and tubulo-interstitial compartments, collectively resulting in progression of proteinuria and renal failure. The puzzle of mechanisms underlying DN pathogenesis involves complex interplay between haemodynamic and metabolic factors (i.e., systemic and intraglomerular pressure, activation of vasoactive hormone pathways, induction of inflammatory and prosclerotic cytokines) and is still far from being fully understood. Several studies suggested involvement of heparanase in DN [4, 5]. Heparanase is the only known mammalian endoglycosidase that cleaves heparan sulfate (HS) [6-8] the principle polysaccharide associated with the cell surface and extracellular matrix (ECM) of a wide range of tissues [9]. HS binds to and assembles structural basement membrane (BM) proteins, thus contributing to BM integrity and barrier function. In addition, HS moieties in the ECM sequester heparin-binding growth factors, cytokines, and chemokines, thereby controlling their accessibility, function and mode of action [6, 9]. Enzymatic degradation of HS by heparanase leads to disassembly of ECM barriers and release of HS-bound bioactive molecules [10, 11], and is therefore involved in fundamental biological phenomena associated with tissue remodeling, including morphogenesis, inflammation, angiogenesis and cancer [6, 7, 12]. The findings linking heparanase to DN and other proteinuric disorders include elevated levels of heparanase in the kidneys and urine of DN patients [13, 14], induction of glomerular heparanase expression in murine models of streptozotocin (STZ)-induced diabetes [14] and passive Heymann nephritis [15], as well as *in vitro* studies demonstrating that hyperglycemic conditions enhance heparanase expression in rat and human glomerular epithelial cells [16]. Induction of glomerular heparanase in the course of diabetes may interfere with kidney function primarily through degradation of HS in the glomerular basement membrane (GBM). Indeed, GBM, along with fenestrated glomerular endothelium and podocyte foot processes/slit diaphragms, serves as the key functional component of the kidney filtration barrier, while HS represents a chief polysaccharide constituent of the GBM [17, 18], playing a key space-filling and molecular-sieving role in the GBM. Degradation/loss of HS in the GBM was tightly linked to the pathophysiology of DN (reviewed in [19]), yet, several recent reports challenged the importance of HS degradation in DN development [20, 21] and causative involvement of heparanase in DN has not been demonstrated.

Our research was undertaken to elucidate the biological significance and regulation of heparanase in DN pathogenesis. Applying heparanase null (*Hpse-KO*) mouse model [22] we

demonstrated that unlike their *wt* littermates, *Hpse-KO* mice fail to develop albuminuria and renal damage in response to STZ-induced diabetes. Investigating the precise molecular mechanism underlying heparanase induction under hyperglycemic conditions, we revealed that Early Growth Response 1 (Egr1) transcription factor critically regulates heparanase overexpression. To block excessive heparanase, brought about in hyperglycemic mice by Egr1, we used a specific heparanase inhibitor SST0001 (non-anticoagulant N-acetylated, glycol split heparin = ^{100}NA,RO-H) [23, 24]. Administration of SST0001 resulted in a marked decrease in the extent of albuminuria and renal damage in diabetic mice. Taken together, our results validate the role of heparanase in the pathogenesis of DN, reveal the molecular mechanism underlying induction of the enzyme in diabetic kidney and attest the enzyme as a promising therapeutic target in DN patients.

Materials and methods

Animals

BALB/c, DBA-2, C57Bl/6J (Harlan Laboratories; Jerusalem, Israel) and heparanase-null *Hpse*-KO mice [22] were kept under pathogen-free conditions; all experiments were performed in accordance with the Hebrew University IACUC.

STZ-induced diabetes

Mice were injected intraperitoneally with 40 mg/kg body weight STZ in 100 mM citrate buffer (pH 4.6) for five consecutive days (following an overnight fast). Mice received 0.4 IU of insulin every other day when their blood glucose levels rose above 350 mg/dl. Blood glucose levels were measured using Ascensia ELITE Blood Glucose Meter (Bayer, Germany). Subcutaneous injections of compound SST0001 (300 µg in 100 µl saline, twice a day) were given to mice in the experimental group. Mice in the control group were injected with saline alone. For urine collection mice were placed in metabolic cages for 24 hours. Urinary albumin was measured using ELISA kit (Bethyl laboratories Inc, Montgomery, TX). Creatinine was measured using VITROS system 5.1, FS chemistry (Johnson & Johnson). Animals were sacrificed on indicated time points, half of each kidney was snap-frozen for RNA preparation/protein extraction and another half processed for histology and immunostaining.

Renal histopathology

Kidney tissue was immersion-fixed in 4% paraformaldehyde in PBS, routinely processed, and 2-4 µm sections were stained with Masson's trichrome and periodic acid-Schiff (PAS). A

semiquantitative score was used to evaluate the extent of glomerular mesangial expansion essentially as described in [25]. Briefly, mesangial matrix expansion for each glomerulus was graded from 1 to 4 as follows: 1 represents no lesion, 2 sclerosis of >25% of the glomerulus, while 3 and 4 represent sclerosis of > 50% and >75% of the glomerulus, respectively. A whole kidney sclerosis index was obtained by averaging scores from all glomeruli on one section. Four mice per experimental condition were analyzed and >50 glomeruli were assessed per mouse. Immunohistochemistry of the paraffin-embedded and cryostat kidney sections was performed as previously described [26, 27].

Antibodies

The following antibodies were used: anti-F4/80 (Serotec); anti-TGFβ (s.c.146); anti-Egr1 (s.c-588, Santa Cruz Biotechnology, Santa Cruz, CA); anti-HS HS4C3 [27]; anti-heparanase #733 [28] and #01385-126 (kindly provided by Dr. P. Kussie; ImClone Systems Inc., New York, NY) [29].

Cell culture

Human embryonic kidney cells 293 (HEK-293) were cultured at 37°C in a 5% CO_2 humidified incubator in complete DMEM medium with 10% FCS. HK-160 primary human epithelial kidney cells (kindly provided by Dr. A. Katz, Bikur Holim Hospital, Jerusalem, Israel) were cultured at 37°C in a 8% CO_2 humidified incubator in complete RPMI medium with 20% FCS. In some experiments, cells at 60-80% confluence were maintained for 24 hours in serum-free medium and then incubated in absence or presence of increasing concentrations of glucose, as indicated.

Reverse transcription and real time PCR

RNA isolation, reverse transcription and real-time quantitative PCR were performed as previously described [26]. Mouse RNA polymerase IIA and L-19, and human Gus-β primers were used as internal standards. Primer sequences are shown in Table 1.

Immunoblotting

Equal protein aliquots (20 µg) were subjected to SDS-PAGE (10% acrylamide) under reducing conditions. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore Corporation) and probed with the anti-Egr1 rabbit polyclonal antibody s.c-588 (Santa Cruse) (1:200), followed by horseradish peroxidase-conjugated secondary antibody (KPL) and a chemiluminescent substrate (iNtron Biotechnology), as described [26]. Membrane was stripped and incubated with anti-β-actin (1:1000) or anti-lamin (1:1000) antibodies to ensure equal protein load.

Table 1: Primers used in real-time PCR.

Target gene	Primer sequence
mEgr1	(F) 5'-CCTTTTCTGACATCGCTCTGAA-3'
	(R) 5'-CGAGTCGTTTGGCTGGGATA-3'
mHPSE	(F) 5'-GGAGCAAACTCCGAGTGTATC-3'
	(R) 5'-CAGAATTTGACCGTTCAGTTGG-3'
mHPSE	(F) 5'-CAAGAAGGAATCAACCTTTGAAG-3'
	(R) 5'-GTAGTCCAGGAGCAACTGAG-3'
mPolRIIA	(F) 5'-GTCCTCTACTCATGCTGTCTTGG-3'
	(R) 5'-AAATGCCTGTATCCCAATCAAG-3'
mL-19	(F) 5'-GAATGGCTCAACAGGTAAACA-3'
	(R) 5'-GGGTTCCAGAGTCAAGTTCAG-3'
hEgr	(F) 5'-GAGCAGCCCTACGAGC-3'
	(R) 5'-AGCGGCCAGTATAGGT-3'
hHPSE	(F) 5'-GTTCTAATGCTCAGTTGCTCCT-3'
	(R) 5'-ACTGCGACCCATTGATGAAA-3'
hGUS-β	(F) 5'-CACAAGAGTGGTGCTGAGGA-3'
	(R) 5'-GTATTGGATGGTCCCTGGTG-3'

Heparanase activity assay

For measurements of heparanase enzymatic activity, tissue lysates were incubated (16-36 hours, 37°C, pH 6.2) on dishes coated with sulfate-labeled ECM, and analyzed as described [8]. Nearly intact heparan sulfate proteoglycans are eluted just after the void volume (peak I, $K_{av} < 0.2$, fractions 1-10) and HS degradation fragments are eluted later with $0.5 < K_{av} < 0.8$ (peak II, fractions 15-35) (8). These fragments were shown to be degradation products of HS as they were 5-6 fold smaller than intact HS side chains, resistant to further digestion with papain and chondroitinase ABC, and susceptible to deamination by nitrous acid.

Chromatin immunoprecipitation (ChIP)

Cross-linking of proteins to DNA, chromatin isolation and sonication was performed as described [30]. ChIP was performed with 5 µg of anti-Egr1 (s.c H-250) or anti-LAMP1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) preincubated with magnetic bead-conjugated rabbit IgG (Dynal Biotech ASA, Norway) at 4°C, overnight with rotation. Elution of immune complexes, reverse cross-linking and DNA extraction were carried out as previously described [30]. PCR analysis was performed using 5 µl of immunoprecipitated chromatin or input chromatin, using Titanium *Taq* PCR kit (Clontech, Mountain View, CA). Amplifications (38 cycles) were performed using specific primer set that covers functional Egr1 binding site in the heparanase promoter [31]. The following primers were used: Hpse promoter forward 5'-TTCGTAAGTGAACGTCACCG-3', reverse 5'-CTTCTGCATCCCTCCCACT-3';
L-19 forward 5'-ATGCCAACTCTCGTCAACAG-3', reverse 5'-GCGCTTTCGTGCTTCCTT-3'.

Statistical analysis

Values are expressed as mean ± SEM. Statistical analysis was performed using t-test or Mann-Whitney test. A p value of < 0.05 was considered significant.

Results

Heparanase KO mice fail to develop DN in response to streptozotocin induced diabetes

Although several findings link heparanase to DN (reviewed in [5]), direct mechanistic demonstration of the role of the enzyme in the pathogenesis of this disease was not available. Here, to provide decisive evidence that heparanase is causally involved in the pathogenesis of DN, we utilized the model of STZ-induced DN [32] in heparanase null *(Hpse-KO)* mice and their *wt* littermates. The *Hpse-KO* mice are fertile, viable and show normal gross appearance [22], including kidney size and renal histology. Blood glucose, serum creatinine, urea nitrogen (not shown) and urinary albumin excretion (Figure 1C) measured in healthy *Hpse-KO* mice were not different from those in *wt* littermates. *Hpse-KO* and *wt* mice were made hyperglycemic applying multiple low dose STZ administration (40 mg/kg/day for 5 days), to mitigate the nonspecific renal toxicity of STZ [32]. By week 2 of the experiment, 100% of the *wt* and KO mice developed diabetes as revealed by increased blood glucose levels (>300 mg/dL). Blood glucose levels were maintained at <350 mg/dL by treatment with insulin (0.4U/mouse), administered every other day. Mice were kept diabetic for 16 weeks and their urine samples were collected for analysis of albumin excretion. It should be noted that the body weight and blood glucose concentrations measured throughout the experiment (Table 2), as well as the amount of insulin required to maintain glycemic control, did not differ between *Hpse-KO* and *wt* mice.

Table 2: Average blood glucose levels and body weight of *Hpse-KO* and *wt* mice on week 16 of the experiment.

	Hpse-KO mice		**wt mice**	
	Non-diabetic	**Diabetic**	**Non-diabetic**	**Diabetic**
Glucose (mg/dl)	96.09 ± 3.51	305.89 ± 19.09	93.57 ± 3.51	312.93 ± 17.24
Mouse weight (g)	28 ± 1.1	29.8 ± 1.5	28 ± 1.74	28.4 ± 1.8

Following 16 weeks of the experiment, mice were sacrificed, their kidneys excised and heparanase expression in the renal cortex was assessed by quantitative real-time PCR (qRT-PCR). A marked increase in heparanase mRNA was readily detected in the renal

cortex of diabetic *wt* mice, as compared to non-diabetic *wt* mice (p = 0.0493, Figure 1A). As expected, no heparanase mRNA was detected in the kidneys of either diabetic or non-diabetic *Hpse-KO* mice (Figure 1A). In agreement with the increased expression of heparanase, immunofluorescence staining with anti-HS antibody HS4C3 [27] revealed statistically significant decrease in HS content along the GBM in the kidneys of diabetic vs. non diabetic *wt* mice, whereas no change in HS content was detected in the kidneys of diabetic *Hpse-KO* mice, as compared to their non-diabetic littermates (Figure 1B). Examination of albumin excretion in urine samples revealed a 5-fold increase in urine albumin excretion (quantified as µg albumin/mg creatinine) in diabetic *wt* mice vs. their non-diabetic controls, while no increase in urine albumin excretion was noted in diabetic *Hpse-KO* mice compared to non-diabetic *Hpse-KO* mice (Figure 1C). The occurrence of albuminuria was paralleled by glomerular morphological changes characteristic of DN (i.e., mesangial matrix expansion) in diabetic *wt* kidney tissue, exemplified by >2 fold increase in glomerular expansion score, as compared to normal glomerular architecture preserved in kidney tissue derived from diabetic *Hpse-KO* mice (Figure 2A-B). Along with mesangial matrix expansion in glomeruli, tubulointerstitial injury is considered as a major feature of DN and an important predictor of renal dysfunction [33]. During progression of DN, the renal tubule is exposed to glomerular effluent, which includes, in addition to glycation end products and glucose, large quantities of protein. Excessive protein load to the proximal tubule ultimately leads to peritubular inflammation and fibrosis [33]. In agreement with this notion, massive interstitial fibrosis (as indicated by increased collagen deposition) was detected in the kidney of diabetic *wt* but not in diabetic *Hpse-KO* mice, by Masson's trichrome staining (Figure 2C). Transforming growth factor-β (TGF-β) is implicated in cellular processes characteristic of DN, including glomerular hypertrophy with mesangial matrix expansion, renal tubular injury and interstitial fibrosis [34-36]. Immunohistochemical evaluation of TGF-β in kidney tissue specimens revealed a marked increase in TGF-β in diabetic *wt* mice vs. their non-diabetic controls (Figure 2D, left panels). No increase in TGF-β levels was noted in diabetic vs. non-diabetic *Hpse-KO* mice (Figure 2D, right panels). In agreement, 2-fold increase in TGF-β mRNA expression was detected by qRT-PCR in the kidney of diabetic vs. non-diabetic *wt* mice, while no induction of TGF-β mRNA was noted in kidneys of diabetic *Hpse-KO* mice as compared to their non-diabetic littermates (not shown). Macrophage accumulation is another characteristic feature of diabetic kidney disease, associated with both experimental and human DN [37-39]. Moreover, the intensity of the interstitial macrophage infiltrate is proportional to the rate of subsequent decline in renal function [39]. This notion led us to examine the degree of macrophage infiltration in *wt* and *Hpse-KO* diabetic kidney. Applying immunostaining with antibody directed against F4/80 (mouse macrophage specific marker [40]), we detected increased macrophage infiltration in the diabetic kidney of *wt* vs. *Hpse-KO*

mice (Figure 2E). Quantification of F4/80-positive macrophages per microscopic field, based on six sections from 3 independent mice of each group, revealed a 6-fold increase in macrophage accumulation in the cortex of diabetic vs. non-diabetic kidney in *wt* mice (95.3 ±15.8 vs. 15.9 ±10.5 macrophages/field, p=0.001), while no statistically significant difference in macrophage infiltration was noted in the kidneys of diabetic vs. non-diabetic *Hpse-KO* mice (63.9 ±27.7 vs. 40.9 ±10.3 macrophages/field, p>0.2).

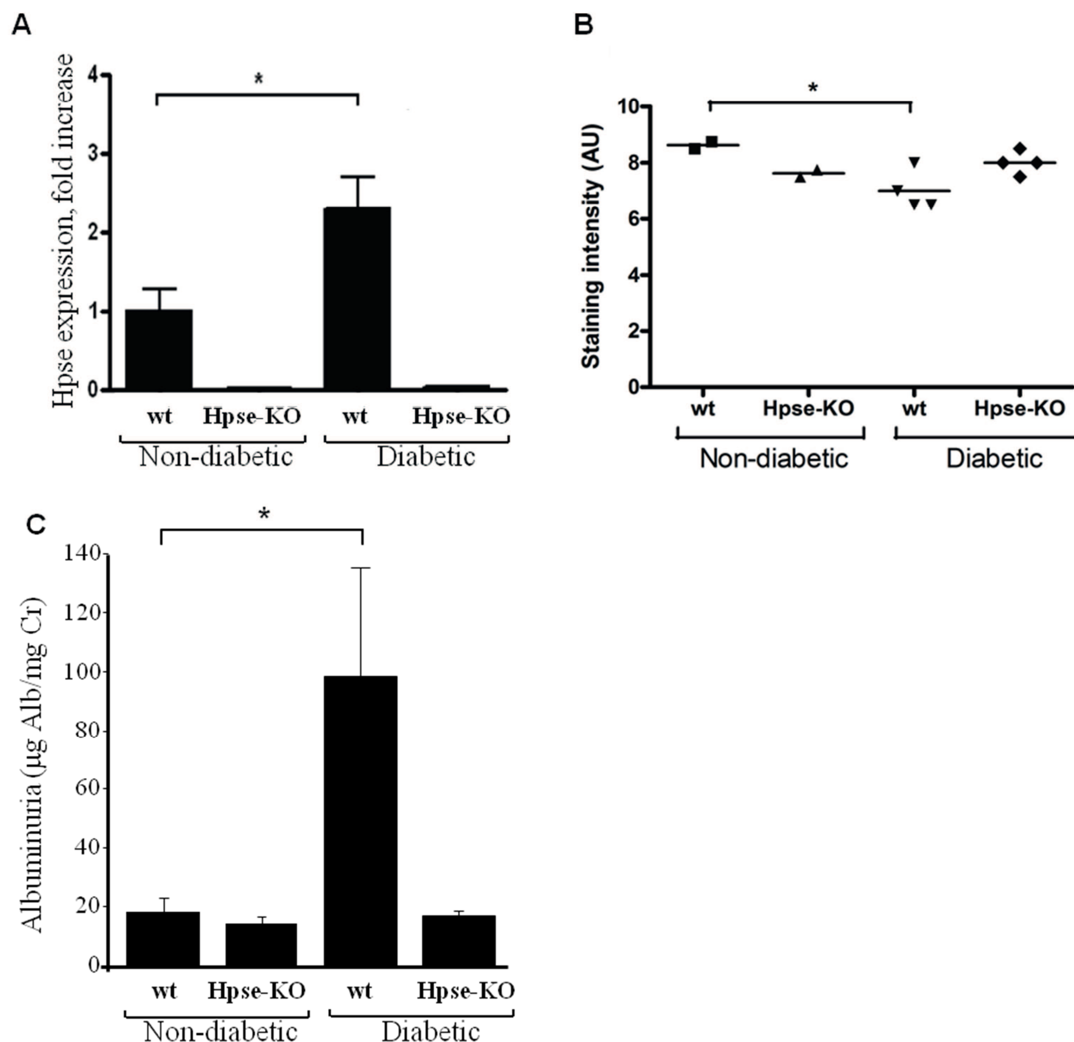

Figure 1: Changes in heparanase expression, HS content and urinary albumin excretion in *wt* and *Hpse-KO* mice in response to STZ induced diabetes. Kidney tissue samples were harvested from diabetic *wt* and *Hpse-KO* mice at 16 weeks after induction of diabetes and from their age-matched non-diabetic littermates. (**A**) Heparanase expression in non-diabetic and diabetic kidney lysates was assessed by qRT-PCR (n=5 per experimental condition). (**B**) Semi-quantitative analysis of the glomerular HS recognized by antibody HS4C3. Staining intensity of HS was scored by three independent observers on a scale between 0 and 10 in arbitrary units (a.u.) expressed as means ± SEM (2 non-diabetic and 4 diabetic mice per genotype). (**C**) Urinary albumin excretion. 24 hour urine samples were collected from diabetic *wt* and *Hpse-KO* mice 16 weeks after induction of diabetes and from age-matched non-diabetic littermates. The experiment was repeated 3 times, n ≥ 5 per experimental condition. Albuminuria is expressed as µg albumin/mg creatinine. Urinary albumin (Alb) and creatinine (Cr) were determined as described in 'Methods'. *p< 0.05.

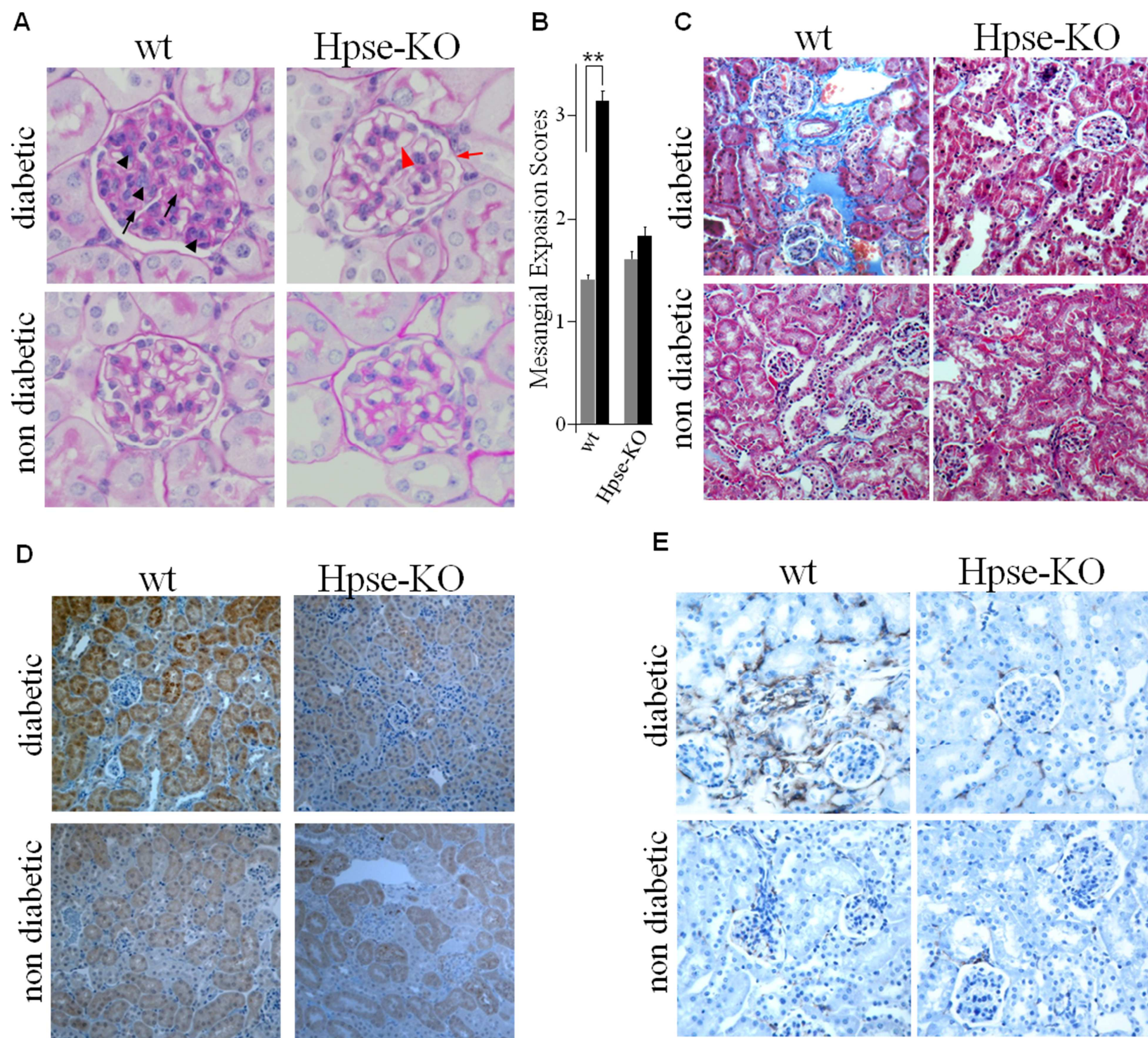

Figure 2: Histopathological changes in the kidneys of *wt* and *Hpse-KO* mice in response to STZ induced diabetes. Kidney sections obtained from diabetic *wt* and *Hpse-KO* mice at 16 weeks after induction of diabetes and from age-matched non-diabetic littermates were processed for staining with periodic acid-Schiff (**A**), Masson-Trichrome (**C**) and for immunohistochemistry with antibodies directed against TGF-β1(**D**) and F4/80 (**E**). Representative microphotographs are shown, n ≥ 4 mice per experimental condition. (**A**,**B**) Glomerular mesangial expansion. (**A**) Representative glomerular histopathology. Note mesangial matrix expansion (black arrow), focal hypercellurarity (black arrowhead) and absence of visible clear vascular space in diabetic *wt* kidney (top, left), as compared to diabetic *Hpse-KO* kidney (top, right) displaying normal-looking glomerular structure, clear Bowman's capsule (red arrow), clear vascular space (red arrowhead) and normal tuft architecture, resembling that of non-diabetic kidney (bottom). Original magnification X400. (**B**) Glomerular mesangial expansion scores in diabetic (black bars) *wt* and *Hpse-KO* mice and the age-matched non-diabetic littermates (grey bars). The scores were determined using light microscopy at x400, as described in 'Methods'. **p< 0.001. (**C**) Representative Masson's trichrome-stained kidney sections. Note higher degree of tubulointerstitial fibrosis (blue staining) in diabetic kidneys derived from *wt* vs. *Hpa-KO* mice. (**D**,**E**) Immunohistochemistry with antibodies directed against TGF-β1 (D) and F4/80 (**E**), reveals induction of TGF-β1 (**D**) and increased accumulation of macrophages (**E**) in diabetic *wt* kidney (top, left), as compared to both diabetic *Hpse-KO* (top, right) and *wt* non-diabetic (bottom, left) kidney.

Induction of heparanase in diabetic kidney is mediated by Egr-1 transcription factor

Causal involvement of heparanase in DN, along with the increased levels of heparanase observed in diabetic kidney tissue specimens [14] and in the urine and plasma of diabetic patients [13] prompted us to investigate how this enzyme is regulated under diabetic conditions. Highly relevant to DN setting, heparanase expression is induced in renal epithelial cells by high glucose [16]. However, the precise mechanism responsible for induction of heparanase by high glucose has not been fully elucidated. Among several factors controlling heparanase expression, the Early Growth Response 1 (Egr1) transcription factor is implicated in inducible transcription of the heparanase gene in immune cells and many (but not all) cancer cell types [31, 41, 42]. In light of the previously reported inducibility of Egr1 by glucose [43], it is logical to assume that when glomerular cells are exposed to high glucose levels (i.e., in the kidneys of diabetic patients), glucose-induced Egr1 may affect heparanase gene promoter activity, leading to over-expression of heparanase. To test this hypothesis, we first investigated the effect of glucose on Egr1 levels in kidney-derived cells. Human embryonic kidney (HEK-293) cells were exposed to increasing concentrations of glucose. Western blot analysis and qRT- PCR revealed a dose dependent increase in Egr1 expression following exposure to glucose (Figure 3A). Similar results were obtained with HK-160 human kidney epithelial cells (not shown). In agreement, a significant increase in Egr1 mRNA and protein levels was revealed in mouse kidneys of diabetic C57BL/6 mice 10 weeks after induction of diabetes by STZ, as compared to healthy mice (Figure 3C and not shown), corroborating the relevance of the *in vitro* findings. This increase in Egr1 levels was invariably associated with induction of heparanase expression both *in vitro* (Figure 3B) and in the kidney tissue of diabetic mice, as demonstrated by real-time PCR, immunohistochemistry, immunoblotting and enzymatic activity assay (Figure 3D).

These observations are in further support of our assumption that similar to immune and some cancer cells, in kidney tissue Egr1 acts as an activator of heparanase transcription. To further validate this hypothesis we applied the ChIP approach to study the effect of elevated levels of glucose on occupancy of the heparanase promoter by Egr1 in HEK-293 cells. For this purpose, HEK 293 cells were incubated in the presence of increasing concentrations of glucose (0; 1; and 4.5 g/l). Following chemical cross-linking, chromatin was isolated, sonicated to ~500-bp fragments and immunoprecipitated with anti-Egr1 antibody. DNA obtained from the immunoprecipitated chromatin was amplified using heparanase promoter-specific primers, as well as a set of primers specific to the unrelated L-19 gene sequence, to normalize for equal chromatin amounts. As shown in figure 3E (top panels), markedly increased occupancy of the heparanase gene promoter by Egr1 was detected in cells that were incubated with high glucose. No enrichment was observed when antibody directed

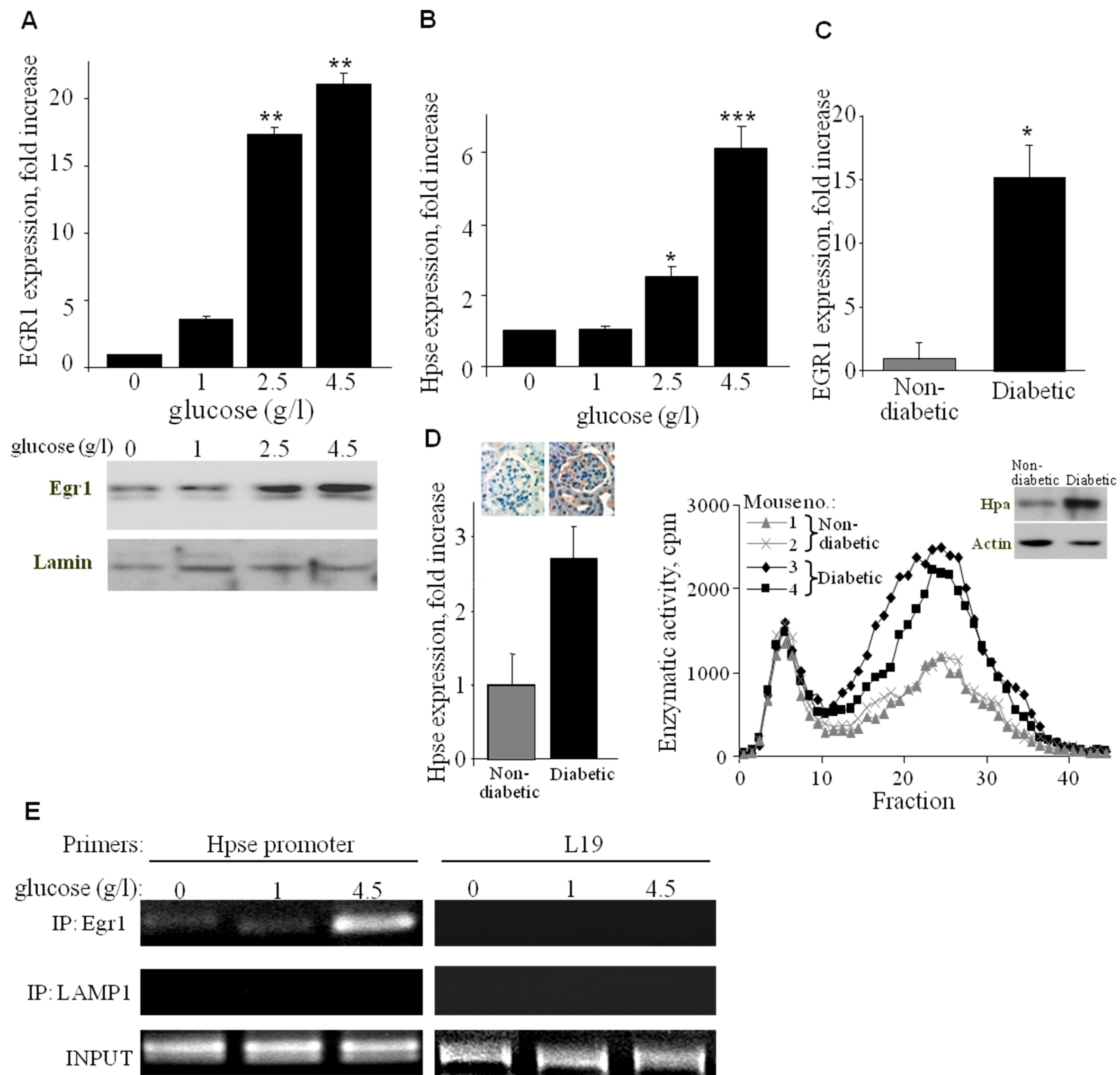

Figure 3: Egr1 mediates glucose-induced over-expression of heparanase in the kidney. (**A**,**B**) Effects of glucose on Egr1 and heparanase expression *in vitro*. HEK 293 cells were incubated (1.5 hours) in triplicates in the absence or presence of increasing concentrations of glucose (1, 2.5 and 4.5 g/l). (**A**) Egr1 expression was assessed by immunoblotting of nuclear extracts (bottom) and qRT-PCR (top). (**B**) Heparanase mRNA expression was assessed by qRT-PCR. (**C**, **D**) Egr1 induction in the kidneys of diabetic mice correlates with over-expression of heparanase. (**C**) Kidney tissue was harvested from non-diabetic and diabetic mice 10 weeks after induction of diabetes by STZ and Egr1 mRNA expression was determined by qRT-PCR. Error bars represent ±SE (n= 4 mice for each group). (**D**) Heparanase expression in non-diabetic and diabetic kidney lysates was assessed by qRT-PCR (left), immunostaining (left, inset), enzymatic activity assay (right) and immunoblotting (right, inset). (**E**) Increased recruitment of Egr1 to the heparanase promoter in the presence of high glucose. Following cross-linking of proteins to DNA, chromatin was isolated from HEK 293 cells incubated (1.5 hours) in the presence of increasing concentrations of glucose (0, 1, and 4.5 g/l), sonicated into fragments of average length ≤500 bp and immunoprecipitated with antibodies against Egr1 (top panels), or an unrelated protein LAMP1 (middle panels). The final DNA extractions were amplified using primer set that covers functional Egr1 binding site in the heparanase promoter (left panels), or primer set specific to unrelated L-19 gene sequence, used as control (right panel). Samples were equilibrated for DNA loading amounts using primers specific to the heparanase promoter and DNA that was PCR amplified from chromatin preparations before immunoprecipitation (lower panels). The results are representative of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

against an irrelevant protein (LAMP1) was used for the ChIP analysis (Figure 3E, middle panels).

Finally, to validate the ability of Egr1 to induce heparanase expression in our system, HEK-293 cells were co-transfected with plasmids encoding for luciferase (LUC) driven by heparanase promoter [44] together with either Egr1 expressing vector (pEgr1) or empty pcDNA3 vector and incubated in the absence or presence of 4.5 g/l glucose. Luciferase activity was measured in cell lysates 24 hours post transfection and normalized with β-galactosidase. A 3-fold increase in heparanase promoter activity was detected in cells (incubated in medium without glucose) that were co-transfected with the Egr1 expressing vector, as compared to cells co-transfected with empty vector ($p<0.05$; Figure 4). Notably, both co-transfection with pEgr1 or incubation with high glucose levels activated heparanase promoter to a similar extent (Figure 4), in further support of the role of Egr1 in glucose-induced heparanase expression. Collectively these results demonstrate that in the presence of elevated glucose levels Egr1 directly binds to the heparanase promoter and stimulates its transcriptional activity.

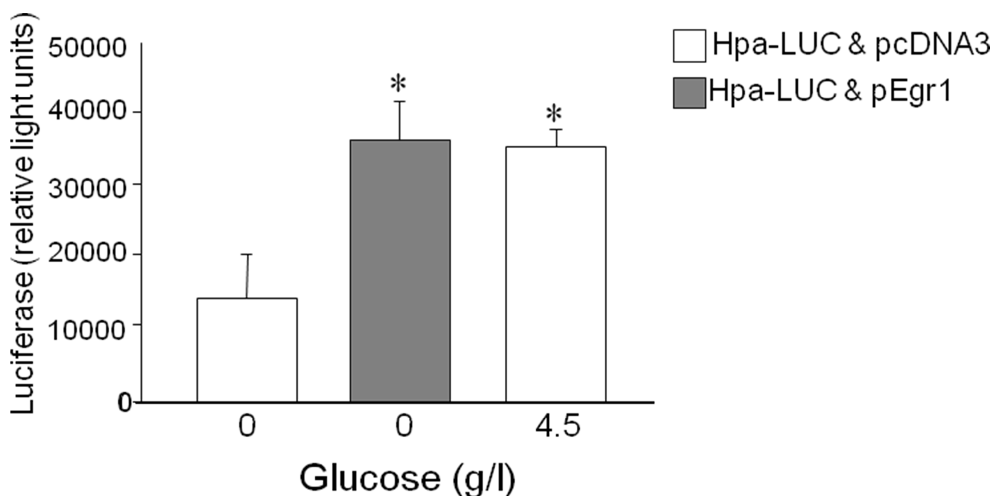

Figure 4: Egr1 expression and high glucose levels activate heparanase promoter in HEK 293 cells. HEK 293 cells were co-transfected with plasmid encoding for luciferase (LUC) driven by the heparanase promoter (Hpa-LUC) reporter gene construct and with either Egr1 expressing vector (pEgr1) (filled bar), or empty plasmid (pcDNA3) (empty bars), and incubated (24 hours) in the absence or presence of glucose (4.5 g/l). LUC activity in cell lysates was measured 24 hours later and normalized with β-Gal. Error bars represent ±SE.*$p<0.05$.

Specific heparanase inhibitor decreases albuminuria in mouse models of DN

The above-described results led us to hypothesize that inhibition of excessive heparanase (induced in diabetic kidney via Egr1 dependent mechanism) may prevent the progression of DN in a manner similar to that observed in *Hpse-KO* mice (Figure 1-2). To validate this hypothesis, we investigated the effect of the specific heparanase inhibitor SST0001 on the

development of proteinuria in diabetic mice. Compound SST0001 is 100% N-acetylated, 25% glycol-split heparin that effectively inhibits heparanase enzymatic activity *in vitro* and, unlike unmodified heparin, is devoid of anticoagulant and pro-angiogenic activities [23, 24, 45]. The effectiveness of SST0001 in suppressing the biological activity of heparanase *in vivo* was demonstrated in models of heparanase-driven processes other than DN, such as inflammation, tumor growth and metastatic spread [24, 26, 46]. To test the effect of heparanase inhibition on DN, we utilized BALB/c mouse strain, known to be particularly prone to DN [25]. Diabetes was induced by multiple low dose STZ administration (40 mg/kg/day for 5 days). Five days after the last STZ administration, diabetic mice (blood glucose levels >300 mg/dL) were divided into 2 groups (n=7) and treated with either SST0001 (administered i.p. twice a day, 300 µg per injection) or vehicle (saline) alone. Blood glucose levels in experimental mice were maintained at ~300 mg/dL by treatment with 0.4U insulin, administered every other day. As expected, 12 weeks after induction of diabetes, a marked increase in 24 h albumin excretion was detected in the urine of vehicle treated diabetic BALB/c mice (Figure 5, black bars). Importantly, SST0001 treatment resulted in a statistically significant ($p < 0.0351$) two-fold decrease in 24 h albumin excretion (Figure 5, gray bars). The difference between 24 h albumin excretion in SST0001-treated vs. vehicle-treated mice was maintained on week 16 of the experiment as well, although it did not reach statistical significance (not shown). In addition, assessment of renal function in vehicle treated diabetic mice on experimental week 16 revealed a significant increase in serum creatinine ($p=0.016$) and blood urea nitrogen BUN ($p=0.0171$) (Figure 5B, black bars), as compared to healthy non-diabetic mice, while in SST0001 treated diabetic mice the corresponding values did not increased significantly compared to those observed in non-diabetic mice (Figure 5B, gray bars). In a similar manner, treatment with SST0001 resulted in a ≥ 2 fold decrease in 24 h urinary albumin excretion in two additional *in vivo* experimental systems: STZ-induced type 1 DN in DBA-2 mice (data not shown), which, along with BALB/c strain, is considered as one of the most useful platforms for DN modeling [25], and in type 2 DN in obese db/db mice ($p=0.010$) (data not shown), regarded as one of the best genetic models of diabetic renal disease [47], further validating heparanase inhibition as a possible therapeutic approach in DN.

Discussion

Diabetes mellitus and its complications represent one of the most important health problems worldwide, which is likely to worsen to critical levels in the next decades [3]. Therefore, DN (one of the most relevant complications secondary to diabetes) is becoming one of the

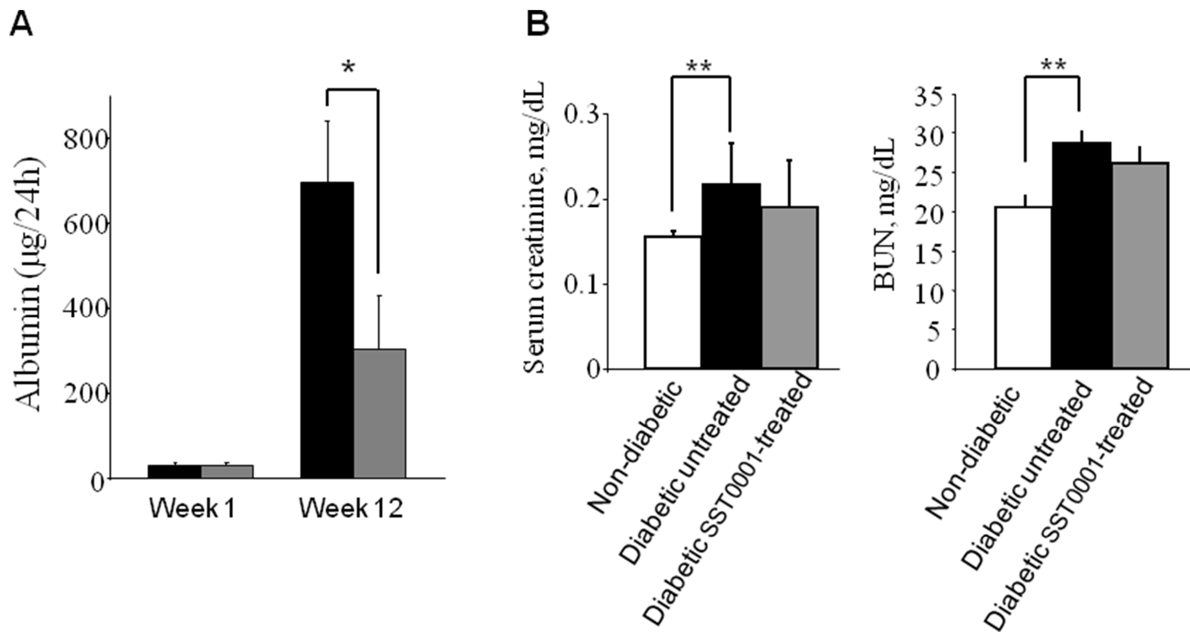

Figure 5: Effect of SST0001 on albuminuria and renal damage in diabetic BALB/c mice. Mice were made hyperglycemic applying multiple low dose STZ as described in 'Methods' and either remained untreated (black bars) or treated with compound SST0001 (300 µg/mouse injected twice a day i.p.) for 12 weeks (grey bar).(**A**) 24-hour albumin excretion on week 1 and 12 after the onset of diabetes was monitored by ELISA. Seven mice per experimental condition were used. Treatment of diabetic mice with SST0001 resulted in more than 2 fold decrease in urine albumin (*$p=0.035$). (**B**) Vehicle treated diabetic mice exhibited a significant increase in serum creatinine (left, **$p=0.0157$) and BUN (right, **$P=0.0171$), as compared to healthy mice (n= 5 mice per group). The corresponding values in SST0001 treated diabetic mice did not increased significantly compared to basal levels.

primary medical concerns [1-3]. Thus, more data about the identity of downstream effectors responsible for DN pathogenesis are needed to properly address the disease treatment. Considerable progress has been made in deciphering the role of multiple signaling pathways leading to kidney damage in diabetic patients. Much less is known about the exact role of ECM-degrading enzymes in DN pathophysiology. Here we demonstrate for the first time the essential involvement of heparanase in experimental DN and describe a molecular mechanism underlying heparanase induction under hyperglycemic conditions.

Despite the lack of experimental data directly confirming causative role of heparanase in the pathogenesis of DN, several reports pointed on a possible link between heparanase and DN [4, 5]. On the other hand, relationship between heparanase and DN progression was disputed in recent publications, opposing the involvement of the enzyme in diabetic kidney disease [20, 21]. In light of this controversy, recent generation of heparanase-null (*Hpse-KO*) mice [22] offered a unique opportunity to assess the relevance of heparanase to the development of DN. Importantly, all *Hpse-KO* mice utilized in our experiments failed to develop proteinuria in response to STZ-induced diabetes, and their urinary albumin excretion rate remained at the same level as before the onset of diabetes. In contrast, >5-fold increase

in urinary excretion rate of albumin was detected in *wt* mice following STZ-induced diabetes. In addition, heparanase deficiency resulted in amelioration of mesangial matrix expansion, macrophage infiltration, tubulointerstitial fibrosis and TGF-β over-expression in the diabetic kidneys of *Hpse-KO* vs. *wt* mice. In further support of a causal involvement of heparanase in DN are our findings showing lower degree of albuminuria in type 1 and type 2 diabetic mice treated with the heparanase inhibitor SST0001 vs. mice treated with vehicle alone. Amelioration of albuminuria by SST0001 represents a proof of concept for heparanase inhibition as a relevant therapeutic approach in DN and warrants further studies aimed at identifying the most effective dose and schedule administration schemes for SST0001 treatment, toward future translation to clinical setting.

In addition, elucidation of the precise molecular mechanism(s) underlying heparanase induction and pathogenic action in diabetic kidney is critically important toward effective implementation of anti-heparanase treatment modalities in the future. Here we report that the transcription factor Egr1 mediates over-expression of heparanase in diabetic kidney. In agreement with the previously reported glucose responsiveness of Egr1 in other cell types [48], we have demonstrated dose-dependent induction of Egr1 expression in cultured kidney cells *in vitro*, and in diabetic kidney *in vivo*. Interestingly, Egr1 can both induce and repress the expression of heparanase, depending on the tissue/cell type; it was shown to activate heparanase expression in T lymphocytes and carcinomas of the breast, prostate and colon, but to inhibit heparanase transcription in melanoma and pancreatic carcinoma cells [31, 49]. Transactivation studies using Egr1 expression vector, co-transfected with a heparanase promoter - reporter construct, showed that in kidney cells Egr1 acts to stimulate heparanase transcription. At the same experimental setting, ChIP analysis revealed increased occupancy of the heparanase promoter by Egr1 in the presence of elevated glucose levels, further confirming the role of Egr1 in glucose-dependent induction of heparanase in the kidney.

A limitation of the present study is that our findings do not provide enough information regarding the exact mode of heparanase action in DN pathogenesis. In the past it was generally accepted that induction of glomerular heparanase in the course of diabetes may contribute to DN progression primarily through degradation of HS in the GBM. Findings supporting importance of HS integrity in permselectivity of the glomerular filtration (reviewed in [19]), include: i) occurrence of massive proteinuria following administration of monoclonal anti-HS antibody to rats, ii) increased GBM permeability as a result of HS removal, and iii) decreased GBM HS content in human/experimental diabetes, which inversely correlated with the degree of proteinuria. Nevertheless, the impact of GBM-residing HS and its enzymatic degradation on DN development was questioned by several recent studies, showing no change in glomerular HS content/structure in early human and experimental DN [20, 21]. These reports challenged the notion that heparanase-mediated loss of HS in the GBM is the

primary mechanism implicating the enzyme in DN. In parallel, it was suggested that additional mechanisms (i.e., changes in glomerular cell-GBM interactions due to loss of HS, or release of HS-bound growth factors, cytokines and bioactive HS fragments in the glomeruli) may drive heparanase-mediated renal failure [4, 5]. In addition, our unpublished results indicate that a previously unappreciated mechanism (i.e., heparanase-driven activation of macrophages leading to unresolved inflammation [26]) may be critically important in DN, as well as in additional kidney pathologies previously linked to heparanase [4, 5]. The important role of chronic inflammation and macrophages in DN [37-39, 50], taken together with the reduced number of macrophages infiltrating *Hpse-KO* diabetic kidneys (Figure 2E) and the recently revealed ability of heparanase to facilitate macrophage activation by lipopolysaccharide [26] and by components of the diabetic milieu (i.e., glucose, our unpublished data), strongly suggest that under diabetic conditions, heparanase, induced in the kidney epithelium via Egr1 dependent mechanism, sustains continuous activation of kidney-damaging macrophages, thus fostering DN development and progression. Studies are underway to accurately evaluate the pathophysiological significance of each of the above-mentioned heparanase-dependent mechanisms in DN progression and thus better define future combination treatment options, as well as target patient populations in whom future anti-heparanase therapies could be particularly beneficial.

Acknowledgements

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References

1. Ibrahim, H.N. and T.H. Hostetter, *Diabetic nephropathy.* J Am Soc Nephrol, 1997. **8**(3): p. 487-93.
2. Gilbertson, D.T., et al., *Projecting the number of patients with end-stage renal disease in the United States to the year 2015.* J Am Soc Nephrol, 2005. **16**(12): p. 3736-41.
3. Atkins, R.C. and P. Zimmet, *Diabetic kidney disease: act now or pay later.* J Am Soc Hypertens, 2010. **4**(1): p. 3-6.
4. Szymczak, M., J. Kuzniar, and M. Klinger, *The role of heparanase in diseases of the glomeruli.* Arch Immunol Ther Exp (Warsz), 2010. **58**(1): p. 45-56.
5. van den Hoven, M.J., et al., *Heparanase in glomerular diseases.* Kidney Int, 2007. **72**(5): p. 543-8.
6. Vreys, V. and G. David, *Mammalian heparanase: what is the message?* J Cell Mol Med, 2007. **11**(3): p. 427-52.
7. Parish, C.R., C. Freeman, and M.D. Hulett, *Heparanase: a key enzyme involved in cell invasion.* Biochim Biophys Acta, 2001. **1471**(3): p. M99-108.
8. Vlodavsky, I., et al., *Mammalian heparanase: gene cloning, expression and function in tumor progression and metastasis.* Nat Med, 1999. **5**(7): p. 793-802.
9. Iozzo, R.V., *Heparan sulfate proteoglycans: intricate molecules with intriguing functions.* J Clin Invest, 2001. **108**(2): p. 165-7.
10. Elkin, M., et al., *Heparanase as mediator of angiogenesis: mode of action.* Faseb J, 2001. **15**(9): p. 1661-3.
11. Kato, M., et al., *Physiological degradation converts the soluble syndecan-1 ectodomain from an inhibitor to a potent activator of FGF-2.* Nat Med, 1998. **4**(6): p. 691-7.
12. Li, J.P. and I. Vlodavsky, *Heparin, heparan sulfate and heparanase in inflammatory reactions.* Thromb Haemost, 2009. **102**(5): p. 823-8.
13. Katz, A., et al., *Involvement of human heparanase in the pathogenesis of diabetic nephropathy.* Isr Med Assoc J, 2002. **4**(11): p. 996-1002.
14. van den Hoven, M.J., et al., *Increased expression of heparanase in overt diabetic nephropathy.* Kidney Int, 2006. **70**(12): p. 2100-8.
15. Levidiotis, V., et al., *A synthetic heparanase inhibitor reduces proteinuria in passive Heymann nephritis.* J Am Soc Nephrol, 2004. **15**(11): p. 2882-92.
16. Maxhimer, J.B., et al., *Heparanase-1 gene expression and regulation by high glucose in renal epithelial cells: a potential role in the pathogenesis of proteinuria in diabetic patients.* Diabetes, 2005. **54**(7): p. 2172-8.
17. Conde-Knape, K., *Heparan sulfate proteoglycans in experimental models of diabetes: a role for perlecan in diabetes complications.* Diabetes Metab Res Rev, 2001. **17**(6): p. 412-21.
18. Miner, J.H., *Renal basement membrane components.* Kidney Int, 1999. **56**(6): p. 2016-24.
19. Raats, C.J., J. Van Den Born, and J.H. Berden, *Glomerular heparan sulfate alterations: mechanisms and relevance for proteinuria.* Kidney Int, 2000. **57**(2): p. 385-400.
20. van den Born, J., et al., *No change in glomerular heparan sulfate structure in early human and experimental diabetic nephropathy.* J Biol Chem, 2006. **281**(40): p. 29606-13.
21. Harvey, S.J. and J.H. Miner, *Revisiting the glomerular charge barrier in the molecular era.* Curr Opin Nephrol Hypertens, 2008. **17**(4): p. 393-8.
22. Zcharia, E., et al., *Newly generated heparanase knock-out mice unravel co-regulation of heparanase and matrix metalloproteinases.* PLoS ONE, 2009. **4**(4): p. e5181.
23. Naggi, A., et al., *Modulation of the heparanase-inhibiting activity of heparin through selective desulfation, graded N-acetylation, and glycol splitting.* J Biol Chem, 2005. **280**(13): p. 12103-13.

24. Ritchie, J.P., et al., *SST0001, a chemically modified heparin, inhibits myeloma growth and angiogenesis via disruption of the heparanase/syndecan-1 axis.* Clin Cancer Res. **17**(6): p. 1382-93.
25. Qi, Z., et al., *Characterization of susceptibility of inbred mouse strains to diabetic nephropathy.* Diabetes, 2005. **54**(9): p. 2628-37.
26. Lerner, I., et al., *Heparanase powers a chronic inflammatory circuit that promotes colitis-associated tumorigenesis in mice.* J Clin Invest, 2011. **121**(5): p. 1709-21.
27. van Kuppevelt, T.H., et al., *Generation and application of type-specific anti-heparan sulfate antibodies using phage display technology. Further evidence for heparan sulfate heterogeneity in the kidney.* J Biol Chem, 1998. **273**(21): p. 12960-6.
28. Doviner, V., et al., *Spatial and temporal heparanase expression in colon mucosa throughout the adenoma-carcinoma sequence.* Mod Pathol, 2006. **19**(6): p. 878-88.
29. Kelly, T., et al., *High heparanase activity in multiple myeloma is associated with elevated microvessel density.* Cancer Res, 2003. **63**(24): p. 8749-56.
30. Cohen, I., et al., *Tamoxifen induces heparanase expression in estrogen receptor-positive breast cancer.* Clin Cancer Res, 2007. **13**(14): p. 4069-77.
31. de Mestre, A.M., et al., *Early growth response gene 1 (EGR1) regulates heparanase gene transcription in tumor cells.* J Biol Chem, 2005. **280**(42): p. 35136-47.
32. Breyer, M.D., et al., *Mouse models of diabetic nephropathy.* J Am Soc Nephrol, 2005. **16**(1): p. 27-45.
33. Gilbert, R.E. and M.E. Cooper, *The tubulointerstitium in progressive diabetic kidney disease: more than an aftermath of glomerular injury?* Kidney Int, 1999. **56**(5): p. 1627-37.
34. Ziyadeh, F.N., *Mediators of diabetic renal disease: the case for tgf-Beta as the major mediator.* J Am Soc Nephrol, 2004. **15 Suppl 1**: p. S55-7.
35. Lu, A., et al., *Blockade of TSP1-Dependent TGF-beta Activity Reduces Renal Injury and Proteinuria in a Murine Model of Diabetic Nephropathy.* Am J Pathol. **178**(6): p. 2573-86.
36. Yamamoto, T., et al., *Expression of transforming growth factor beta is elevated in human and experimental diabetic nephropathy.* Proc Natl Acad Sci U S A, 1993. **90**(5): p. 1814-8.
37. Tesch, G.H., *Macrophages and diabetic nephropathy.* Semin Nephrol, 2010. **30**(3): p. 290-301.
38. Tuttle, K.R., *Linking metabolism and immunology: diabetic nephropathy is an inflammatory disease.* J Am Soc Nephrol, 2005. **16**(6): p. 1537-8.
39. Nguyen, D., et al., *Macrophage accumulation in human progressive diabetic nephropathy.* Nephrology (Carlton), 2006. **11**(3): p. 226-31.
40. Starkey, P.M., L. Turley, and S. Gordon, *The mouse macrophage-specific glycoprotein defined by monoclonal antibody F4/80: characterization, biosynthesis and demonstration of a rat analogue.* Immunology, 1987. **60**(1): p. 117-22.
41. Ogishima, T., et al., *Increased heparanase expression is caused by promoter hypomethylation and up-regulation of transcriptional factor early growth response-1 in human prostate cancer.* Clin Cancer Res, 2005. **11**(3): p. 1028-36.
42. de Mestre, A.M., et al., *Expression of the heparan sulfate-degrading enzyme heparanase is induced in infiltrating CD4+ T cells in experimental autoimmune encephalomyelitis and regulated at the level of transcription by early growth response gene 1.* J Leukoc Biol, 2007. **82**(5): p. 1289-300.
43. Hasan, R.N., S. Phukan, and S. Harada, *Differential regulation of early growth response gene-1 expression by insulin and glucose in vascular endothelial cells.* Arterioscler Thromb Vasc Biol, 2003. **23**(6): p. 988-93.
44. Elkin, M., et al., *Regulation of heparanase gene expression by estrogen in breast cancer.* Cancer Res, 2003. **63**(24): p. 8821-6.
45. Casu, B., I. Vlodavsky, and R.D. Sanderson, *Non-anticoagulant heparins and inhibition of cancer.* Pathophysiol Haemost Thromb, 2008. **36**(3-4): p. 195-203.

46. Yang, Y., et al., *The syndecan-1 heparan sulfate proteoglycan is a viable target for myeloma therapy.* Blood, 2007. **110**(6): p. 2041-8.
47. Allen, T.J., M.E. Cooper, and H.Y. Lan, *Use of genetic mouse models in the study of diabetic nephropathy.* Curr Diab Rep, 2004. **4**(6): p. 435-40.
48. Josefsen, K., et al., *Glucose induces early growth response gene (Egr-1) expression in pancreatic beta cells.* Diabetologia, 1999. **42**(2): p. 195-203.
49. Meirovitz, A., et al., *Role of heparanase in radiation-enhanced invasiveness of pancreatic carcinoma.* Cancer Res, 2011. **71**(7): p. 2772-80.
50. Navarro-Gonzalez, J.F. and C. Mora-Fernandez, *The role of inflammatory cytokines in diabetic nephropathy.* J Am Soc Nephrol, 2008. **19**(3): p. 433-42.

Chapter 3

Cathepsin L is crucial for the development of early experimental diabetic nephropathy

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Abstract

Proteinuria is one of the first clinical signs of diabetic nephropathy (DN) and an independent predictor for the progression to renal failure. Cathepsin L, a lysosomal cysteine protease, can be involved in the development of proteinuria by degradation of proteins that are important for normal podocyte architecture, such as the CD2-associated protein, synaptopodin and dynamin. Cathepsin L also activates heparanase, a heparan sulfate (HS) endoglycosidase previously shown to be crucial for the development of DN. In the current study, we evaluated the exact mode of action of cathepsin L in the development of proteinuria in streptozotocin-induced diabetes. Cathepsin L-deficient mice, in contrast to their WT littermates, failed to develop albuminuria, mesangial matrix expansion, tubulointerstitial fibrosis, renal macrophage influx and showed a normal renal function. In WT mice the early development of albuminuria correlated with the activation of heparanase and loss of HS expression, whereas loss of synaptopodin expression and podocyte damage occurred at a later stage. Our results indicate that cathepsin L is causally involved in the pathogenesis of experimental DN. Most likely, cathepsin L-dependent heparanase activation is crucial for the development of albuminuria and renal damage.

Introduction

Diabetic nephropathy (DN) is the leading cause of end-stage-renal-disease in the Western world [1, 2]. Proteinuria is one of the first clinical signs of DN and microalbuminuria is an independent risk factor for the progression of renal failure [3]. Proteinuria is caused by damage to the glomerular filtration barrier (GFB), which consists of glomerular endothelial cells covered by a glycocalyx, the glomerular basement membrane (GBM) and podocytes. Damage to any of these layers can eventually result in the development of proteinuria, and therefore all layers need to be intact to maintain a normal filtration barrier [4, 5].

Cathepsin L is a lysosomal cysteine protease involved in the breakdown of proteins and the activation of enzymes. Cathepsin L-deficient mice are fertile, develop normal and have no visible or renal phenotype. However, some reports have described that cathepsin L-deficient mice display periodic hair loss, skin thickening, bone and heart defects, an enhanced susceptibility to bacterial infections, and reduced serum levels of glucose [6-9]. Cathepsin L expression is increased in many glomerular diseases, such as membranous glomerulonephritis (MGN), minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS) and DN [10]. Previous studies associated the induction of cathepsin L expression in podocytes, either by lypopolysaccharide (LPS) or puromycin aminonucleoside (PAN), with the development of proteinuria [10, 11]. Importantly, treatment with cathepsin inhibitor cysteine-type cathepsins trans-epoxysuccinyl-*L*-leucylamido-(4-guanidino)butane (E-64) reduced proteinuria in rats with accelerated anti-GBM disease [12]. Recent studies suggest that the onset of proteinuria reflects a migratory event in the foot processes of the podocyte that is associated with the activation of cathepsin L [11]. Three known substrates of cathepsin L in the podocyte are the CD2-associated protein (CD2AP), synaptopodin and dynamin [10, 13, 14]. CD2AP, synaptopodin and dynamin are proteins that are crucial for the normal architecture of the cytoskeleton of the podocyte, and cathepsin L-mediated degradation of CD2AP, synaptopodin and dynamin has been associated with the reorganization of the actin cytoskeleton, foot process effacement, proteinuria and renal failure [10, 14-19].

In addition to the breakdown of CD2AP, synaptopodin and dynamin, cathepsin L is also involved in the activation of heparanase. Heparanase is an endo-β(1,4)-D-glucuronidase that cleaves the negatively charged heparan sulfate (HS) side chains of HS proteoglycans (HSPGs), which are abundantly expressed in basement membranes and at the surface of various cell types. HSPGs are involved in cell-cell and cell-matrix interaction, and function as a (co)receptor for the binding of various bioactive molecules, such as chemokines, cytokines and growth factors [20, 21]. The expression of HS is decreased in many (experimental) glomerular diseases, and this decrease inversely correlates with the level of proteinuria [5,

22, 23]. Heparanase is synthesized as a pre-proheparanase of 68 kDa, which is processed into a proheparanase by removing the signal peptide in the endoplasmatic reticulum. Proheparanase is further processed in the lysosomes into an active heparanase heterodimer by cathepsin L-mediated cleaving of a 10 kDa linker domain [24]. We recently showed that heparanase is crucial for the development of proteinuria in DN [25]. In response to streptozotocin-induced diabetes, heparanase-deficient mice, in contrast to their wild type (WT) littermates, failed to develop proteinuria and renal failure. In addition, treatment with the heparanase inhibitor SST0001 reduced proteinuria and improved renal function [25]. Moreover, we showed that heparanase, induced by high glucose and activated by cathepsin L, contributes to the inflammatory cascade during the pathogenesis of DN, by sustaining macrophage activation [26].

As stated, cathepsin L may be involved in the pathogenesis of proteinuria at several levels, including the degradation of CD2AP, synaptopodin and dynamin, and the activation of heparanase. In the current study, we evaluated the role of cathepsin L in the development of proteinuria by inducing diabetes in cathepsin L-deficient mice and their WT littermates. Our results indicate that cathepsin L is causally involved in the pathogenesis of proteinuria in DN. Most likely, cathepsin L-mediated activation of heparanase results in the loss of glomerular HS expression and the development of proteinuria.

Materials and methods

Animals

C57BL/6n mice (Charles River Laboratories, Wilmington, MA, USA) and cathepsin L-deficient mice in a C57BL/6n background [7] were kept under pathogen-free conditions and housed in a temperature-controlled room with a 12-hour light/dark cycle with *ad libitum* access to food and water. All experiments were approved by the Animal Ethical Committee of the Radboud University Nijmegen.

Streptozotocin-induced diabetes

6-8-week-old WT and cathepsin L-deficient mice were injected intraperitoneally with 50 mg/kg streptozotocin (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) in 250 mM sodium citrate buffer (pH 4.5) or sodium citrate buffer alone for 5 consecutive days, after a 4 hour fasting period. Blood glucose levels were measured twice a week using the Accu-Chek Aviva Blood Glucose Meter (Roche Diagnostics, Almere, The Netherlands). Mice were implanted half an insulin pellet (LinShin Canada, Toronto, Canada) when their blood glucose level raised above 25 mmol/l. Mice were sacrificed after 4, 8 and 16 weeks, and kidneys and

blood were collected. Collected kidneys were snap frozen in liquid nitrogen. Urine was collected after 18 hours in metabolic cages. Six mice were used per time-point. Urinary albumin was measured by radial immunodiffusion (Mancini) and blood urea nitrogen concentrations were determined routinely in our clinical diagnostic facility. Plasma creatinine concentrations were measured by HPLC as described previously [27].

RNA isolation and real-time PCR

Total RNA was isolated from renal cortex using the RNeasy mini-kit (Qiagen, Venlo, The Netherlands). Reversed transcription of 1 µg RNA was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). Quantitative real-time PCR was performed using SYBR Green SuperMix (Roche Diagnostics, Mannheim, Germany) with gene-specific primers (10µM; Isogen Life Sciene, De Meern, The Netherlands) (Table 1) on the CFX real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Gene expression levels were quantified by the delta-delta C_T method using RNA polymerase II as the housekeeping gene.

Table 1: Primers used in real-time PCR.

Target gene	Primer sequence
RNA polymerase II	(F) 5’-AGCCCACGATATACACCACAG-3'
	(R) 5’-AGCTGGGACTGTAGGAAGGACTA-3’
Heparanase	(F) 5’-GAGCGGAGCAAACTCCGAGTGTATC-3’
	(R) 5’-GATCCAGAATTTGACCGTTCAGTTGG-3’
Cathepsin L	(F) 5’-TCTGTTGCTATGGACGCAAG-3’
	(R) 5’-CCGGTCTTTGGCTATTTTGA-3’
TGF-β1	(F) 5’-GAGCGACGTCACTGGAGTTGTACG-3’
	(R) 5’-GATCCCGTTGATTTCCACGTGGAG-3’
CTGF	(F) 5’-GTCCAGACCACAGAGTGGAG-3’
	(R) 5’-CTCCAGGTCAGCTTCGCAG-3’
TNF-α	(F) 5’-CATCTTCTCAAAATTCGAGTGACAA-3’
	(R) 5’-TGGGAGTAGACAACGTACAACCC-3’
IFN-γ	(F) 5’-GGTGACCTTGTGACAAGCTC-3’
	(R) 5’-TGCTGTGTGGTCTGTCTGTC-3’

TGF-β1, transforming growth factor-β1; CTGF, connective tissue growth factor; TNF-α, tumor necrosis factor-α; INF-γ, interferon-γ. F, forward; R, reverse.

Immunofluorescence staining

Immunofluorescence staining was performed on renal cryosections (2 µm) as described [28]. Primary antibodies included anti-CD68 (MCA1957, Serotec, Oxford, UK), anti-heparanase (HPA1, ProsPecTany, Rehovot, Israel), anti-synaptopodin (G1D4, Progen, Heidelberg,

Germany), anti-desmin (Y-20, Santa Cruz Biotechnology, Dellas, Texas, USA), anti-WT1 (C-19, Santa Cruz), anti-VCAM-1 (Novus Biologicals, Abingdon, UK) and the VSV-tagged anti-HS antibody HS4C3 (recognizing N-, 2-O, 3-O and 6-O sulfation) [29]. Appropriate secondary antibodies included Alexa 488-conjugated antibodies (Invitrogen Life Technologies, Breda, The Netherlands) and the anti-VSV-Cy3 antibody (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands). For visualization of the capillary loops we used the hamster anti-agrin antibody (MI91) [30], which is recognized by anti-hamster Cy-3-labeled antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Glomerular heparanase, HS, synaptopodin, desmin and VCAM-1 were scored semiquantitatively for linearity and/or intensity on a scale between 0 and 5 (0= no staining, 2.5= 50% staining and 5= 100% staining) for heparanase, desmin and VCAM-1, and a scale between 0 and 10 (0= no staining, 5= 50% staining, 10= 100% staining) for HS and synaptopodin, respectively. Tubulointerstitial influx of macrophages was scored semiquantitatively from 0 to 5 (0: no macrophage influx; 1: macrophage influx <5% in area; 2: macrophage influx 5-15% in area; 3: macrophage influx 15-25% in area; 4: macrophage influx 25-50% in area; 5: macrophage influx >50% in area). Tubulointerstitial macrophage score was obtained by averaging scores from 10 randomly chosen microscopic fields per mouse. The number of podocytes per glomerulus was determined by counting the number WT-1 positive cells per 50 glomeruli and averaging the numbers. Scoring was performed by two independent investigators on blinded sections using a Leica CTR6000 microscope.

Renal histology

Renal tissue was fixed in 4% buffered formaldehyde and embedded in paraffin. Sections (5 µm) were deparaffinized and stained with periodic acid-Schiff and Masson’s trichrome. Mesangial matrix expansion was scored semiquantitatively as described previously [25]. Briefly, mesangial matrix expansion in each glomerulus was scored from 1 to 4 (1: no mesangial expansion; 2: mesangial expansion in 25%-50% of the glomerulus; 3: mesangial expansion in 50%-75% of the glomerulus; 4: mesangial expansion in >75% of the glomerulus). Mesangial expansion score was obtained by averaging scores from 50 glomeruli in one section. Tubulointerstitial fibrosis was scored semiquantitatively from 1 to 4 (1: no fibrosis; 2: mild fibrosis (10-30% in area); 3: moderate fibrosis (30-50% in area); 4: severe fibrosis (>50% in area)) as described [31]. Tubulointerstitial fibrosis score was obtained by averaging scores from 10 microscopic fields per mouse section.

Cathepsin L and heparanase activity assays

Cathepsin L activity in renal cortex was determined using a commercially available assay (Abcam, Cambridge, UK) according to manufacturer’s instructions.

Heparanase activity in renal cortex was determined using a commercially available assay (AMS Biotechnology, Abingdon, UK) according to manufacturer's instructions.

Determination of glomular ultrastructure by Transmission Electron Microscopy

Small pieces of cortex were fixed in 2.5% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer (pH 7.4). After an overnight incubation at 4ºC, samples were washed in cacodylate buffer. Samples were postfixed in Palade-buffered 2% OsO_4 for 1 hour, dehydrated, and embedded in Epon 812, Luft's procedure (Merck, Darmstadt, Germany). Ultrathin sections were stained with 4% uranyl acetate for 45 minutes and subsequently with lead citrate for 5 minutes at room temperature. Sections were analyzed with a Jeol JEM 1400 electron microscope (JEOL, Tokyo, Japan).

Determination of urinary IgG levels by ELISA

Mouse IgG levels were measured using a commercially available assay (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions.

Statistical analysis

Values are expressed as mean ± SEM. Significance was determined by a one-way ANOVA and *post hoc* analysis with Tukey's multiple comparison test. Comparison of expression between two different groups was evaluated using the Student's *t*-test. Statistical analysis was performed using GraphPad Prism 5.03 (GraphPad Software, Inc., San Diego, CA, USA). A p-value of ≤ 0.05 was considered statistically significant.

Results

Cathepsin L expression and activity are increased in response to STZ-induced diabetes

Although recent studies suggest that cathepsin L is involved in the pathogenesis of proteinuria, the exact role of cathepsin L in the pathogenesis of DN is still unknown. To evaluate the role of cathepsin L in the pathogenesis of proteinuria in DN, we induced type 1 diabetes in WT and cathepsin L-deficient mice following the low-dose streptozotocin induction protocol to avoid the non-specific renal toxicity effects of streptozotocin [32]. One week after induction of diabetes, all mice developed diabetes, as measured by their blood glucose levels (>16 mmol/l). Blood glucose levels remained high throughout the experiment (Table 2). Mice were sacrificed 4, 8 and 16 weeks after the induction of diabetes. Blood glucose levels (Table 2), body weight (Table 2) and the amount of insulin required to

maintain glycemic control did not differ between diabetic WT and diabetic cathepsin L-deficient mice (data not shown). As described previously, non-diabetic cathepsin L-deficient mice have a better glucose control than non-diabetic WT mice [6]. Cortical cathepsin L mRNA expression in WT mice was significantly increased 16 weeks after the induction of diabetes (Figure 1A). Cortical cathepsin L activity was increased 4 and 8 weeks after the induction of diabetes, but comparable to control after 16 weeks (Figure 1B). As expected, cathepsin L was not expressed or active in the cathepsin L-deficient mice (Figure 1A-B).

Table 2: Average blood glucose levels and body weight of WT and CTSL-KO mice at week 4, 8 and 16.

	WT mice		**CTSL-KO mice**	
	Non-diabetic	**Diabetic**	**Non-diabetic**	**Diabetic**
4 weeks				
Blood glucose (mmol/l)	8.4 ± 1.9	21.2 ± 8.6	6.2 ± 0.4	21.7 ± 3.8
Body weight (g)	24.2 ± 3.7	23.9 ± 3.4	23.9 ± 2.7	23.3 ± 1.0
8 weeks				
Blood glucose (mmol/l)	7.9 ± 0.9	22.3 ± 3.0	6.8 ± 1.3	22.8 ± 2.0
Body weight (g)	25.3 ± 3.8	23.2 ± 2.8	24.9 ± 1.4	24.5 ± 0.6
16 weeks				
Blood glucose (mmol/l)	7.8 ± 0.7	21.3 ± 3.1	6.6 ± 1.3	21.3 ± 3.4
Body weight (g)	30.2 ± 5.5	23.8 ± 3.2	26.3 ± 1.8	24.9 ± 1.8

WT, wild type; CTSL-KO, cathepsin L-deficient. The mean ± SD of six mice per group are depicted.

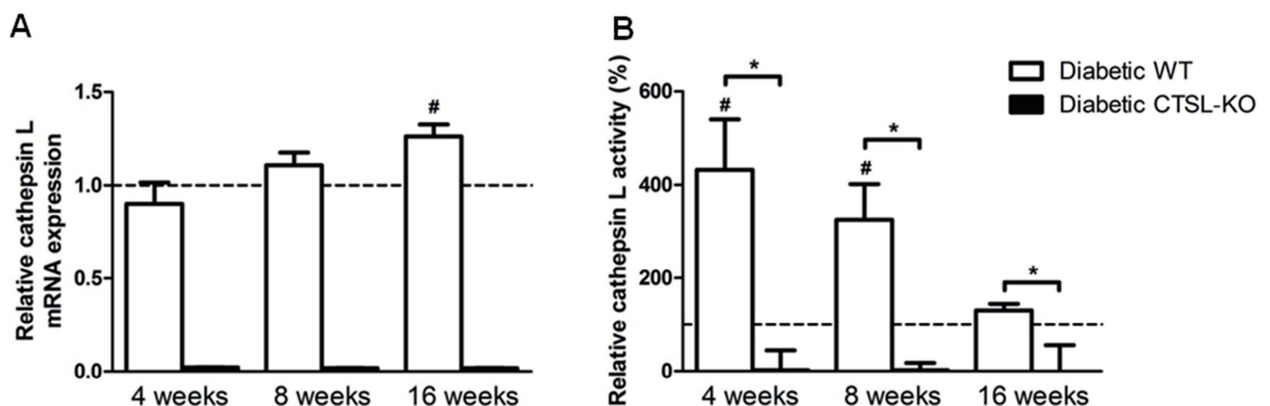

Figure 1: Streptozotocin-induced diabetes increased cortical cathepsin L expression and activity in WT mice. (**A**) Cathepsin L mRNA expression in WT mice was significantly increased 16 weeks after the induction of diabetes. (**B**) Cathepsin L activity in WT mice was significantly increased 4 and 8 weeks after induction of diabetes. Cathepsin L was not expressed or active in the cathepsin L-deficient mice. 5-6 mice per group were used for analysis. Dotted lines indicate relative cathepsin L mRNA expression or activity of non-diabetic control mice. #*P*<0.05 versus non-diabetic control. **P*<0.05 versus diabetic WT mice. WT; wild type. CTSL-KO; cathepsin L-deficient.

Cathepsin L-deficient mice fail to develop albuminuria and show better renal function after induction of experimental DN

Diabetic WT mice developed a significant albuminuria (4-fold increase in albumin-creatinine ratio *versus* non-diabetic controls), starting at week 4 and continuing until week 16, whereas diabetic cathepsin L-deficient mice failed to develop significant albuminuria (Figure 2A). Next we evaluated whether albuminuria in diabetic WT mice was caused by glomerular damage. The development of albuminuria in diabetic WT mice was accompanied by an increased IgG excretion in the urine (Figure 2B), indicating that the development of albuminuria in the diabetic WT mice is caused by glomerular damage. Renal function, as measured by the blood urea nitrogen (BUN) concentration and plasma creatinine concentration, was impaired 16 weeks after induction of diabetes in the WT mice, but remained normal in the cathepsin L-deficient mice (Figure 2C-D).

Cathepsin L-deficient mice fail to develop DN in response to STZ-induced diabetes

Characteristics features of DN include mesangial matrix expansion, tubulointerstitial fibrosis and macrophage infiltration. WT mice developed mild mesangial matrix expansion 16 weeks after the induction of diabetes, whereas diabetic cathepsin L-deficient mice did not develop mesangial matrix expansion (Figure 3A). A mild, but progressive tubulointerstitial fibrosis, as indicated by an increased collagen deposition, was observed in WT mice 8 and 16 weeks after the induction of diabetes, which was significantly reduced in the diabetic cathepsin L-deficient mice (Figure 3B). The mRNA expression of the cytokine transforming growth factor (TGF)-β1, which is involved in the induction of fibrotic processes [33, 34], was significantly increased in the diabetic WT mice after 8 and 16 weeks, whereas TGF-β1 expression remained normal in the cathepsin L-deficient mice, except for week 4 (Table 3). The expression of connective tissue growth factor (CTGF), another fibrogenic protein [35], was significantly higher in the diabetic WT mice versus diabetic cathepsin L-deficient mice (Table 3).

Macrophage accumulation in the diabetic kidney may predict a decline in renal function [36]. Tubulointerstitial macrophage infiltration was significantly increased in diabetic WT mice, starting at week 4 and continuing until week 16, which was not observed in diabetic cathepsin L-deficient mice (Figure 3C). Macrophages can be activated by cytokines such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ. We observed an increased TNF-α and INF-γ mRNA expression in the diabetic WT mice, whereas TNF-α and IFN-γ mRNA expression were normal in the diabetic cathepsin L-deficient mice (Table 3).

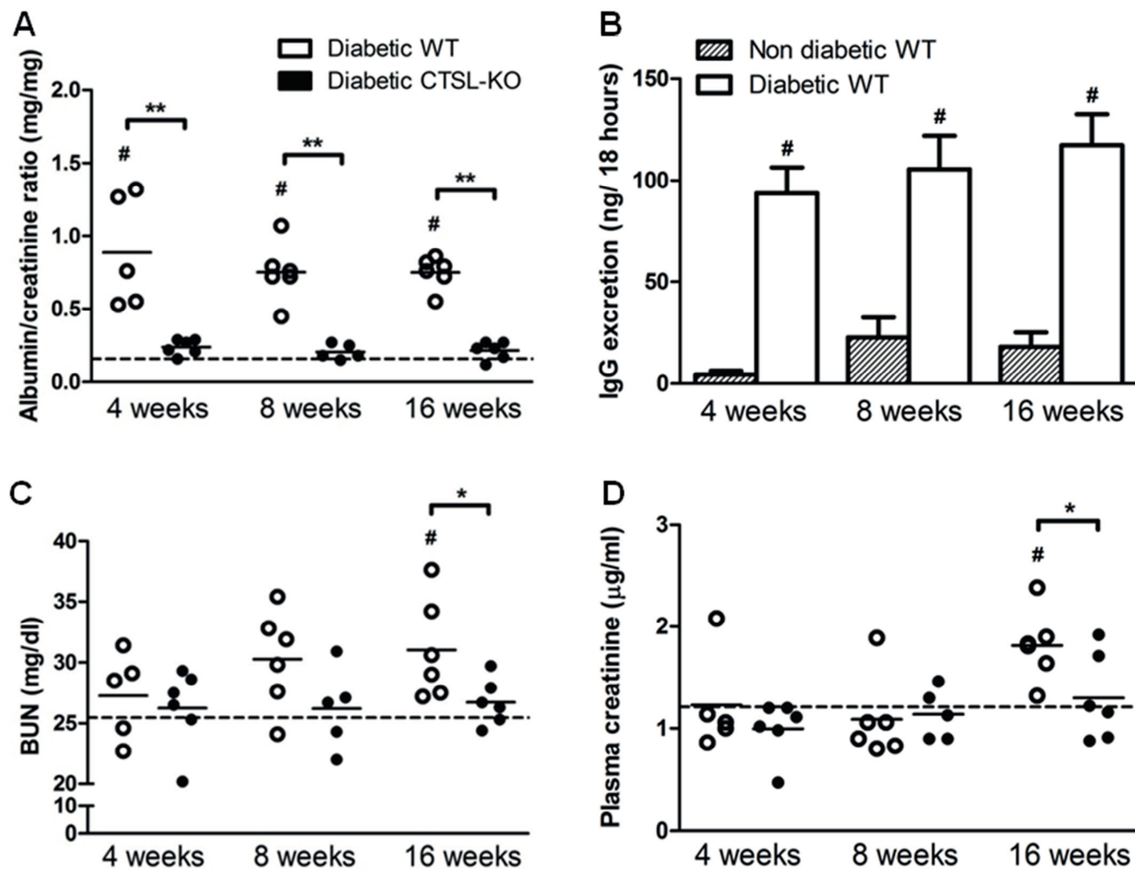

Figure 2: Cathepsin L-deficient mice show better renal function during streptozotocin-induced diabetes. (**A**) The albumin/creatinine ratio was significantly increased in diabetic WT mice compared with non-diabetic WT mice 4, 8, and 16 weeks after induction of diabetes. Cathepsin L-deficient mice failed to develop albuminuria at these time points. (**B**) IgG excretion in the urine, as measured by an IgG ELISA, was significantly increased in diabetic WT mice compared with non-diabetic WT mice 4, 8, and 16 weeks after induction of diabetes. (**C**) Plasma blood urea nitrogen (BUN) levels and (**D**) plasma creatinine levels were significantly increased in diabetic WT mice compared with non-diabetic WT mice 16 weeks after induction of diabetes. Plasma BUN and plasma creatinine levels were normal in the diabetic cathepsin L-deficient mice. 5-6 mice per group were used for analysis. Dotted lines indicate the average albumin-creatinine ratio and BUN of non-diabetic control mice. #*P*<0.05 versus non-diabetic control. **P*<0.05 and ***P*<0.01 versus diabetic WT mice. WT; wild type. CTSL-KO; cathepsin L-deficient.

Table 3: Quantitative mRNA expression of TGF-β1, CTGF, TNF-α and IFN-γ in renal cortex of WT and cathepsin L-deficient mice during type 1 diabetes[a].

	4 weeks		**8 weeks**		**16 weeks**	
	WT	**CTSL-KO**	**WT**	**CTSL-KO**	**WT**	**CTSL-KO**
TGF-β1	0.93 ± 0.08	1.60 ± 0.20*#	1.75 ± 0.15*	1.12 ± 0.12#	1.79 ± 0.23**	1.20 ± 0.11#
CTGF	2.58 ± 0.31**	1.47 ± 0.13*#	2.46 ± 0.56*	1.23 ± 0.11#	2.35 ± 0.46**	1.19 ± 0.14#
TNF-α	2.09 ± 0.45**	1.32 ± 0.11#	2.37 ± 0.47*	1.35 ± 0.18#	1.93 ± 0.20**	1.12 ± 0.16#
IFN-γ	1.74 ± 0.22*	1.18 ± 0.21	2.43 ± 0.53*	1.16 ± 0.19#	2.24 ± 0.23**	1.27 ± 0.18#

TGF-β1, transforming growth factor-β1; CTGF, connective tissue growth factor; TNF-α, tumor necrosis factor-α; INF-γ, interferon-γ; WT, wild type; CTSL-KO, cathepsin L-deficient. The mean ± SEM of six mice per group are depicted. **P*<0.05 and ***P*<0.01 versus control mice; #*P*<0.05 CTSL-KO versus WT mice. [a]The relative expression compared with non-diabetic WT or non-diabetic CTSL-KO mice is depicted, with both non-diabetic WT and non-diabetic CTSL-KO mice set at 1.

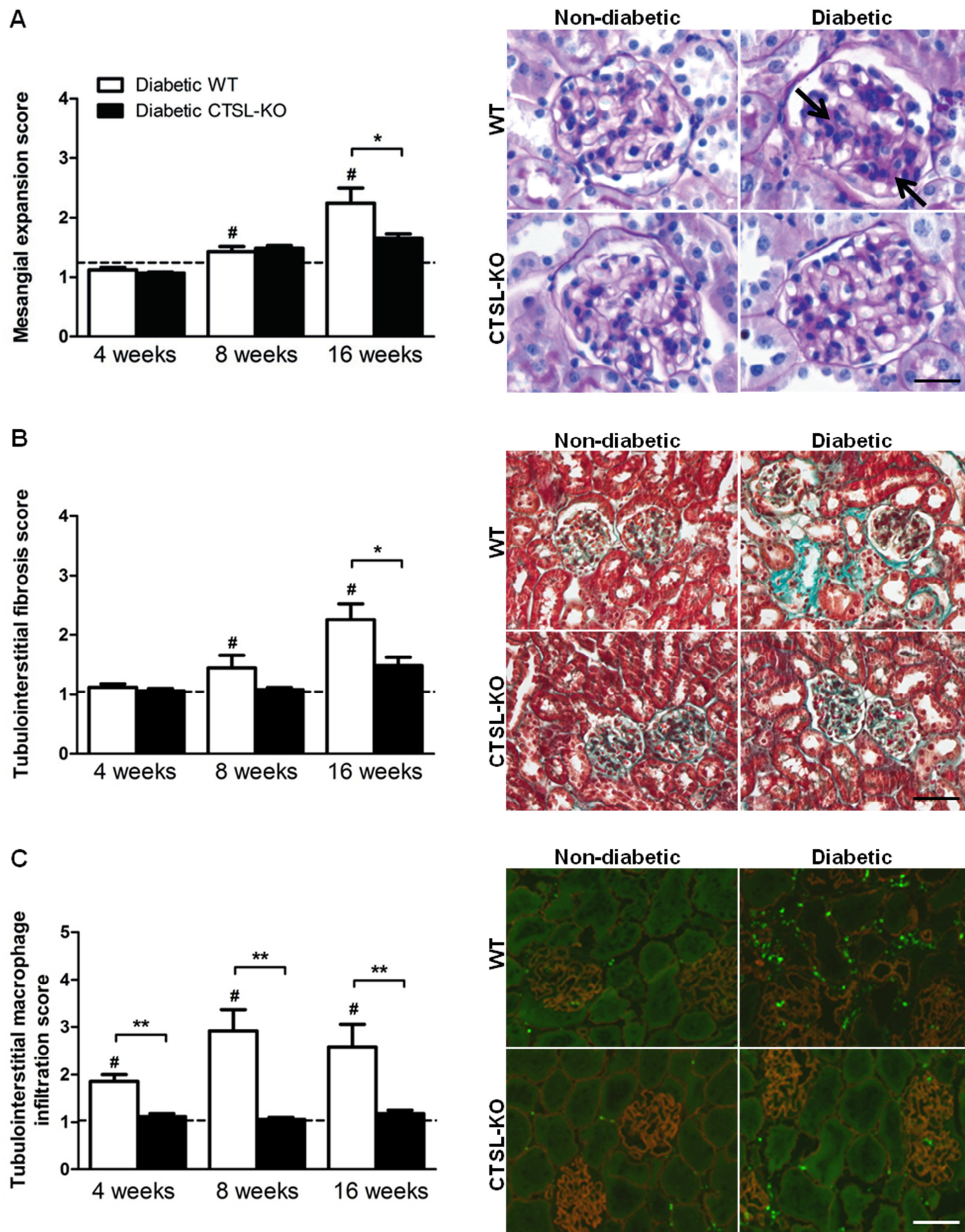

Figure 3: Cathepsin L-deficient mice fail to develop mesangial matrix expansion, tubulointerstitial fibrosis and macrophage infiltration in experimental diabetes. Kidney sections of diabetic WT and cathepsin L-deficient mice were stained with (**A**) periodic acid-Schiff, (**B**) Masson-Trichome and (**C**) CD68. Representative pictures of mice at t=16 weeks are shown. (**A**) Diabetic WT mice developed mild mesangial matrix expansion (black arrow) after 16 weeks, which was significantly reduced in the diabetic cathepsin L-deficient mice (scale bar: 20 µm). (**B**) Diabetic WT mice developed mild tubulointerstitial fibrosis (green staining) after 8 and 16 weeks, which was significantly reduced in the diabetic cathepsin L-deficient mice (scale bar: 40 µm). (**C**) Tubulointerstitial macrophage infiltration (green) was significantly increased in diabetic WT mice after 4, 8 and 16 weeks, but normal in diabetic cathepsin L-deficient mice (scale bar: 40 µm). 5-6 mice per group were used for analysis. See methods section for scoring parameters. Dotted lines indicate mesangial expansion score, tubulointerstitial fibrosis score or tubulointerstitial macrophage infiltration score of non-diabetic control mice. $^{\#}P<0.05$ versus non-diabetic control. $^{*}P<0.05$ and $^{**}P<0.01$ versus diabetic WT mice. WT; wild type. CTSL-KO; cathepsin L-deficient.

Cathepsin L deficiency preserves glomerular HS expression in experimental DN

Next, we evaluated the effect of cathepsin L deficiency on glomerular heparanase and HS expression in experimental DN. Heparanase mRNA expression in kidney cortex was significantly increased 4, 8, and 16 weeks after the induction of diabetes in both WT and cathepsin L-deficient mice (Figure 4A). Notably, the higher heparanase mRNA expression in WT mice compared to cathepsin L-deficient mice may be explained by a higher presence of tubulointerstitial macrophages in WT mice. In WT mice, the increased heparanase mRNA expression correlated with an increased glomerular heparanase protein expression (Figure 4B) and heparanase activity (Figure 4C). The anti-heparanase antibody we used specifically recognizes the active form of heparanase. Therefore, as expected, no heparanase protein expression or activity could be observed in the cathepsin L-deficient mice. Glomerular HS expression in diabetic WT mice was reduced, starting at week 4 and continuing until week 16, whereas glomerular HS expression was preserved in the diabetic cathepsin L-deficient mice (Figure 4D).

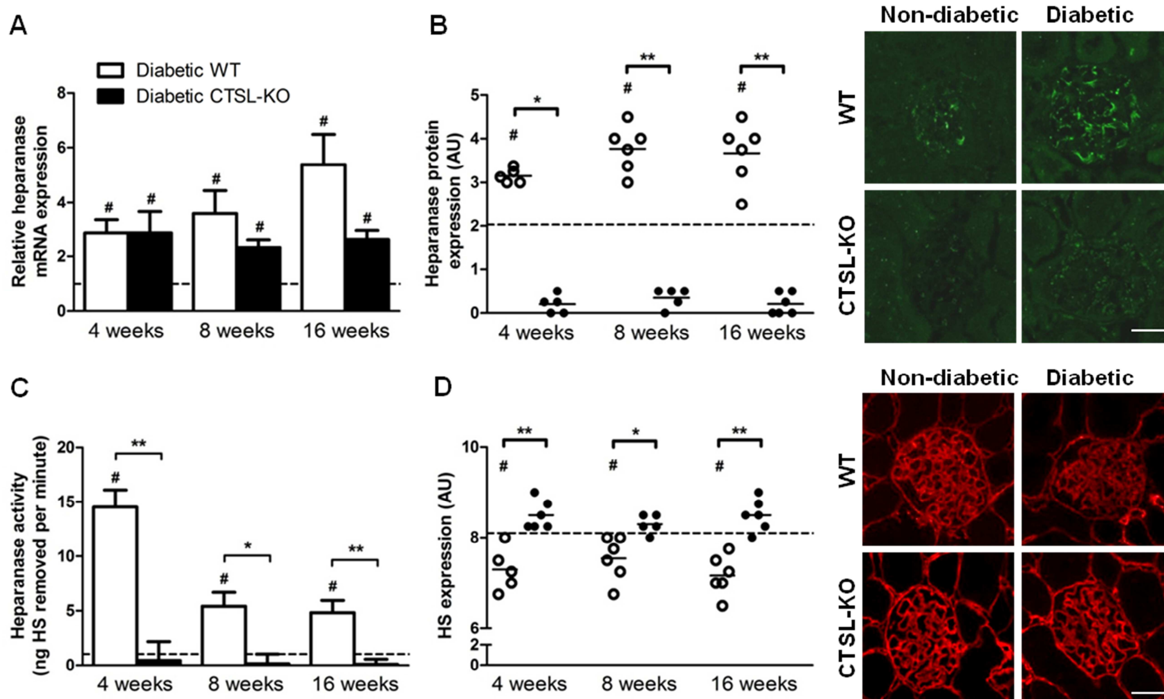

Figure 4: Cathepsin L deficiency preserves glomerular HS expression in experimental diabetes. By induction of diabetes in WT mice, (**A**) cortical heparanase mRNA expression, (**B**) glomerular heparanase protein expression, and (**C**) cortical heparanase activity were significantly increased, whereas (**D**) glomerular HS expression was significantly reduced. Heparanase mRNA expression was significantly increased in the diabetic cathepsin L-deficient mice, but no heparanase protein expression or activity was observed in the cathepsin L-deficient mice. Glomerular HS expression was preserved in the diabetic cathepsin L-deficient mice. (**B**,**D**) Representative images showing glomerular heparanase protein expression and glomerular HS expression of mice at t=16 weeks, respectively, as determined by immunofluorescence staining (scale bar: 20 µm). 5-6 mice per group were used for analysis. Dotted lines indicate heparanase/HS expression/activity of non-diabetic control mice. $^{\#}P<0.05$ versus non-diabetic control. $^{*}P<0.05$ and $^{**}P<0.01$ versus diabetic WT mice. WT; wild type. CTSL-KO; cathepsin L-deficient. AU; arbitrary units.

Cathepsin L deficiency reduces podocyte and endothelial damage in experimental DN

As outlined, cathepsin L is also involved in the degradation of the podocyte protein synaptopodin, which may be another contributing factor in the development of DN and albuminuria. Synaptopodin protein expression in WT mice was significantly reduced 8 and 16 weeks after induction of diabetes, whereas synaptopodin expression was preserved in the diabetic-cathepsin L-deficient mice (Figure 5A). Despite the reduced synaptopodin protein expression, we could not observe significant podocyte foot process effacement (Figure 6A) or podocyte loss (Figure 6B) in diabetic WT mice, although partial foot process effacement could be recognized in diabetic WT mice (Figure 6A). Furthermore, there appeared no clear loss of endothelial fenestrations 16 weeks after the induction of diabetes (Figure 6A). However, the expression of the podocyte damage marker desmin, a protein that is not directly targeted by cathepsin L, was significantly increased 16 weeks after induction of diabetes in WT mice, whereas desmin protein expression remained normal in the diabetic cathepsin L-deficient mice (Figure 5B).

In the early stages of DN that is mimicked by the streptozotocin model, glomerular endothelial injury is the most prominent phenotype and develops prior to podocyte injury. To assess endothelial activation, we determined the glomerular expression of vascular cell adhesion molecule-1 (VCAM-1). VCAM-1 expression was significantly increased 4 weeks after induction of diabetes in WT mice, whereas VCAM-1 expression was normal in the diabetic cathepsin L-deficient mice (Figure 5C). No significant increase in VCAM-1 expression was observed 8 and 16 weeks after the induction of diabetes in WT mice. However, VCAM-1 expression was significantly reduced in diabetic cathepsin L-deficient mice versus diabetic WT mice 16 weeks after the induction of diabetes (Figure 5C).

Discussion

In this study we showed that cathepsin L is crucial for the development of DN in experimental diabetes. Diabetic cathepsin L-deficient mice failed to develop albuminuria, had a normal renal function and less mesangial matrix expansion, tubulointerstitial fibrosis and macrophage accumulation. In WT mice, we showed that the development of albuminuria was associated with the induction of heparanase activity and loss of HS expression, whereas loss of synaptopodin expression and podocyte injury was observed at a later stage.

The present study is the first study to show that cathepsin L is essential for the development of DN. A previous study showed that cathepsin L is essential for the development of type 1 diabetes in autoimmune diabetes-prone NOD mice [37]. Cathepsin L-deficient mice showed

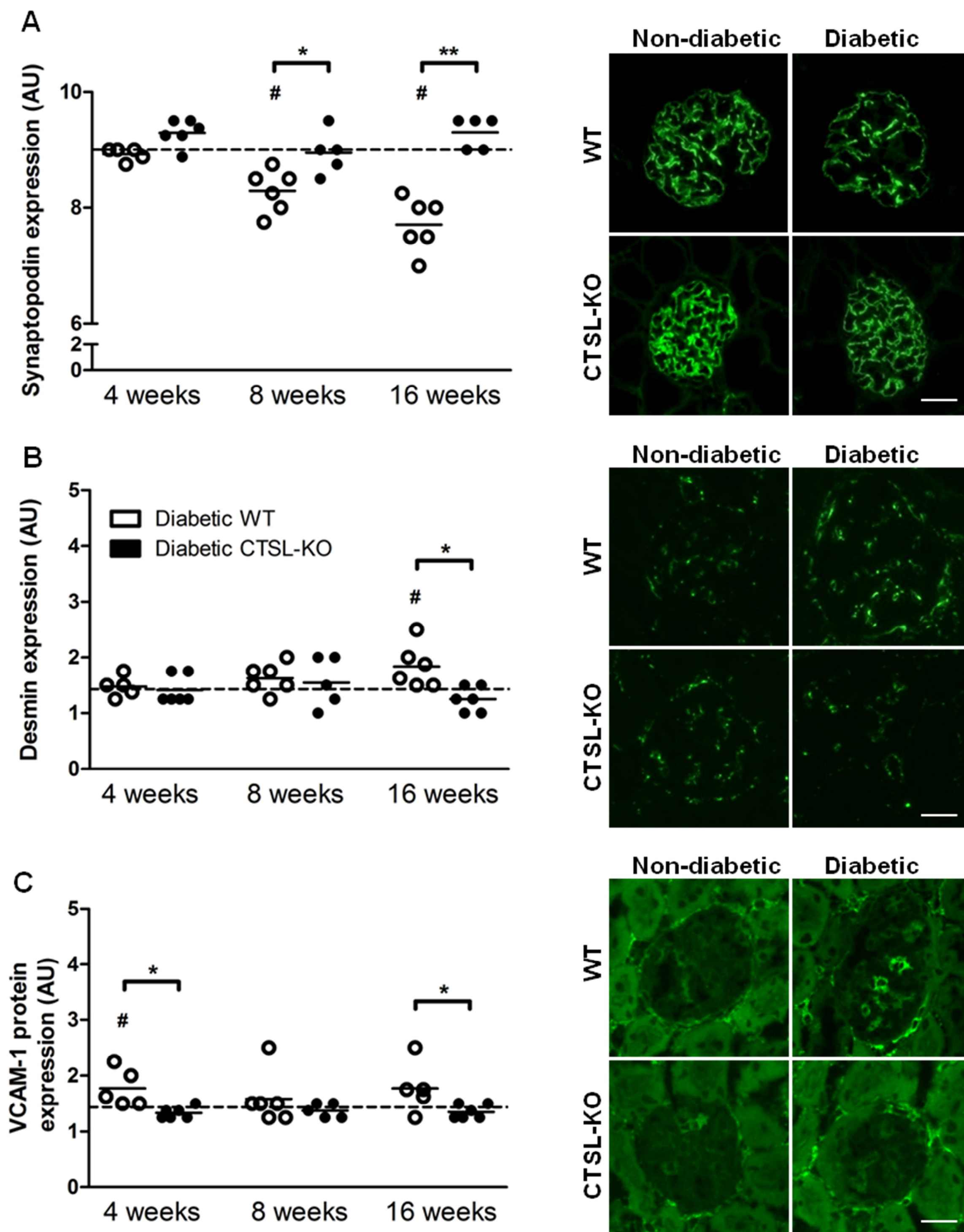

Figure 5: Cathepsin L deficiency prevents podocyte and glomerular endothelial injury in experimental diabetes. (**A**) synaptopodin protein expression was reduced 8 and 16 weeks after streptozotocin-induced diabetes in WT mice. Synaptopodin expression was normal in the diabetic cathepsin L-deficient mice. (**B**) Desmin protein expression was increased 16 weeks after induction of diabetes in WT mice. Desmin expression was normal in the diabetic cathepsin L-deficient mice. (**C**) VCAM-1 expression was increased 4 weeks after induction of diabetes in WT mice, but normal in the diabetic cathepsin L-deficient mice. Representative picture of mice at t=4 weeks for VCAM-1 and t=16 weeks for synaptopodin and desmin are shown. 5-6 mice per group were used for analysis. Dotted lines indicate synaptopodin/desmin/VCAM-1 expression of non-diabetic control mice. Scale bar: 20 µm. $^{\#}P<0.05$ versus non-diabetic control. $^{*}P<0.05$ and $^{**}P<0.01$ versus diabetic WT mice. WT; wild type. CTSL-KO; cathepsin L-deficient. VCAM-1; vascular cell adhesion molecule-1. AU; arbitrary units.

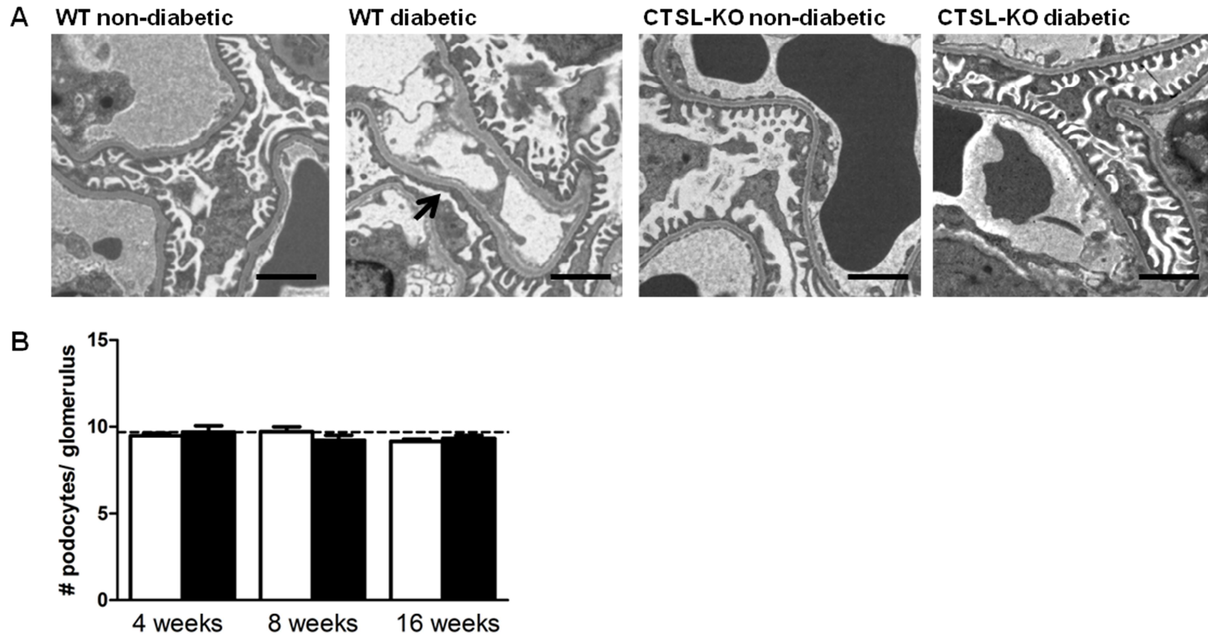

Figure 6: Glomerular ultrastructure and the number of podocytes per glomerulus is normal in both diabetic WT and diabetic cathepsin L-deficient mice. (**A**) Glomerular ultrastructure, as visualized by transmission electron microscopy, was normal in both diabetic WT and diabetic cathepsin L-deficient mice 16 weeks after induction of diabetes, although partial foot process effacement could be recognized in the diabetic WT mice (black arrow). Scale bar: 2 µm. (**B**) The number of podocytes per glomerulus, as visualized by immunofluorescence staining, was not affected by induction of diabetes in both WT and cathepsin L-deficient mice. Dotted lines indicate the number of podocytes of non-diabetic control mice. WT; wild type. CTSL-KO; cathepsin L-deficient.

an increased proportion of regulatory T cells compared with their WT littermates, protecting them to develop type 1 diabetes. In the present study we were able to induce type 1 diabetes in the cathepsin L-deficient mice, as indicated by increased blood glucose levels. In contrast to the previous study, we induced type 1 diabetes by streptozotocin, a drug that enters beta cells in the pancreas via the glucose transporter (GLUT)-2 and damages the DNA [38-40]. A seeming limitation of the streptozotocin-induced model in our hands may be the lack of development of overt DN accompanied with overt podocyte foot process effacement. However, our model may be very relevant for the development of microalbuminuria in humans, which is an early and specific predictor for the development of overt DN and macroalbuminuria [41-44]. Importantly, we show that diabetic cathepsin L-deficient mice failed to develop albuminuria, in contrast to their WT littermates.

Previous studies showed that cathepsin L plays a role in the development of proteinuria, as treatment with the cysteine-cathepsin inhibitor E-64 reduced proteinuria in rats with accelerated anti-GBM disease [12]. Moreover, cathepsin L-deficient mice are protected from LPS-induced foot process effacement and proteinuria [10]. Two substrates of cathepsin L, synaptopodin and dynamin, are key stabilizers of the podocyte actin cytoskeleton [10, 13]. *In*

vivo synaptopodin gene delivery or podocyte-specific transgenic expression of a synaptopodin mutant lacking cathepsin L cleavage sites protects mice from LPS-induced proteinuria, suggesting that cleavage of synaptopodin by cathepsin L is required for the induction of foot process effacement [13, 45]. In addition, synaptopodin can control dynamin levels [13]. In the present study we observed no development of foot process effacement, although diabetic WT mice did develop albuminuria. Previous studies showed that proteinuria can occur without foot process effacement and that foot process effacement is not correlated with the level of proteinuria [46-49]. Moreover, we observed a reduced synaptopodin protein expression 8 weeks after induction of diabetes, and podocyte injury, as indicated by an increased desmin expression, and 16 weeks after induction of diabetes, whereas mice developed already a significant albuminuria 4 weeks after streptozotocin-induced diabetes. This suggest that synaptopodin is not involved in the initial development of proteinuria in experimental DN, which may be relevant for the microalbuminuric phase in the human setting. However, our findings do not exclude a role for synaptopodin at a later stage in the development of proteinuria and overt DN.

In addition to albuminuria, we showed that cathepsin L is involved in the development of mesangial matrix expansion and tubulointerstitial fibrosis. This is most likely caused by loss of heparanase activity, as diabetic heparanase-deficient mice also failed to develop mesangial matrix expansion and tubulointerstitial fibrosis [25]. Moreover, a recent study showed that heparanase is a key player in renal fibrosis, as it regulates TGF-β expression and activity and subsequently epithelial-mesenchymal transition (EMT) [50, 51]. In addition, under diabetic conditions heparanase induces macrophage activation [26], which has been suggested to drive renal fibrosis [52, 53].

Induction of heparanase activity, loss of HS expression and macrophage accumulation correlated with the development of albuminuria. Previously, we showed that heparanase is essential for the development of experimental DN, as heparanase-deficient mice failed to develop albuminuria and DN after streptozotocin-induced diabetes [25]. Moreover, we showed that the heparanase-deficient mice had a preserved glomerular HS expression. In a follow-up study, we showed that under diabetic conditions heparanase, activated by cathepsin L of tubular origin, sustains continuous activation of macrophages, resulting in an increased production of TNF-α. No increase in macrophage activation or TNF-α production was observed in the heparanase-deficient mice [26]. Similar results we have now observed in the diabetic cathepsin L-deficient mice, which also showed no increase in macrophage infiltration and TNF-α expression, most likely caused by loss of heparanase activity. Whether proteinuria mediated by heparanase in DN is caused by loss of HS expression in the GFB, the activation of macrophages or both should be addressed in future research.

Heparanase is expressed by several cells in the glomerulus, including endothelial cells and podocytes, but also by several cells of the immune system, such as macrophages, leukocytes and platelets. The activation of heparanase by cathepsin L can occur both intracellular and extracellular. After activation, heparanase is able to cleave HS on both endothelial cells and podocytes, resulting in a reduced HS expression on both cell types. We and others previously showed that loss of HS in the endothelial glycocalyx is essential for the development of proteinuria in experimental DN [54-56]. Other studies showed that loss of podocyte HS resulted in the development of foot process effacement, suggesting that podocyte damage may be secondary to loss of HS [57, 58]. However, the presence of synaptopodin is also essential to maintain a normal podocyte architecture [13], and future studies should address whether podocyte damage is caused by loss of HS, cathepsin L-mediated degradation of synaptopodin, or a combination of both.

The important role of cathepsin L in the development of proteinuria is most likely not limited to DN. Previous studies showed that the expression of cathepsin L is increased in several other glomerular diseases, such as MGN, MCD and FSGS [10]. Treatment with a cathepsin L inhibitor reduced proteinuria in a rat model for glomerulonephritis [12]. We recently showed that heparanase is essential for the development of experimental glomerulonephritis [59]. Moreover, we showed that the expression of heparanase is increased in adriamycin-induced nephropathy, an experimental model for FSGS [60, 61]. Together, these studies suggest that cathepsin L may play an important role in the development of proteinuria in other glomerular diseases by the activation of heparanase. But, as mentioned, cathepsin L-mediated degradation of synaptopodin and dynamin may also play an important role in the development of proteinuria.

Not much is known about the role of other cathepsins in the development of DN. Only for the cysteine proteases cathepsin B and S there are studies indicating that they play a role in the development of DN. Cathepsin B plays a role in the degradation of fibronectin. In DN there is an inadequate cathepsin B activity, which results in an insufficient degradation of fibronection and a progressive accumulation of extracellular matrix [62]. Cathepsin S is involved in the induction of proteinuria and glomerular endothelial cell injury in type 2 diabetic mice, by activating the protease-activated receptor-2 (PAR2) [63].

In conclusion, in this study we showed that cathepsin L is causally involved in the development of experimental DN. Most likely, cathepsin L-deficiency is essential for heparanase activity, leading to the development of albuminuria, mesangial matrix expansion, tubulointerstitial fibrosis and macrophage accumulation. The current study suggests that targeting cathepsin L or heparanase represents an attractive therapeutic target for the treatment of DN.

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References

1. Ritz, E., et al., *End-stage renal failure in type 2 diabetes: A medical catastrophe of worldwide dimensions.* Am J Kidney Dis, 1999. **34**(5): p. 795-808.
2. Gilbertson, D.T., et al., *Projecting the number of patients with end-stage renal disease in the United States to the year 2015.* J Am Soc Nephrol, 2005. **16**(12): p. 3736-41.
3. Tryggvason, K. and E. Pettersson, *Causes and consequences of proteinuria: the kidney filtration barrier and progressive renal failure.* J Intern Med, 2003. **254**(3): p. 216-24.
4. Patrakka, J. and K. Tryggvason, *Molecular make-up of the glomerular filtration barrier.* Biochemical and biophysical research communications, 2010. **396**(1): p. 164-9.
5. Garsen, M., et al., *The role of heparanase and the endothelial glycocalyx in the development of proteinuria.* Nephrol Dial Transplant, 2014. **29**(1): p. 49-55.
6. Yang, M., et al., *Cathepsin L activity controls adipogenesis and glucose tolerance.* Nat Cell Biol, 2007. **9**(8): p. 970-7.
7. Roth, W., et al., *Cathepsin L deficiency as molecular defect of furless: hyperproliferation of keratinocytes and pertubation of hair follicle cycling.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2000. **14**(13): p. 2075-86.
8. Potts, W., et al., *Cathepsin L-deficient mice exhibit abnormal skin and bone development and show increased resistance to osteoporosis following ovariectomy.* Int J Exp Pathol, 2004. **85**(2): p. 85-96.
9. Honey, K., et al., *Cathepsin L regulates CD4+ T cell selection independently of its effect on invariant chain: a role in the generation of positively selecting peptide ligands.* J Exp Med, 2002. **195**(10): p. 1349-58.
10. Sever, S., et al., *Proteolytic processing of dynamin by cytoplasmic cathepsin L is a mechanism for proteinuric kidney disease.* J Clin Invest, 2007. **117**(8): p. 2095-104.
11. Reiser, J., et al., *Podocyte migration during nephrotic syndrome requires a coordinated interplay between cathepsin L and alpha3 integrin.* J Biol Chem, 2004. **279**(33): p. 34827-32.
12. Baricos, W.H., et al., *Evidence suggesting a role for cathepsin L in an experimental model of glomerulonephritis.* Archives of biochemistry and biophysics, 1991. **288**(2): p. 468-72.
13. Faul, C., et al., *The actin cytoskeleton of kidney podocytes is a direct target of the antiproteinuric effect of cyclosporine A.* Nat Med, 2008. **14**(9): p. 931-8.
14. Yaddanapudi, S., et al., *CD2AP in mouse and human podocytes controls a proteolytic program that regulates cytoskeletal structure and cellular survival.* J Clin Invest, 2011. **121**(10): p. 3965-80.
15. Kim, J.M., et al., *CD2-associated protein haploinsufficiency is linked to glomerular disease susceptibility.* Science, 2003. **300**(5623): p. 1298-300.
16. Huber, T.B., et al., *Bigenic mouse models of focal segmental glomerulosclerosis involving pairwise interaction of CD2AP, Fyn, and synaptopodin.* J Clin Invest, 2006. **116**(5): p. 1337-45.
17. Lehtonen, S., F. Zhao, and E. Lehtonen, *CD2-associated protein directly interacts with the actin cytoskeleton.* Am J Physiol Renal Physiol, 2002. **283**(4): p. F734-43.
18. Mundel, P. and J. Reiser, *Proteinuria: an enzymatic disease of the podocyte?* Kidney international, 2010. **77**(7): p. 571-80.
19. Reiser, J., B. Adair, and T. Reinheckel, *Specialized roles for cysteine cathepsins in health and disease.* J Clin Invest, 2010. **120**(10): p. 3421-31.
20. Bernfield, M., et al., *Functions of cell surface heparan sulfate proteoglycans.* Annu Rev Biochem, 1999. **68**: p. 729-77.

21. Rops, A.L., et al., *Heparan sulfate proteoglycans in glomerular inflammation.* Kidney Int, 2004. **65**(3): p. 768-85.
22. van den Born, J., et al., *Distribution of GBM heparan sulfate proteoglycan core protein and side chains in human glomerular diseases.* Kidney international, 1993. **43**(2): p. 454-63.
23. van den Hoven, M.J., et al., *Heparanase in glomerular diseases.* Kidney international, 2007. **72**(5): p. 543-8.
24. Abboud-Jarrous, G., et al., *Cathepsin L is responsible for processing and activation of proheparanase through multiple cleavages of a linker segment.* J Biol Chem, 2008. **283**(26): p. 18167-76.
25. Gil, N., et al., *Heparanase is essential for the development of diabetic nephropathy in mice.* Diabetes, 2012. **61**(1): p. 208-16.
26. Goldberg, R., et al., *Role of heparanase-driven inflammatory cascade in pathogenesis of diabetic nephropathy.* Diabetes, 2014. **63**(12): p. 4302-13.
27. Dunn, S.R., et al., *Utility of endogenous creatinine clearance as a measure of renal function in mice.* Kidney Int, 2004. **65**(5): p. 1959-67.
28. Rops, A.L., et al., *Syndecan-1 deficiency aggravates anti-glomerular basement membrane nephritis.* Kidney Int, 2007. **72**(10): p. 1204-15.
29. van Kuppevelt, T.H., et al., *Generation and application of type-specific anti-heparan sulfate antibodies using phage display technology. Further evidence for heparan sulfate heterogeneity in the kidney.* J Biol Chem, 1998. **273**(21): p. 12960-6.
30. Raats, C.J., et al., *Differential expression of agrin in renal basement membranes as revealed by domain-specific antibodies.* J Biol Chem, 1998. **273**(28): p. 17832-8.
31. Oba, S., et al., *miR-200b precursor can ameliorate renal tubulointerstitial fibrosis.* PLoS One, 2010. **5**(10): p. e13614.
32. Breyer, M.D., et al., *Mouse models of diabetic nephropathy.* J Am Soc Nephrol, 2005. **16**(1): p. 27-45.
33. Ziyadeh, F.N., *Mediators of diabetic renal disease: the case for tgf-Beta as the major mediator.* J Am Soc Nephrol, 2004. **15 Suppl 1**: p. S55-7.
34. Yamamoto, T., et al., *Sustained expression of TGF-beta 1 underlies development of progressive kidney fibrosis.* Kidney Int, 1994. **45**(3): p. 916-27.
35. Wang, S., et al., *Connective tissue growth factor in tubulointerstitial injury of diabetic nephropathy.* Kidney Int, 2001. **60**(1): p. 96-105.
36. Tesch, G.H., *Macrophages and diabetic nephropathy.* Semin Nephrol, 2010. **30**(3): p. 290-301.
37. Maehr, R., et al., *Cathepsin L is essential for onset of autoimmune diabetes in NOD mice.* J Clin Invest, 2005. **115**(10): p. 2934-43.
38. Schnedl, W.J., et al., *STZ transport and cytotoxicity. Specific enhancement in GLUT2-expressing cells.* Diabetes, 1994. **43**(11): p. 1326-33.
39. Kroncke, K.D., et al., *Nitric oxide generation during cellular metabolization of the diabetogenic N-methyl-N-nitroso-urea streptozotozin contributes to islet cell DNA damage.* Biol Chem Hoppe Seyler, 1995. **376**(3): p. 179-85.
40. Yamamoto, H., Y. Uchigata, and H. Okamoto, *Streptozotocin and alloxan induce DNA strand breaks and poly(ADP-ribose) synthetase in pancreatic islets.* Nature, 1981. **294**(5838): p. 284-6.
41. Basi, S., et al., *Microalbuminuria in type 2 diabetes and hypertension: a marker, treatment target, or innocent bystander?* Diabetes Care, 2008. **31 Suppl 2**: p. S194-201.
42. Moriya, T., et al., *Diabetic retinopathy and microalbuminuria can predict macroalbuminuria and renal function decline in Japanese type 2 diabetic patients: Japan Diabetes Complications Study.* Diabetes Care, 2013. **36**(9): p. 2803-9.
43. Bruno, G., et al., *Progression to overt nephropathy in type 2 diabetes: the Casale Monferrato Study.* Diabetes Care, 2003. **26**(7): p. 2150-5.

44. Rossing, P., P. Hougaard, and H.H. Parving, *Progression of microalbuminuria in type 1 diabetes: ten-year prospective observational study.* Kidney Int, 2005. **68**(4): p. 1446-50.
45. Kistler, A.D., et al., *Enzymatic disease of the podocyte.* Pediatr Nephrol, 2010. **25**(6): p. 1017-23.
46. Kalluri, R., *Proteinuria with and without renal glomerular podocyte effacement.* J Am Soc Nephrol, 2006. **17**(9): p. 2383-9.
47. Jarad, G., et al., *Proteinuria precedes podocyte abnormalities inLamb2-/- mice, implicating the glomerular basement membrane as an albumin barrier.* J Clin Invest, 2006. **116**(8): p. 2272-9.
48. Topham, P.S., et al., *Nephritogenic mAb 5-1-6 is directed at the extracellular domain of rat nephrin.* J Clin Invest, 1999. **104**(11): p. 1559-66.
49. van den Berg, J.G., et al., *Podocyte foot process effacement is not correlated with the level of proteinuria in human glomerulopathies.* Kidney Int, 2004. **66**(5): p. 1901-6.
50. Masola, V., et al., *Heparanase is a key player in renal fibrosis by regulating TGF-beta expression and activity.* Biochim Biophys Acta, 2014. **1843**(9): p. 2122-8.
51. Masola, V., et al., *Impact of heparanase on renal fibrosis.* J Transl Med, 2015. **13**: p. 181.
52. Ko, G.J., et al., *Macrophages contribute to the development of renal fibrosis following ischaemia/reperfusion-induced acute kidney injury.* Nephrol Dial Transplant, 2008. **23**(3): p. 842-52.
53. Chow, F.Y., et al., *Macrophages in streptozotocin-induced diabetic nephropathy: potential role in renal fibrosis.* Nephrol Dial Transplant, 2004. **19**(12): p. 2987-96.
54. Singh, A., et al., *Glomerular endothelial glycocalyx constitutes a barrier to protein permeability.* J Am Soc Nephrol, 2007. **18**(11): p. 2885-93.
55. Salmon, A.H., et al., *Loss of the endothelial glycocalyx links albuminuria and vascular dysfunction.* J Am Soc Nephrol, 2012. **23**(8): p. 1339-50.
56. Garsen, M., et al., *Endothelin-1 Induces Proteinuria by Heparanase-Mediated Disruption of the Glomerular Glycocalyx.* J Am Soc Nephrol, 2016.
57. Chen, S., et al., *Podocytes require the engagement of cell surface heparan sulfate proteoglycans for adhesion to extracellular matrices.* Kidney Int, 2010. **78**(11): p. 1088-99.
58. Chen, S., et al., *Loss of heparan sulfate glycosaminoglycan assembly in podocytes does not lead to proteinuria.* Kidney Int, 2008. **74**(3): p. 289-99.
59. Garsen, M., et al., *Heparanase Is Essential for the Development of Acute Experimental Glomerulonephritis.* Am J Pathol, 2016. **186**(4): p. 805-15.
60. Kramer, A., et al., *Induction of glomerular heparanase expression in rats with adriamycin nephropathy is regulated by reactive oxygen species and the renin-angiotensin system.* J Am Soc Nephrol, 2006. **17**(9): p. 2513-20.
61. Garsen, M., et al., *Vitamin D attenuates proteinuria by inhibition of heparanase expression in the podocyte.* J Pathol, 2015. **237**(4): p. 472-81.
62. Wyczalkowska-Tomasik, A., et al., *Strong association between fibronectin accumulation and lowered cathepsin B activity in glomeruli of diabetic rats.* J Physiol Pharmacol, 2012. **63**(5): p. 525-30.
63. Kumar Vr, S., et al., *Cathepsin S Cleavage of Protease-Activated Receptor-2 on Endothelial Cells Promotes Microvascular Diabetes Complications.* J Am Soc Nephrol, 2016. **27**(6): p. 1635-49.

Chapter 4

Heparanase is essential for the development of acute experimental glomerulonephritis

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Abstract

Heparanase, a heparan sulfate (HS)-specific endoglucuronidase, mediates the onset of proteinuria and renal damage during experimental diabetic nephropathy. Glomerular heparanase expression is increased in the majority of proteinuric diseases. Here, we evaluated the role of heparanase in two models of experimental glomerulonephritis, being anti-glomerular basement membrane and lipopolysaccharide (LPS)-induced glomerulonephritis, in wild type (WT) and heparanase-deficient mice. Induction of experimental glomerulonephritis led to an increased heparanase expression in WT mice, which was associated with a decreased glomerular expression of a highly sulfated HS domain and albuminuria. Albuminuria was reduced in the heparanase-deficient mice in both models of experimental glomerulonephritis, which was accompanied by a better renal function and less renal damage. Notably, glomerular HS expression was preserved in the heparanase-deficient mice. Glomerular leukocyte and macrophage influx was reduced in the heparanase-deficient mice, which was accompanied by a reduced expression of both Th1 and Th2 cytokines. *In vitro*, tumor necrosis factor (TNF)-α and LPS directly induced heparanase expression and increased transendothelial albumin passage. Our study shows that heparanase contributes to proteinuria and renal damage in experimental glomerulonephritis by decreasing glomerular HS expression, enhancing renal leukocyte and macrophage influx, and affecting the local cytokine milieu.

Introduction

Proteinuria is a hallmark of many glomerular diseases and an independent risk factor for the progression of renal failure [1]. Heparan sulfate (HS) is a highly negatively charged glycosaminoglycan (GAG) that is covalently attached to a core protein, so called HS proteoglycans (HSPGs). In seminal papers Kanwar and Farquhar demonstrated the presence of HS in the glomerular filtration barrier (GFB), which is composed of glomerular endothelial cells covered by a glycocalyx, the glomerular basement membrane (GBM) and podocytes [2-4]. Due to its negative charge, HS seems to play an important role in the charge-selective permeability of the GFB [5]. Removal of HS with bacterial heparinase leads to a dramatic increase in glomerular permeability for neutral and cationic macromolecules [6]. Genetic targeting of HS in the GFB compromised permselectivity and barrier function to a lesser extent [7-9], although the development of proteinuria has been associated with a reduced expression of HS in the GFB [3, 5, 10]. The HS chain is composed of up to 150 α(1-4)-glucuronate-β(1,4)-N-acetyl-glucosamine disaccharide units that can be modified extensively. HS is characterized by an enormous structural diversity, which dictates the binding of several soluble ligands, such as cytokines, chemokines and growth factors [11, 12]. In addition, specific endothelial HS domains mediate the trafficking of leukocytes [13].

In many human and experimental glomerular diseases the reduced expression of HS in the GFB is associated with an increased expression of heparanase [3, 14]. Heparanase is an endo-β(1,4)-D-glucuronidase that can cleave HS side chains. Heparanase is synthesized as a pre-proheparanase of 68 kDa. To gain its biological activity, pre-proheparanase is processed in the endoplasmatic reticulum, where the signal peptide is removed, and further processed in lysosomes, where cathepsin L cleaves off a linker domain to form the active form of heparanase [14, 15]. Outside the kidney heparanase is involved in cancer progression, in particular metastasis and neovascularization [16-18]. Heparanase is also involved in the pathogenesis of several inflammatory disorders, such as inflammatory lung injury, rheumatoid arthritis and chronic colitis [19-22]. Recently, we demonstrated that heparanase is essential for the development of proteinuria in experimental diabetic nephropathy (DN) [23]. In streptozotocin-induced diabetes, heparanase-deficient mice failed to develop proteinuria and renal damage, in contrast to their wild type (WT) littermates. In addition, proteinuria was reduced and renal function improved by treatment with the heparanase inhibitor SST0001 [23]. In a follow-up study, we showed that heparanase contributes to the inflammatory cascade during the pathogenesis of diabetic nephropathy [24]. Although heparanase plays a crucial role in the development of diabetic nephropathy, the exact role of heparanase in inflammatory glomerular diseases, such as glomerulonephritis, is still unknown.

Glomerulonephritis is characterized by the influx of inflammatory cells, proteinuria, hematuria and a decline in renal function. Previous studies revealed that heparanase may be involved in the development of proteinuria in passive Heymann nephritis (PHN) and in a model of accelerated anti-GBM disease, as treatment of rats with a polyclonal antibody against heparanase or the heparanase inhibitor PI-88 reduced proteinuria in both experimental diseases [25-27]. In addition, it has been recently described that lipopolysaccharide (LPS)-induced glomerulonephritis involves an increased heparanase expression and a damaged glomerular endothelium, which is in large part mediated by tumor necrosis factor (TNF)-α [28]. Interestingly, heparanase inhibition prevented glycocalyx loss and neutrophil adhesion during sepsis-induced acute lung injury, indicating that heparanase may also be involved in the influx of inflammatory cells [19]. In another recent study, severe systemic sepsis was induced by cecal ligation and puncture, and it was suggested that heparanase mediated early renal dysfunction. Unfortunately, in the latter study there was no direct proof for a reduced HS expression in the GFB mediated by heparanase, whereas the applied anti-HS antibody (3G10) suggests involvement of bacterial-derived heparinases instead of mammalian heparanase [29].
In order to elucidate the role of heparanase in the development of glomerulonephritis, we evaluated the involvement of heparanase in two experimental glomerulonephritis models, anti-GBM and LPS-induced glomerulonephritis, in WT and heparanase-deficient mice. Our results indicate that heparanase drives anti-GBM and LPS-induced glomerulonephritis by enhancing the renal influx of inflammatory cells and by influencing the local cytokine production.

Materials and methods

Animals

C57Bl/6 (Harlan Laboratories, Jerusalem, Israel) and heparanase knockout (KO) mice [30] in a C57Bl/6 background were kept under pathogen-free conditions, housed in a temperature-controlled room with a 12-hour light/dark cycle, and had *ad libitum* access to food and water. All animal experiments were performed in accordance with, and approved by, the Hebrew University Institutional Animal Care and Use Committee.

Induction of anti-GBM and LPS glomerulonephritis and determination of albuminuria and blood urea nitrogen

Experimental anti-GBM glomerulonephritis was induced as previously described [31]. Wild type (WT) and heparanase-deficient mice, 14- to 15-weeks-old, were injected in the tail vein

with 7 mg rabbit anti-mouse GBM IgG serum. Mice were sacrificed after 2 hours, 1 day and 4 days to collect kidneys and blood. LPS glomerulonephritis was induced in 14- to 15-weeks-old WT and heparanase-deficient mice by an intraperitoneal injection with 80 µg LPS (O111:B4; Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands). Mice were sacrificed after 2 days to collect kidneys and blood. Eight mice were used per time-point. Urine was collected through a bladder punction or after 24 hours in metabolic cages. Collected kidneys were snap frozen in liquid nitrogen. Urinary albumin was measured by radial immunodiffusion (Mancini) and blood urea nitrogen and urinary creatinine concentrations were determined routinely in our clinical diagnostic facility.

Immunofluorescence staining

Immunofluorescence staining was performed on 2 µM thick cryosections as described [31]. Directly labeled antibodies included rat anti-mouse GR-1 (RB6.8C5)-FITC (BD Biosciences, Alphen aan de Rijn, The Netherlands) and rat anti-mouse CD41-Alexa 488 (ITK Diagnostics, Uithoorn, The Netherlands). Unlabeled primary antibodies included CD68 (MCA1957) (Serotec, Oxford, UK), and the VSV-tagged anti-HS antibodies HS4C3, AO4B08, EW4G2, and EW3D10 [31, 32]. Appropriate secondary antibodies include Alexa 488 antibodies (Invitrogen Life Technologies, Breda, The Netherlands) or anti-VSV-Cy3 antibody (Sigma-Aldrich). Capillary loops were visualized with the hamster anti-agrin antibody (MI91) [33], recognized by a Cy-3-labeled antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were postfixed with 1% paraformaldehyde-PBS and embedded in Vectashield mounting medium H-1000 (Brunschwig Chemie, Amsterdam, The Netherlands). HS was scored semi-quantitatively for staining intensities on a scale between 0 and 10 (0= no staining, 5= 50% staining, 10= 100% staining) by two investigators. Glomerular influx of granulocytes and macrophages was determined by counting the number of cells per 50 glomeruli. Glomerular influx of platelets in LPS glomerulonephritis was determined by counting the number of platelets per 50 glomeruli, whereas the number of glomeruli with platelet aggregates per 50 glomeruli was counted for anti-GBM glomerulonephritis. Scoring was performed using a Leica CTR6000 microscope by two independent investigators on blinded sections.

Renal histology

Renal cryosections (2 µM) were fixed in 96% ethanol and stained with periodic acid-Schiff (PAS). Sections were counterstained with hematoxylin and evaluated by an experienced nephropathologist. At least 50 glomeruli per mouse were analyzed for the presence of glomerular lesions and mesangial matrix expansion. The average of glomeruli with lesions or mesangial matrix expansion was calculated.

Heparanase activity assay

The activity of heparanase in renal cortex was determined by a commercially available assay (AMS Biotechnology, Abingdon, UK). Briefly, tissue lysates (100 µl) were mixed 1:1 with reaction buffer and incubated on biotinylated HS-coated plates for 1.5 hours at 37°C. Plates were washed and incubated with Strep-HRP for 60 minutes at room temperature. Peroxidase substrate was added and the reaction was stopped by 0.2 N H_2SO_4. Absorbance was measured at 450 nm.

Cell Culture

Conditionally immortalized mouse glomerular endothelial cells (mGEnC-1) and mouse podocytes (MPC-5) were cultured as previously described [34, 35]. Silencing of heparanase in mGEnC-1 was achieved after transfecting a heparanase shRNA construct (Qiagen, Venlo, The Netherlands) with Lipofectamine 2000 into undifferentiated mGEnC-1 and subsequent selection with G418 (Sigma-Aldrich). Where indicated, differentiated mGEnC-1 and podocytes were stimulated with 10 ng/ml TNF-α (Invitrogen Life Technologies) or 1 µg/ml LPS (Sigma-Aldrich) for 18 hours. All experiments were performed at least twice for confirmation.

Transendothelial albumin passage

mGEnC-1 seeded on polyester membranes in tissue culture inserts (0.4 µm pore size; Corning Incorporated, NY, USA) were after differentiation treated with TNF-α or LPS as outlined. After 18 hours, medium in the insert was replaced by serum free medium (SFM) containing 0.5 mg/ml FITC-labeled BSA (Sigma-Aldrich) and medium in the well was replaced by SFM. Aliquots were removed from the well after 1, 2 and 3 hours and replaced by SFM. Fluorescence was measured with excitation at 495 nm and emission at 520 nm and the amount of albumin passing the endothelial cell monolayer was determined by a set of standard dilutions.

RNA isolation and real-time PCR

Total RNA was isolated from mGEnC-1, MPC-5 podocytes and kidney cortex using the RNeasy mini kit (Qiagen). 1 µg RNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). One-tenth of cDNA was used as template in real-time PCR reaction using SYBR Green SuperMix (Roche Diagnostics, Mannheim, Germany) with gene-specific primers (Isogen Life Science, De Meern, The Netherlands; Table 1) on the CFX real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Gene expression levels were quantified using the delta-delta C_T method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene.

Table 1: Primers used in real-time PCR.

Target gene	Primer sequence
HPSE	(F) 5'-GAGCGGAGCAAACTCCGAGTGTATC-3'
	(R) 5'-GATCCAGAATTTGACCGTTCAGTT-3'
TNF-α	(F) 5'-CATCTTCTCAAAATTCGAGTGACAA-3'
	(R) 5'-TGGGAGTAGACAACGTACAACCC-3'
IFN-γ	(F) 5'-GGTGACCTTGTGACAAGCTC-3'
	(R) 5'-TGCTGTGTGGTCTGTCTGTC-3'
IL-6	(F) 5'-TTCCTCTCTGCAAGAGACT-3'
	(R) 5'-TGTATCTCTCTGAAGGACT-3'
IL-10	(F) 5'-GTGGAGCAGGTGAAGAGTGA-3'
	(R) 5'-TGCAGTTGATGAAGATGTCAAA-3'
IL-12α	(F) 5'-GGGAGAAGCAGACCCTTACAGA-3'
	(R) 5'-GGGTGCTGAAGGCGTGAA-3'
IL-12β	(F) 5'-GGAAGCACGGCAGCAGAATC-3'
	(R) 5'-AACTTGAGGGAGAAGTAGGAATGG-3'
TNFRSF1a	(F) 5'-CCATCATTTGTAGGGATCCC-3'
	(R) 5'-TCTCAGAGCCTCGAGGATAT-3'
TNFRSF1b	(F) 5'-GAAATCCCAGGATGCAGTAG-3'
	(R) 5'-TCAGGCCACTTTGACTGCAA-3'
Sdc-1	(F) 5'-GACTCTGACAACTTCTCTGGCTCT-3'
	(R) 5'-GCTGTGGTGACTCTGACTGTTG-3'

HPSE, heparanase; Sdc-1, syndecan-1; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL, interleukin; TNFRSF, TNF receptor; F, forward; R, reverse.

Statistical analysis

Values are expressed as mean ± SEM. Significance was evaluated by a one-way ANOVA and *post hoc* analysis with Tukey's multiple comparison test. Comparison of expression between two different groups was evaluated using the Student's *t*-test. The non-parametric Spearman's rank test was used to calculate the correlation between HS expression and proteinuria. A 2-way repeated measures ANOVA with Bonferroni post-test was used to evaluate significance for the transendothelial albumin passage experiments. Statistical analysis was performed using GraphPad Prism 5.03 (GraphPad Software, Inc., San Diego, CA, USA). A P-value of ≤ 0.05 was considered statistically significant.

Results

Heparanase expression and activity are increased by induction of experimental glomerulonephritis

Heparanase mRNA expression in kidney cortex was significantly increased 2 hours, 1 day and 4 days after the induction of anti-GBM glomerulonephritis (Figure 1A) and 2 days after the induction of LPS glomerulonephritis (Figure 1B). In WT mice, the increased heparanase expression correlated with an increased heparanase activity after induction of both anti-GBM and LPS glomerulonephritis, although heparanase activity was not significantly increased 2 hours after the induction of anti-GBM glomerulonephritis (Figure 1C and 1D; P = 0.09). Notably, we choose to evaluate day 2 for the LPS-induced model for glomerulonephritis, since we did not observe differences in the urinary albumin/creatinine ratio, nor in renal heparanase activity 1 day after administration of LPS in WT mice (Figure 2).

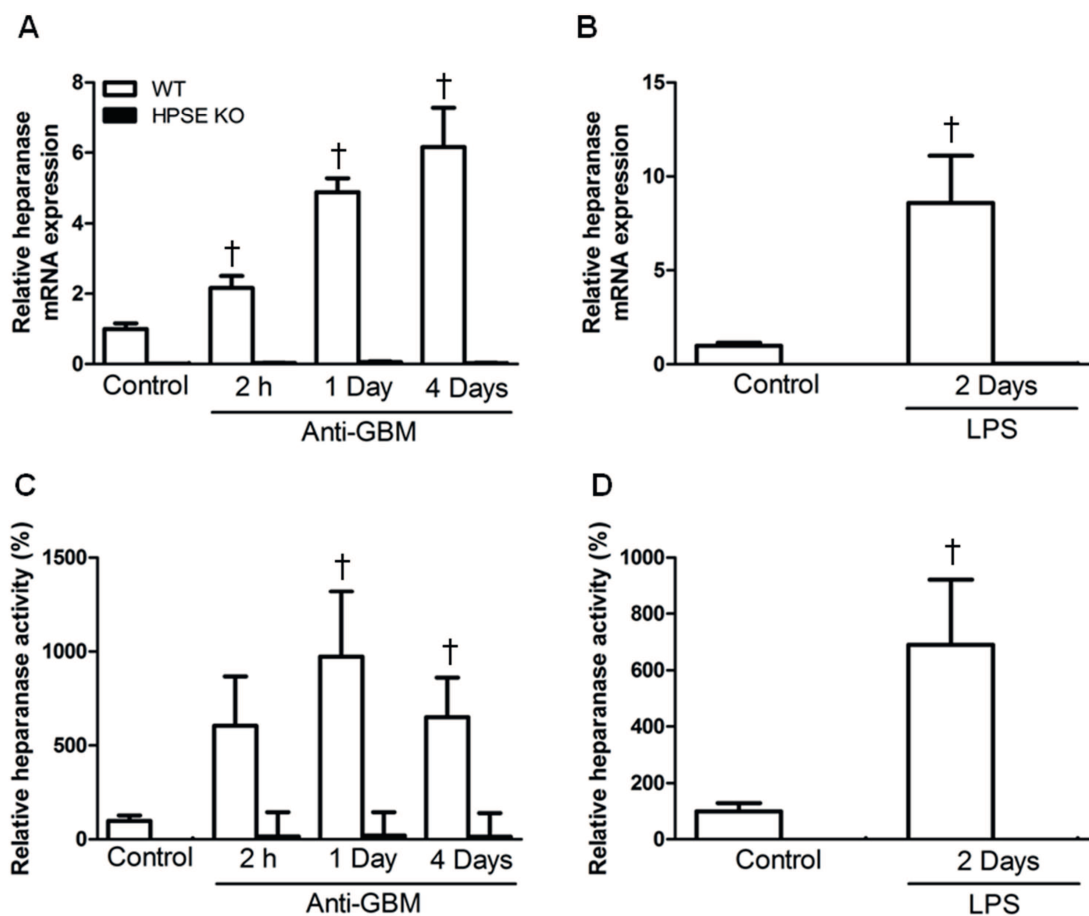

Figure 1: Experimental glomerulonephritis increases renal heparanase expression and activity in WT mice. (**A,B**) Heparanase mRNA expression and (**C,D**) heparanase activity are increased by induction of (**A,C**) anti-GBM glomerulonephritis and (**B,D**) LPS glomerulonephritis. Heparanase was not expressed or active in the heparanase-deficient mice. *P<0.05 versus control. WT; wild type. HPSE-KO; heparanase-deficient.

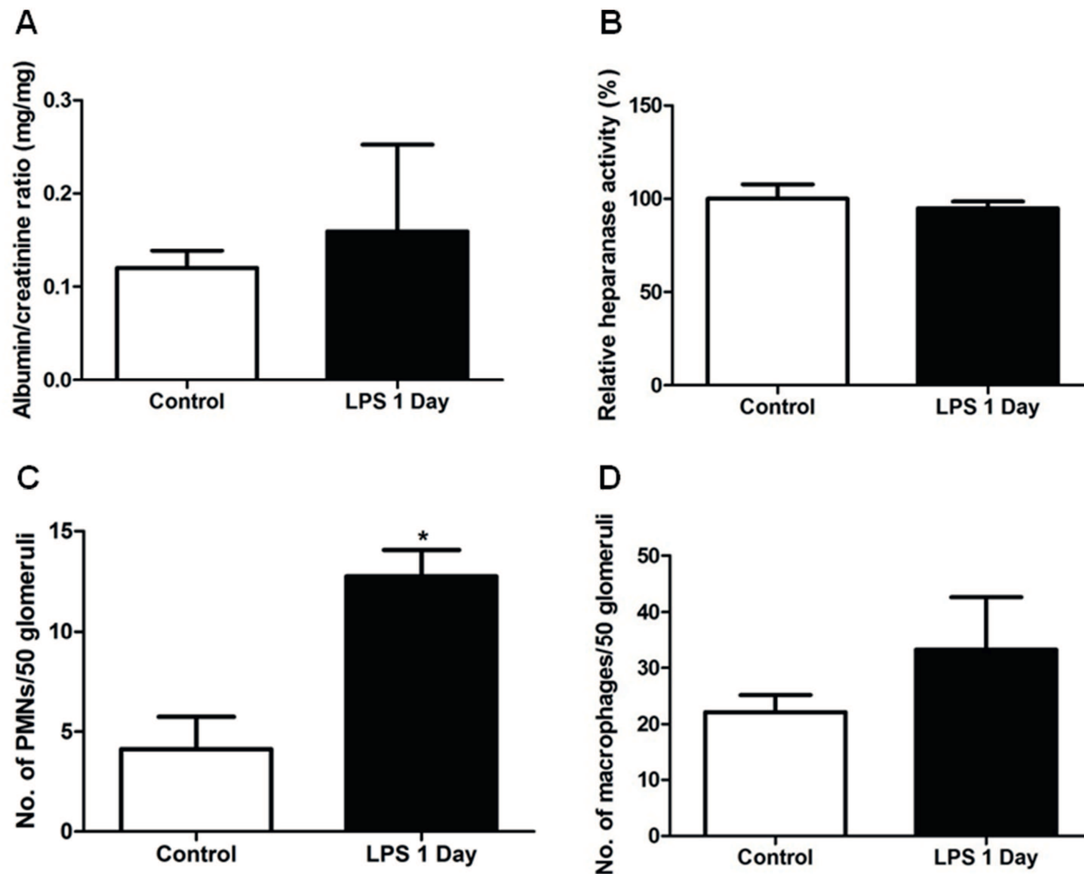

Figure 2: Renal function and heparanase activity are normal 1 day after induction of LPS glomerulonephritis. (**A**) WT mice failed to develop albuminuria 1 day after the induction of LPS glomerulonephritis. (**B**) Heparanase activity is comparable to control 1 day after induction of LPS glomerulonephritis in WT mice. (**C**) Glomerular PMN influx, analyzed by immunofluorescence staining, was significantly increased 1 day after induction of LPS glomerulonephritis in WT mice, whereas (**D**) glomerular macrophage influx was comparable to control 1 day after the induction of LPS glomerulonephritis. $^*P<0.05$ versus control.

Heparanase-deficient mice show better renal function and less renal damage after induction of experimental glomerulonephritis

A significant proteinuria was observed 1 and 4 days after the induction of anti-GBM glomerulonephritis (Figure 3A) and 2 days after the induction of LPS glomerulonephritis in WT mice (Figure 3B). Proteinuria was reduced in the heparanase-deficient mice in both models, although this was borderline significant 4 days after induction of anti-GBM glomerulonephritis ($P = 0.06$). Renal function, as measured by the blood urea nitrogen (BUN) concentration, was impaired 4 days after induction of anti-GBM glomerulonephritis in the WT mice, whereas it was normal in the heparanase-deficient mice (Figure 3C). By induction of LPS glomerulonephritis, renal function was significantly decreased in both WT and heparanase-deficient mice (Figure 3D). However, renal function was significantly better in the heparanase-deficient mice compared to WT mice. WT mice showed significantly more glomerular damage compared to the heparanase-deficient mice, for both the anti-GBM and

LPS model (Figure 4A-C). By induction of anti-GBM glomerulonephritis, WT mice showed significantly more glomerular lesions compared to the heparanase-deficient mice (Figure 4A-B). Moreover, in the LPS model, mesangial matrix expansion, although limited, was only observed in the WT mice and not in the heparanase-deficient mice (Figure 4A,C).

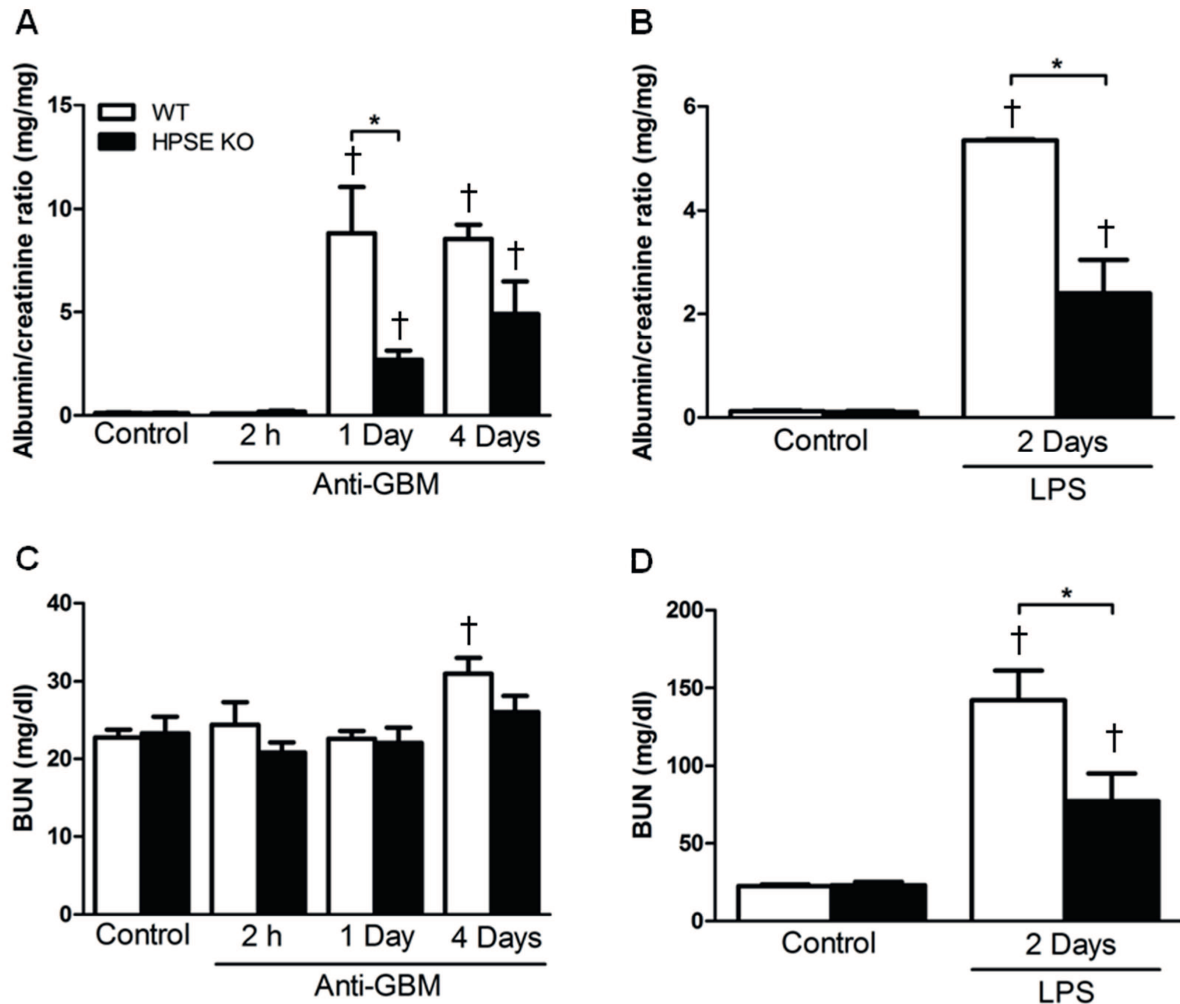

Figure 3: Heparanase-deficient mice show better renal function during anti-GBM and LPS-induced glomerulonephritis. The albumin/creatinine ratio was significantly increased in WT mice compared with untreated control mice (**A**) 1 and 4 days after induction of anti-GBM glomerulonephritis and (**B**) 2 days after induction of LPS glomerulonephritis. Heparanase-deficient mice showed a lower albuminuria at these time points (P = 0.06 versus WT mice 4 days after induction of anti-GBM glomerulonephritis). (**C**) Plasma blood urea nitrogen (BUN) levels were significantly increased in WT mice compared with untreated control mice 4 days after induction of anti-GBM glomerulonephritis. Plasma BUN levels were normal in the heparanase-deficient mice. (**D**) Plasma BUN levels were significantly lower in the heparanase-deficient mice compared with WT mice 2 days after induction of LPS glomerulonephritis. $^{\dagger}P<0.05$ versus control. $^{*}P<0.05$ versus anti-GBM/LPS-injected WT mice. WT; wild type. HPSE-KO; heparanase-deficient.

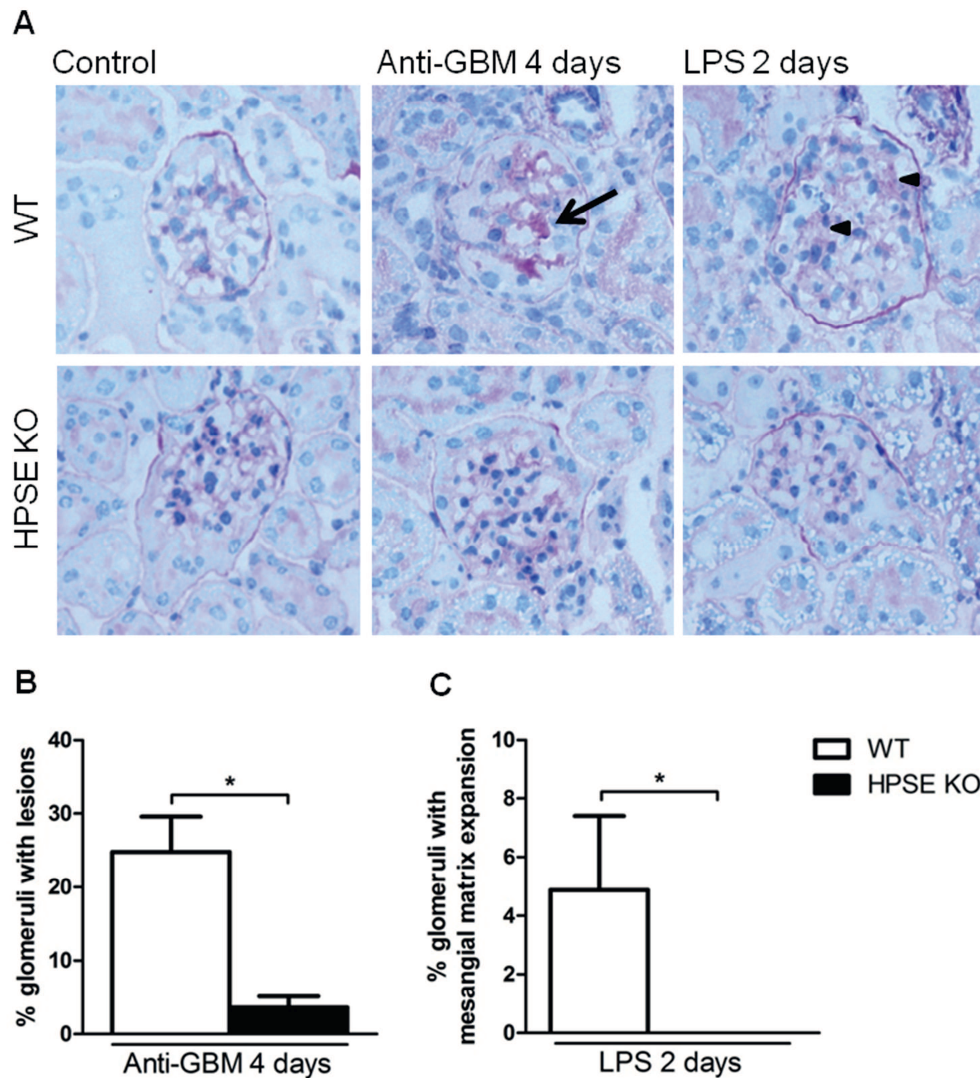

Figure 4: Heparanase-deficient mice show less glomerular injury during anti-GBM and LPS-induced glomerulonephritis. (**A**) Representative pictures of renal histology and semi-quantitative analysis of the percentage of (**B**) glomeruli with lesions (arrow) and (**C**) glomeruli with mesangial matrix expansion (arrowhead), as determined by a PAS stain. WT mice showed significantly more glomerular injury compared with heparanase-deficient mice 2 days after induction of LPS glomerulonephritis and 4 days after induction of anti-GBM glomerulonephritis. (**A**) Magnification x 400. (**B**,**C**) **P*<0.05 versus anti-GBM/LPS-injected WT mice. WT; wild type. HPSE-KO; heparanase-deficient.

Heparanase deficiency preserves glomerular HS expression in experimental glomerulonephritis

As heparanase plays an important role in HS turnover, we determined the glomerular expression of a highly sulfated HS domain recognized by the anti-HS antibody HS4C3. Glomerular HS expression in WT mice was reduced 1 and 4 days after the induction of anti-GBM glomerulonephritis (Figure 5A-B) and 2 days after the induction of LPS glomerulonephritis (Figure 5A,C). Heparanase deficiency preserved HS expression in both models. HS expression inversely correlated with the level of proteinuria (Figure 5D; r = -0.79,

P < 0.0001), which is in line with previous results [10], but also in line with the data on heparanase expression and activity (Figure 1).

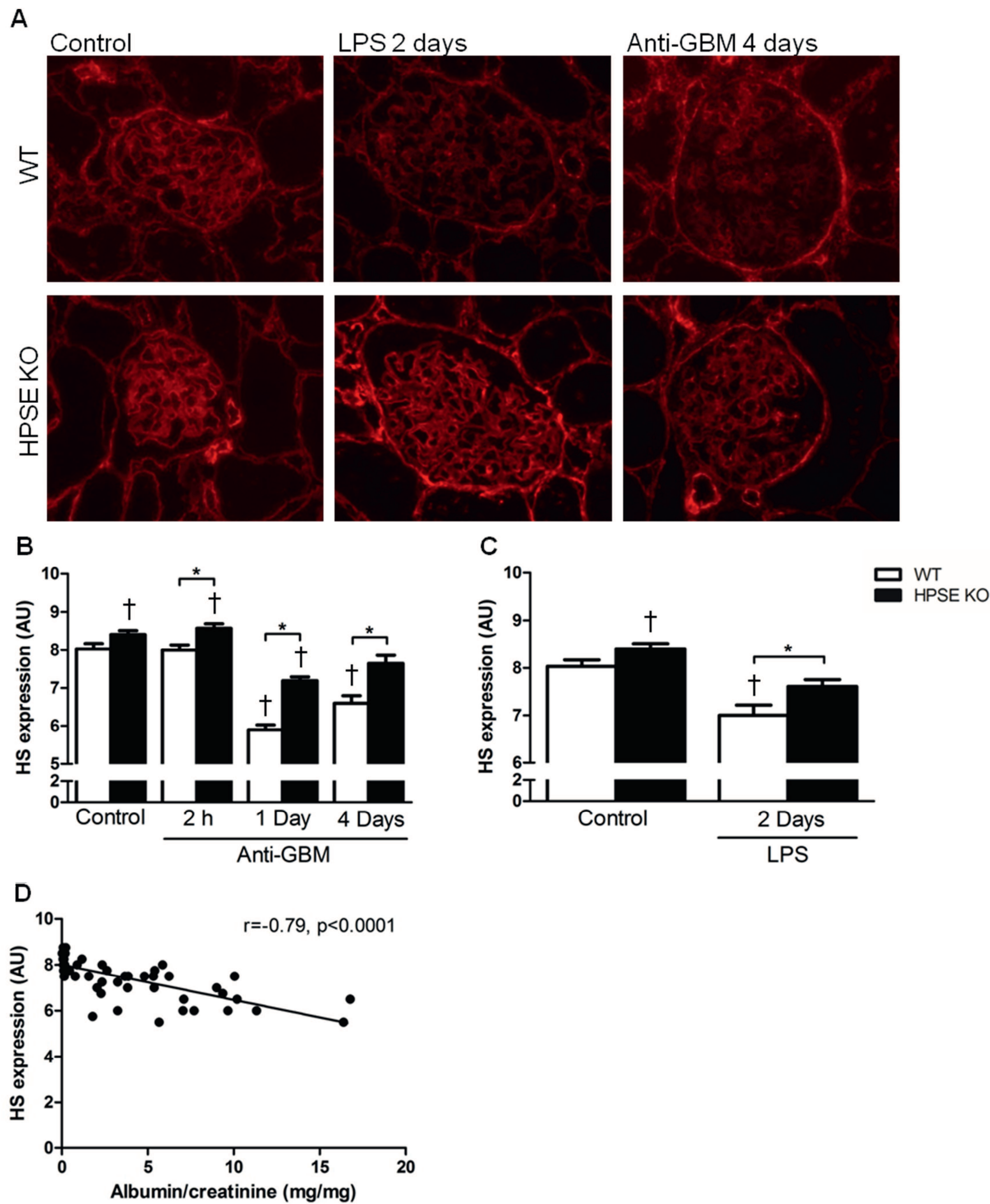

Figure 5: Heparanase deficiency preserves glomerular HS expression in experimental glomerulonephritis. (**A**) Immunofluorescence staining and (**B,C**) semi-quantitative analysis of the glomerular expression of the highly sulfated HS domain recognized by the anti-HS antibody HS4C3 showed a reduced glomerular expression (**B**) 1 and 4 days after the induction of anti-GBM glomerulonephritis and (**C**) 2 days after the induction of LPS glomerulonephritis. HS expression was significantly higher in the heparanase-deficient mice. (**D**) HS expression inversely correlated with the level of proteinuria (r=-0.79, P<0.0001). (**A**) Magnification x 400. (**B,C**) †P<0.05 versus control. *P<0.05 versus anti-GBM/LPS-injected WT mice. AU; arbitrary units. WT; wild type. HPSE-KO; heparanase-deficient.

Heparanase deficiency leads to a reduced glomerular influx of leukocytes and macrophages in experimental glomerulonephritis

Next, we evaluated the role of heparanase on renal inflammation by determining the glomerular influx of polymorphonuclear granulocytes (PMNs) and macrophages. Glomerular PMN influx is an important mediator of glomerular damage and proteinuria in the early heterologous phase of anti-GBM glomerulonephritis. Glomerular PMN influx was maximal 2 hours after the induction of anti-GBM glomerulonephritis in both WT and heparanase-deficient mice and decreased over time (Figure 6A). Notably, after 2 hours, glomerular PMN influx was significantly reduced in the heparanase-deficient mice compared to the WT mice.

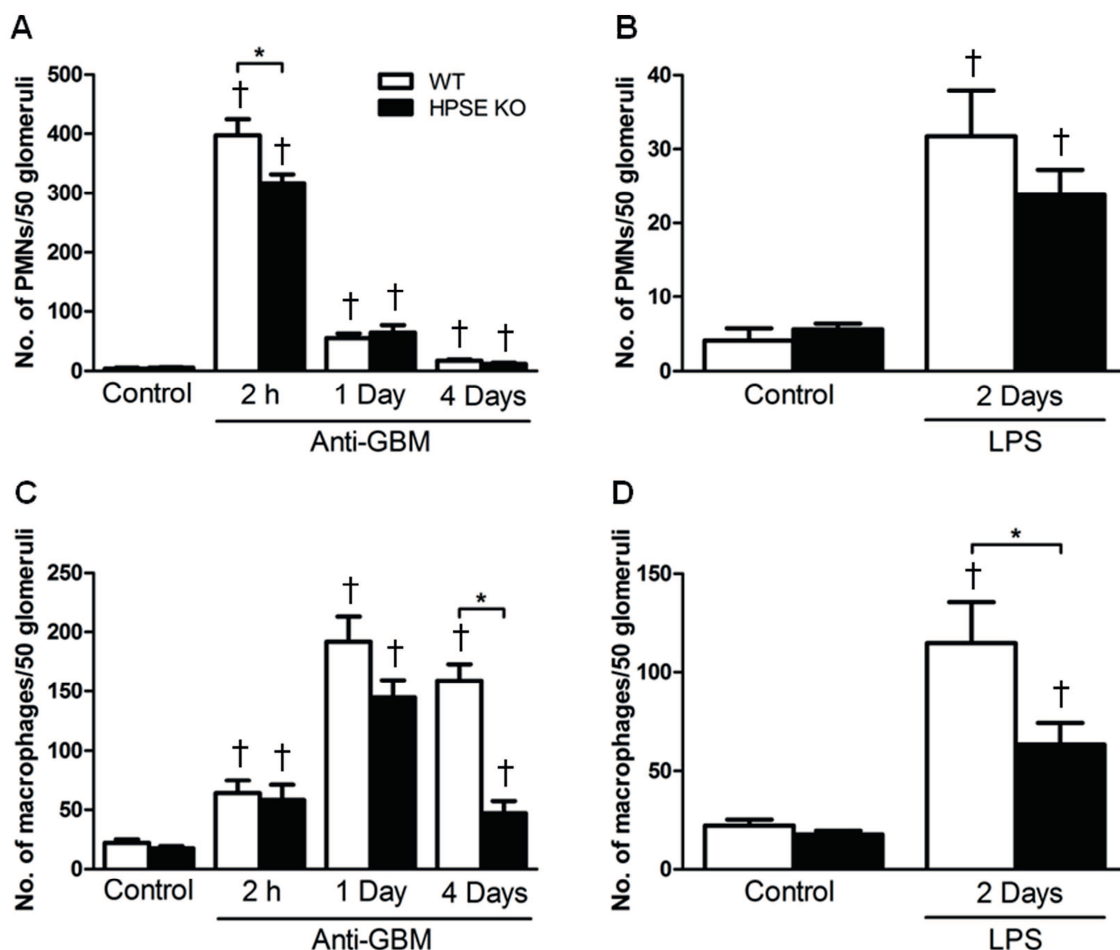

Figure 6: Heparanase deficiency reduces glomerular leukocyte and macrophage influx during experimental glomerulonephritis. (**A**) Glomerular PMN influx, analyzed by immunofluorescence staining, peaked 2 hours after induction of anti-GBM glomerulonephritis in both WT and heparanase-deficient mice, but was significantly lower in the heparanase-deficient mice. (**B**) Glomerular PMN influx was increased by induction of LPS glomerulonephritis, but not significantly different between WT and heparanase-deficient mice. Glomerular macrophage influx was significantly increased after induction of both (**C**) anti-GBM glomerulonephritis and (**D**) LPS glomerulonephritis. Heparanase-deficient mice showed a significantly reduced glomerular macrophage influx 4 days after induction of anti-GBM glomerulonephritis and 2 days after induction of LPS glomerulonephritis. †P<0.05 versus control. *P<0.05 versus anti-GBM/LPS-injected WT mice. WT; wild type. HPSE-KO; heparanase-deficient.

In line with this, the expression of several inflammatory HS domains on endothelium that were previously shown to be important for leukocyte trafficking was also reduced in the heparanase-deficient mice (Figure 7) [13, 31, 36]. Mice with LPS-induced glomerulonephritis did not show significant differences in glomerular PMN influx when comparing WT mice and heparanase-deficient mice (Figure 6B). Glomerular macrophage influx was increased after induction of both anti-GBM and LPS glomerulonephritis (Figure 6C-D). A significant lower number of macrophages was observed in glomeruli of heparanase-deficient mice 4 days after the induction of anti-GBM glomerulonephritis and 2 days after the induction of LPS glomerulonephritis (Figure 6C-D). Taken together, heparanase deficiency significantly reduced glomerular leukocyte influx in anti-GBM glomerulonephritis and glomerular macrophage influx in both models of experimental glomerulonephritis.

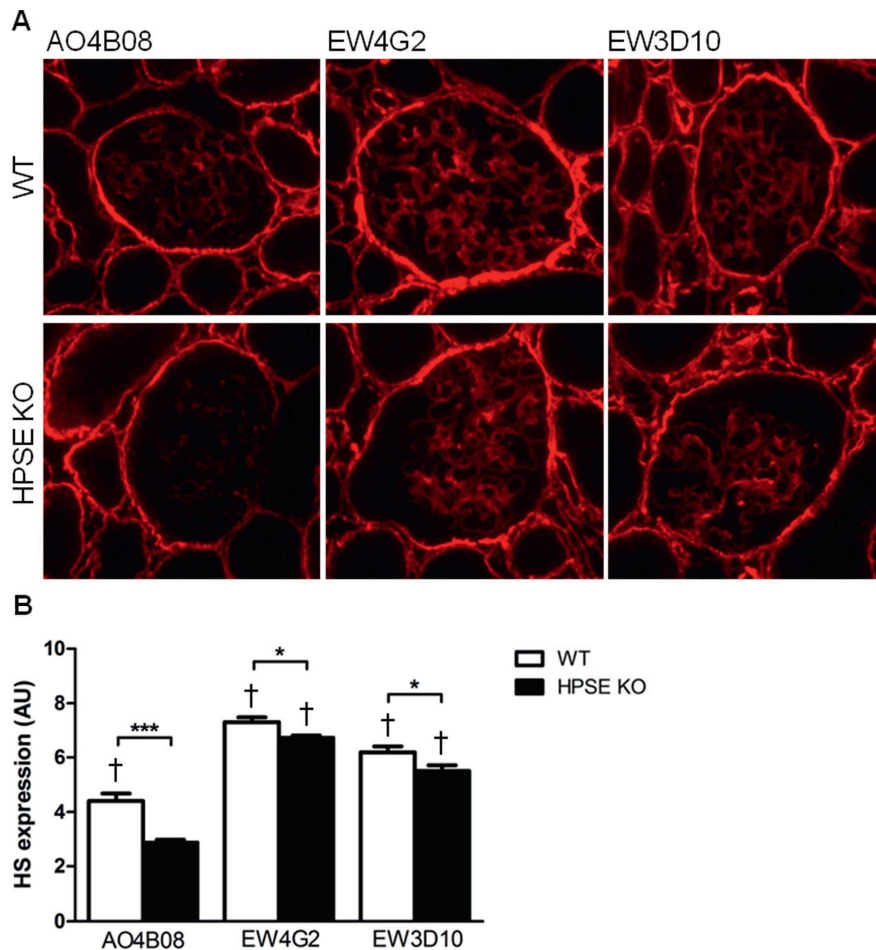

Figure 7: Heparanase deficiency reduces the expression of inflammatory HS domains during anti-GBM-induced glomerulonephritis. (**A**) Immunofluorescence staining and (**B**) semi-quantitative analysis of the glomerular expression of inflammatory HS domains recognized by the anti-HS antibodies AO4B08, EW4G2 and EW3D10 showed a reduced expression of the inflammatory HS domains in the heparanase-deficient mice compared with WT mice 2 hours after induction of anti-GBM glomerulonephritis. (**A**) Magnification x 400. (**B**) $^{\dagger}P<0.05$ versus control. $^{*}P<0.05$ and $^{***}P<0.001$ versus anti-GBM/LPS-injected WT mice. AU; arbitrary units. WT; wild type. HPSE-KO; heparanase-deficient.

The presence of glomerular platelets is decreased in LPS-induced glomerulonephrits and increased in anti-GBM glomerulonephritis

Heparanase during experimental glomerulonephritis may originate from glomerular cells, inflammatory cells and/or platelets. Therefore, we also evaluated the glomerular presence of platelets using the platelet-specific marker CD41. At day 2 after LPS administration the number of platelets was similar for WT and heparanase-deficient mice. Interestingly, in the LPS model the number of platelets was decreased compared to untreated controls in both WT and heparanase-deficient mice (Figure 8), which may suggest that in the LPS model platelet-derived heparanase is not contributing to pathology. In anti-GBM glomerulonephritis in both WT and heparanase-deficient mice, there was a significant increase in the number of glomeruli with aggregates of platelets already 2 hours after administration of anti-GBM serum, which further increased at day 1 and 4 (Figure 8). The number of glomeruli with platelet aggregates was significantly reduced in the heparanase-deficient mice 4 days after induction of anti-GBM glomerulonephritis (Figure 8).

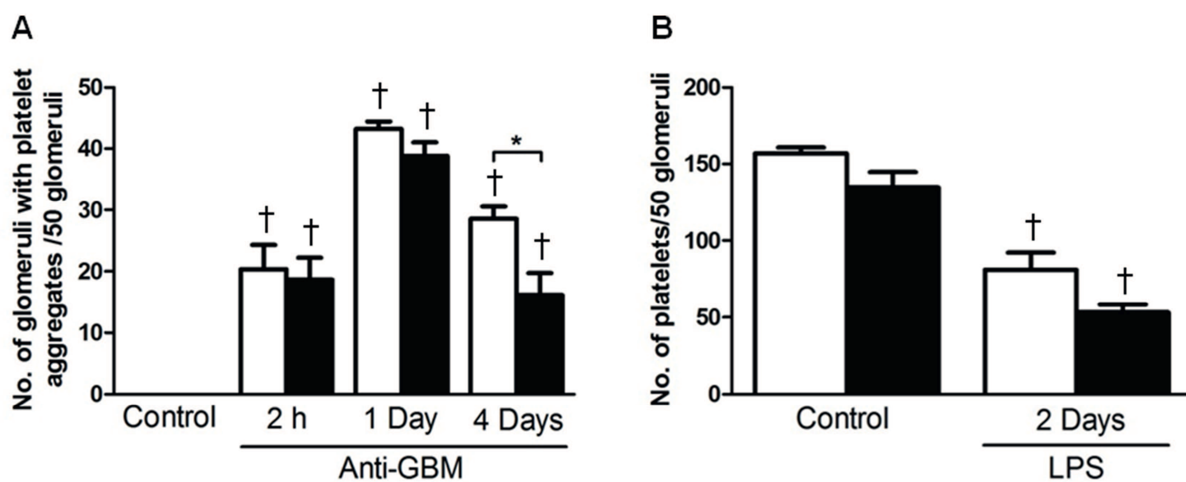

Figure 8: Glomerular platelet influx is increased in anti-GBM glomerulonephritis and reduced in LPS-induced glomerulonephritis. (**A**) Glomerular platelet influx, analyzed by counting the number of glomeruli with platelet aggregates per 50 glomeruli, was significantly increased 2 hours, 1 day and 4 days after induction of anti-GBM glomerulonephritis. The presence of glomerular platelet aggregates was significantly reduced in the heparanase-deficient mice 4 days after induction of anti-GBM glomerulonephritis. (**B**) Glomerular platelet influx, analyzed by counting the number of platelets per 50 glomeruli, was significantly reduced 2 days after induction of LPS glomerulonephritis in both WT and heparanase-deficient mice. †*P*<0.05 versus control. **P*<0.05 versus anti-GBM-injected WT mice. WT; wild type. HPSE-KO; heparanase-deficient.

Heparanase deficiency leads to a reduced expression of Th1 and Th2 cytokines in anti-GBM and LPS glomerulonephritis

As we observed a reduced glomerular influx of leukocytes and macrophages in heparanase-deficient mice after induction of experimental glomerulonephritis in both the anti-GBM and

LPS model, we analyzed the expression of Th1 and Th2 (promoting) cytokines in the renal cortex of these mice (Table 2 and 3). The mRNA expression of Th1 (promoting) cytokines TNF-α, interferon-γ, interleukin (IL)-12α and IL-12β was lower in the heparanase-deficient mice compared to the WT mice in both anti-GBM and LPS-induced glomerulonephritis. In addition, mRNA expression of the Th2 cytokine IL-10 was also lower in the heparanase-deficient mice in both models. The expression of IL-6, which is both a pro- and anti-inflammatory cytokine, was higher in the heparanase-deficient mice compared to the WT mice in the very early phase after induction of anti-GBM glomerulonephritis, but was significantly lower in the heparanase-deficient mice compared to the WT mice after induction of LPS glomerulonephritis. Furthermore, the expression of TNF receptor 1a was lower in the heparanase-deficient mice compared to the WT mice, whereas the expression of TNF receptor 1b was comparable to the WT mice. We previously showed that syndecan-1 deficiency aggravates anti-GBM glomerulonephritis, by skewing the Th1/Th2 response [31]. In line with previous results, in the current study syndecan-1 mRNA expression is increased after induction of anti-GBM and LPS glomerulonephritis in the WT mice, whereas syndecan-1 mRNA expression was lower in the heparanase-deficient mice compared to WT mice in the very early phase of the anti-GBM model, and 10-fold lower in the LPS model (Table 2 and 3). Taken together, heparanase deficiency in experimental glomerulonephritis leads to a less pro-inflammatory cytokine milieu in the kidney.

Table 2: Quantitative mRNA expression of syndecan-1 and Th1 and Th2 cytokines in renal cortex of WT and heparanase-deficient mice during LPS glomerulonephritis[a].

	2 days	
	WT	**HPSE-KO**
TNF-α	18.02 ± 5.69**	6.05 ± 1.13***,†
IFN-γ	1.64 ± 0.38	1.02 ± 0.29
IL-6	1341 ± 764**	282 ± 141*
IL-10	37.92 ± 15.41*	22.80 ± 10.45
IL-12α	4.78 ± 1.66*	0.90 ± 0.25†
IL-12β	1.68 ± 0.79	1.55 ± 0.84
TNFRSF1a	4.25 ± 0.63***	2.29 ± 0.33**,†
TNFRSF1b	9.77 ± 3.08*	14.32 ± 3.96**
Sdc-1	19.51 ± 6.02**	2.10 + 0.42*,†

TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL, interleukin; TNFRSF, TNF receptor; Sdc-1, syndecan-1; WT, wild type; HPSE-KO, heparanase-deficient. The mean ± SEM of eight mice per group are depicted. **P*<0.05, ***P*<0.01, and ****P*<0.001 versus control mice; †*P*<0.05 HPSE-KO versus WT mice. [a]The relative expression compared with untreated WT or HPSE-KO mice is shown, with both untreated WT and untreated HPSE-KO mice set at 1.

Table 3: Quantitative mRNA expression of syndecan-1 and Th1 and Th2 cytokines in renal cortex of WT and heparanase-deficient mice during anti-GBM glomerulonephritis[a].

	2 hours		1 day		4 days	
	WT	**HPSE-KO**	**WT**	**HPSE-KO**	**WT**	**HPSE-KO**
TNF-α	83.84 ± 14.38***	64.77 ± 7.84***	5.14 ± 1.18**	3.89 ± 0.75**	5.08 ± 0.98***	1.87 ± 0.57†
IFN-γ	8.59 ± 0.74***	5.71 ± 1.35**	1.74 ± 0.20**	1.03 ± 0.09†	2.70 ± 0.36***	0.96 ± 0.11†
IL-6	5599 ± 1144**	21269 ± 3328*,†	22.23 ± 4.30**	39.80 ± 8.63	18.49 ± 5.86	12.67 ± 3.47
IL-10	38.70 ± 5.39***	22.85 ± 2.48***,†	5.70 ± 3.42	1.95 ± 0.35	3.41 ± 0.79*	2.50 ± 0.49*
IL-12α	10.65 ± 2.82**	3.47 ± 0.34***,†	1.77 ± 0.30*	0.83 ± 0.08†	2.30 ± 0.28**	0.98 ± 0.24†
IL-12β	63.29 ± 11.39***	92.48 ± 9.03***	1.60 ± 0.30	1.52 ± 0.25	2.17 ± 0.27**	0.67 ± 0.16†
TNFRSF1a	2.68 ± 0.42**	1.34 ± 0.21†	1.89 ± 0.35*	1.01 ± 0.10†	2.12 ± 0.36*	1.78 ± 0.43
TNFRSF1b	9.61 ± 1.78***	12.13 ± 1.52***	1.67 ± 0.45	2.66 ± 0.34***	3.05 ± 0.49**	2.45 ± 0.34**
Sdc-1	2.49 ± 0.47**	0.76 ± 0.15†	1.46 ± 0.47	0.84 ± 0.16	2.78 ± 0.84*	1.55 ± 0.47

TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL, interleukin; TNFRSF, TNF receptor; Sdc-1, syndecan-1; WT, wild type; HPSE-KO, heparanase-deficient. The mean ± SEM of eight mice per group are depicted. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ versus control mice; †$P<0.05$ HPSE-KO versus WT mice.
[a]The relative expression compared with untreated WT or HPSE-KO mice is shown, with both untreated WT and untreated HPSE-KO mice set at 1.

TNF-α and LPS increase transendothelial albumin passage *in vitro*

Next, we evaluated whether TNF-α and LPS regulate heparanase expression in glomerular endothelial cells and podocytes *in vitro*. Activation of mGEnC-1 with TNF-α or LPS resulted in a 2-fold and 1.5-fold increased heparanase mRNA expression, respectively (Figure 9A). Activation of mouse podocytes with TNF-α or LPS resulted in a 2.5-fold and 2-fold increased heparanase mRNA expression, respectively (Figure 9B). At the functional level, activation of mGEnC-1 with TNF-α or LPS resulted in a 1.5 and 1.3-fold increased transendothelial albumin passage, respectively (Figure 9C). Silencing of heparanase expression in mGEnC-1 with shRNA (~60% knockdown) led to a significant reduction in transendothelial albumin passage after activation with TNF-α or LPS compared with mGEnC-1 transfected with a scrambled shRNA (Figure 9D). These data suggest that heparanase mediates transendothelial albumin passage under inflammatory conditions.

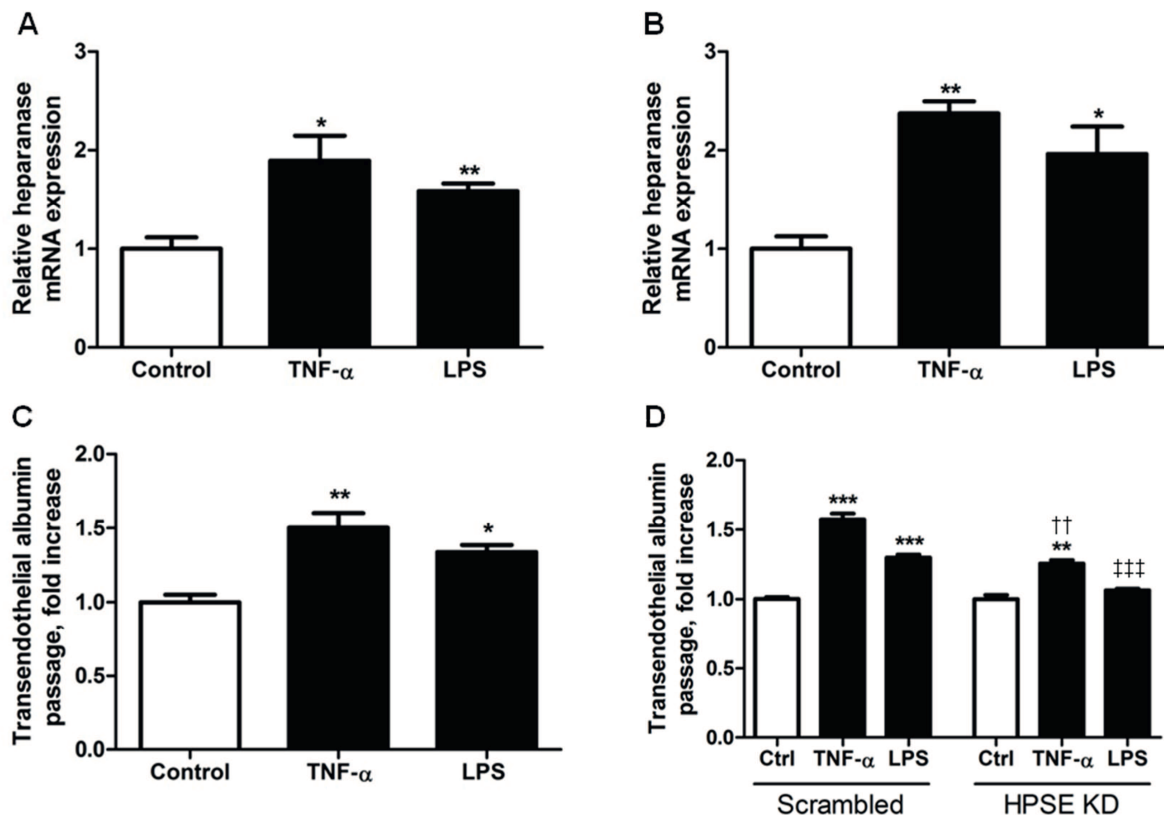

Figure 9: TNF-α and LPS induce heparanase expression and increase transendothelial albumin passage *in vitro*. Activation of (**A**) mouse glomerular endothelial cells (mGEnC-1) and (**B**) mouse podocytes with TNF-α or LPS for 18 hours resulted in an increased heparanase mRNA expression. (**C**) Activation of mGEnC-1 with TNF-α or LPS for 18 hours increased the passage of albumin across the endothelial monolayer 1.5 and 1.3-fold compared to control, respectively. (**D**) Activation of heparanase-silenced mGEnC-1 with TNF-α or LPS for 18 hours showed a lower transendothelial albumin passage compared with scrambled mGEnC-1 activated with TNF-α or LPS, respectively. *P<0.05, **P<0.01 and ***P<0.001 versus control. ††P<0.01 versus TNF-α scrambled. ‡‡‡P<0.001 versus LPS scrambled. Ctrl; control.

Discussion

In this study, we showed that heparanase deficiency ameliorated proteinuria, reduced glomerular damage and reduced the pro-inflammatory cytokine milieu in the kidney during experimental glomerulonephritis. Induction of experimental glomerulonephritis led to an increased heparanase expression and activity in WT mice, which was associated with a decreased glomerular expression of a highly sulfated HS domain and a significant proteinuria 1 and 4 days after induction of anti-GBM glomerulonephritis and 2 days after induction of LPS glomerulonephritis. This is supported by the *in vitro* data, where we showed that TNF-α and LPS directly induced heparanase expression in mouse glomerular endothelial cells and mouse podocytes. Moreover, TNF-α and LPS also increased transendothelial albumin passage *in vitro*, most likely through heparanase-mediated loss of HS expression on glomerular endothelial cells, since silencing of heparanase led to a reduced transendothelial albumin passage. Proteinuria was reduced in the heparanase-deficient mice, which was accompanied by a preserved HS expression in the capillary filter as measured by an anti-HS antibody recognizing a highly sulfated HS domain. On the other hand, the expression of previously identified inflammatory HS domains that mediate binding of leukocytes and chemokines was reduced in heparanase-deficient mice during disease [13, 31, 36]. Our results are in line with previous studies, where treatment of rats with an anti-heparanase antibody or the heparanase inhibitor PI-88 reduced proteinuria in Passive Heymann nephritis and accelerated anti-GBM disease [25-27]. In addition, Lygizos et al. showed that treatment with a heparanase inhibitor attenuated septic acute kidney injury [29].

We previously showed that heparanase deficiency prevented the development of proteinuria and renal damage in experimental DN [23]. However, in contrast to DN, heparanase deficiency did not completely prevent proteinuria during experimental glomerulonephritis in two different models. Whereas heparanase deficiency prevented the influx of macrophages during diabetic nephropathy [23, 24], in the current study neutrophils and macrophages, although reduced, were still present in the glomeruli of heparanase-deficient mice during experimental glomerulonephritis. Since neutrophils and macrophages are important mediators of proteinuria, glomerular damage, and the local cytokine milieu, this may explain the persisting proteinuria in the heparanase-deficient mice during experimental glomerulonephritis albeit lower compared to the WT mice.

PMN influx peaked 2 hours after the induction of anti-GBM glomerulonephritis. PMNs are one of the key determinants of proteinuria and glomerular injury in the early phase of anti-GBM glomerulonephritis. PMN influx was significantly lower in the heparanase-deficient mice, which could result in a reduced release of proteinases, which can destruct the capillary wall [37]. This reduced PMN influx may lead to a reduced proteinuria and to an improved

renal function after 1 and 4 days. Heparanase may affect the glomerular PMN influx in several ways. A first possibility is that heparanase may affect the availability of adhesion molecules. This was previously described for lung injury during sepsis [19]. During acute lung injury, activated heparanase cleaves HS from the pulmonary endothelial glycocalyx, thereby exposing endothelial surface adhesion molecules that allow neutrophil adhesion. A second possible mechanism is by shaping the glomerular endothelial expression of specific inflammatory, *N*- and 6-O-sulfated, HS domains that mediate leukocyte trafficking. We previously showed that the expression of these inflammatory HS domains is increased on activated glomerular endothelial cells and that they enhance leukocyte adhesion *in vitro* [13]. In addition, an increased expression of these inflammatory HS domains was observed 2 hours after induction of anti-GBM glomerulonephritis, at the time leukocyte influx peaked [31, 36]. It can be speculated that heparanase contributes to the shaping of an inflammatory glycocalyx, thereby promoting the presence of inflammatory HS domains mediating PMN binding. Indeed we observed a decreased expression of the inflammatory HS domains in the heparanase-deficient mice. A third possible mechanism may involve the HSPG syndecan-1. A recent study in a myeloma cell line showed that heparanase enhanced the expression and shedding of syndecan-1 [38-40]. We previously showed that syndecan-1 deficiency aggravates anti-GBM glomerulonephritis by increasing leukocyte influx, suggesting that syndecan-1 has anti-inflammatory properties [31]. Loss of heparanase may lead to less shedding of syndecan-1, which may lead to a reduced inflammatory response, thereby reducing the PMN influx.

No significant differences in PMN influx were observed between WT and heparanase-deficient mice after induction of LPS glomerulonephritis. The mechanisms of proteinuria and inflammation for anti-GBM and LPS-induced glomerulonephritis may be different. Whereas the development of our anti-GBM glomerulonephritis model is PMN-dependent, the development of LPS glomerulonephritis is also mediated by a direct effect of LPS on podocytes. Mice deficient of synaptopodin, an actin-associated protein highly expressed by podocytes, show aggravated proteinuria after LPS administration [41]. LPS induces the expression of cytoplasmic cathepsin L [42]. Synaptopodin is a known substrate of cathepsin L [43], and degradation of synaptopodin by cathepsin L resulted in the reorganization of the actin cytoskeleton, foot process effacement and proteinuria. In line with these results, we observed an increased cathepsin L mRNA expression and reduced synaptopodin protein expression (data not shown) after induction of LPS glomerulonephritis. Heparanase-deficiency reduced cathepsin L mRNA expression and increased synaptopodin expression (data not shown).

In addition to PMNs, macrophages may play a role in establishing the inflammatory milieu and the subsequent destruction of the glomerular capillary wall. We observed an increased

glomerular macrophage influx in both models of experimental glomerulonephritis, which was reduced in the heparanase-deficient mice. Macrophages can be activated by cytokines such as interferon-γ and TNF-α, bacterial LPS, extracellular matrix proteins and other chemical mediators. We observed an increased renal expression of the proinflammatory cytokine TNF-α in both experimental models for glomerulonephritis, which was reduced in the heparanase-deficient mice as well. The fact that TNF-α, like LPS, can activate macrophages could explain the increased macrophage influx. Recently, Lerner et al. showed that macrophages are more prone to activation by LPS after they have been pre-treated with heparanase [22]. In intestinal inflammation, macrophages stimulate epithelial cells, resulting in an increased heparanase expression, which is mainly caused by TNF-α. In addition, macrophages can secrete mature cathepsin L, and thereby process and activate heparanase [15]. In turn, this active heparanase could also induce macrophage activation. Similar results were observed in diabetic nephropathy, where high glucose induced heparanase expression [24]. Heparanase is processed by cathepsin L produced by tubular cells and this active heparanase sustains macrophage stimulation, resulting in an increased production of TNF-α. Interestingly, in that study no increase in TNF-α production and macrophage influx was observed in the heparanase-deficient mice. In addition, heparanase expression is normal after induction of LPS glomerulonephritis in TNF receptor 1-deficient mice, which also showed a normal renal function [28]. Although the exact mechanism of macrophage activation by heparanase is not fully understood, toll-like receptors (TLRs) may play an important role [44]. Extracellular HS both inhibits and activates TLR4 signaling and macrophage activation [45-48]. Activated heparanase can cleave HS on the macrophage cell surface and thereby facilitate ligand binding to TLR4 [22]. In addition, this cleaved HS can directly stimulate TLR4 [45, 46, 49, 50], thereby sustaining macrophage activation and promoting renal damage.

In this study we showed an increased heparanase expression by induction of experimental glomerulonephritis *in vivo* and stimulation of mGEnC-1 or mouse podocytes with TNF-α or LPS *in vitro*. Previously, we showed that the early growth response 1 (EGR1) transcription factor can stimulate heparanase expression in kidney cells [23]. Both TNF-α and LPS can induce the expression of EGR1 [22, 51], suggesting that heparanase expression is induced via an EGR1-dependent mechanism.

Our data are not conclusive with respect to the origin of heparanase in our *in vivo* models for experimental glomerulonephritis. We can conclude that in the LPS-induced model for glomerulonephritis heparanase is not derived from platelets, but heparanase may originate from the glomeruli and/or inflammatory cells, such as PMNs and macrophages. In the anti-GBM induced model, heparanase may be derived from platelets as well. Notably, the anti-GBM model is very specifically targeting the glomerulus due to the use of anti-GBM serum,

which specifically attracts inflammatory cells and platelets to the glomeruli. Therefore, it may be suggested that heparanase in anti-GBM glomerulonephritis is locally produced by attracted inflammatory cells and platelets, in addition to local production by glomerular cells like podocytes and glomerular endothelial cells. Nevertheless, the exact contribution of cells and tissue in local heparanase production should be addressed in future research by repeating the LPS-induced and anti-GBM-induced experiments in hematopoietic chimera, i.e. heparanase-deficient bone marrow in WT mice and *vice versa.* In addition, although challenging, experiments could be repeated in WT mice with kidney transplants of heparanase-deficient mice and *vice versa*.

In conclusion, heparanase deficiency significantly ameliorates proteinuria and renal damage during experimental glomerulonephritis by preserving glomerular HS expression, reducing the glomerular influx of leukocytes and macrophages and by affecting the local cytokine milieu. Further studies are required to further precise the underlying mechanisms, but overall, heparanase represents an attractive therapeutic target for the treatment of glomerulonephritis.

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References

1. Tryggvason, K. and E. Pettersson, *Causes and consequences of proteinuria: the kidney filtration barrier and progressive renal failure.* Journal of internal medicine, 2003. **254**(3): p. 216-24.
2. Kanwar, Y.S. and M.G. Farquhar, *Presence of heparan sulfate in the glomerular basement membrane.* Proc Natl Acad Sci U S A, 1979. **76**(3): p. 1303-7.
3. Garsen, M., et al., *The role of heparanase and the endothelial glycocalyx in the development of proteinuria.* Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association, 2014. **29**(1): p. 49-55.
4. Kanwar, Y.S. and M.G. Farquhar, *Isolation of glycosaminoglycans (heparan sulfate) from glomerular basement membranes.* Proc Natl Acad Sci U S A, 1979. **76**(9): p. 4493-7.
5. Groggel, G.C., et al., *Changes in heparan sulfate correlate with increased glomerular permeability.* Kidney international, 1988. **33**(2): p. 517-23.
6. Kanwar, Y.S., A. Linker, and M.G. Farquhar, *Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion.* J Cell Biol, 1980. **86**(2): p. 688-93.
7. van den Hoven, M.J., et al., *Reduction of anionic sites in the glomerular basement membrane by heparanase does not lead to proteinuria.* Kidney international, 2008. **73**(3): p. 278-87.
8. Harvey, S.J., et al., *Disruption of glomerular basement membrane charge through podocyte-specific mutation of agrin does not alter glomerular permselectivity.* Am J Pathol, 2007. **171**(1): p. 139-52.
9. Chen, S., et al., *Loss of heparan sulfate glycosaminoglycan assembly in podocytes does not lead to proteinuria.* Kidney Int, 2008. **74**(3): p. 289-99.
10. van den Born, J., et al., *Distribution of GBM heparan sulfate proteoglycan core protein and side chains in human glomerular diseases.* Kidney international, 1993. **43**(2): p. 454-63.
11. Rops, A.L., et al., *Heparan sulfate proteoglycans in glomerular inflammation.* Kidney international, 2004. **65**(3): p. 768-85.
12. Sarrazin, S., W.C. Lamanna, and J.D. Esko, *Heparan sulfate proteoglycans.* Cold Spring Harbor perspectives in biology, 2011. **3**(7).
13. Rops, A.L., et al., *Heparan sulfate domains on cultured activated glomerular endothelial cells mediate leukocyte trafficking.* Kidney international, 2008. **73**(1): p. 52-62.
14. van den Hoven, M.J., et al., *Heparanase in glomerular diseases.* Kidney international, 2007. **72**(5): p. 543-8.
15. Abboud-Jarrous, G., et al., *Cathepsin L is responsible for processing and activation of proheparanase through multiple cleavages of a linker segment.* The Journal of biological chemistry, 2008. **283**(26): p. 18167-76.
16. Ilan, N., M. Elkin, and I. Vlodavsky, *Regulation, function and clinical significance of heparanase in cancer metastasis and angiogenesis.* The international journal of biochemistry & cell biology, 2006. **38**(12): p. 2018-39.
17. Yang, Y., et al., *Heparanase promotes the spontaneous metastasis of myeloma cells to bone.* Blood, 2005. **105**(3): p. 1303-9.
18. Parish, C.R., C. Freeman, and M.D. Hulett, *Heparanase: a key enzyme involved in cell invasion.* Biochimica et biophysica acta, 2001. **1471**(3): p. M99-108.
19. Schmidt, E.P., et al., *The pulmonary endothelial glycocalyx regulates neutrophil adhesion and lung injury during experimental sepsis.* Nature medicine, 2012. **18**(8): p. 1217-23.

20. Li, R.W., et al., *Dramatic regulation of heparanase activity and angiogenesis gene expression in synovium from patients with rheumatoid arthritis.* Arthritis and rheumatism, 2008. **58**(6): p. 1590-600.
21. Brenchley, P.E., *Antagonising angiogenesis in rheumatoid arthritis.* Annals of the rheumatic diseases, 2001. **60 Suppl 3**: p. iii71-4.
22. Lerner, I., et al., *Heparanase powers a chronic inflammatory circuit that promotes colitis-associated tumorigenesis in mice.* The Journal of clinical investigation, 2011. **121**(5): p. 1709-21.
23. Gil, N., et al., *Heparanase is essential for the development of diabetic nephropathy in mice.* Diabetes, 2012. **61**(1): p. 208-16.
24. Goldberg, R., et al., *Role of heparanase-driven inflammatory cascade in pathogenesis of diabetic nephropathy.* Diabetes, 2014. **63**(12): p. 4302-13.
25. Levidiotis, V., et al., *Heparanase is involved in the pathogenesis of proteinuria as a result of glomerulonephritis.* Journal of the American Society of Nephrology : JASN, 2004. **15**(1): p. 68-78.
26. Levidiotis, V., et al., *A synthetic heparanase inhibitor reduces proteinuria in passive Heymann nephritis.* Journal of the American Society of Nephrology : JASN, 2004. **15**(11): p. 2882-92.
27. Levidiotis, V., et al., *Heparanase inhibition reduces proteinuria in a model of accelerated anti-glomerular basement membrane antibody disease.* Nephrology (Carlton, Vic.), 2005. **10**(2): p. 167-73.
28. Xu, C., et al., *TNF-mediated damage to glomerular endothelium is an important determinant of acute kidney injury in sepsis.* Kidney international, 2014. **85**(1): p. 72-81.
29. Lygizos, M.I., et al., *Heparanase mediates renal dysfunction during early sepsis in mice.* Physiol Rep, 2013. **1**(6): p. e00153.
30. Zcharia, E., et al., *Newly generated heparanase knock-out mice unravel co-regulation of heparanase and matrix metalloproteinases.* PloS one, 2009. **4**(4): p. e5181.
31. Rops, A.L., et al., *Syndecan-1 deficiency aggravates anti-glomerular basement membrane nephritis.* Kidney international, 2007. **72**(10): p. 1204-15.
32. van de Westerlo, E.M., et al., *Human single chain antibodies against heparin: selection, characterization, and effect on coagulation.* Blood, 2002. **99**(7): p. 2427-33.
33. Raats, C.J., et al., *Differential expression of agrin in renal basement membranes as revealed by domain-specific antibodies.* The Journal of biological chemistry, 1998. **273**(28): p. 17832-8.
34. Rops, A.L., et al., *Isolation and characterization of conditionally immortalized mouse glomerular endothelial cell lines.* Kidney international, 2004. **66**(6): p. 2193-201.
35. Mundel, P., et al., *Rearrangements of the cytoskeleton and cell contacts induce process formation during differentiation of conditionally immortalized mouse podocyte cell lines.* Experimental cell research, 1997. **236**(1): p. 248-58.
36. A, L.W.M.M.R., et al., *Modulation of heparan sulfate in the glomerular endothelial glycocalyx decreases leukocyte influx during experimental glomerulonephritis.* Kidney international, 2014. **86**(5): p. 932-42.
37. Assmann, K.J., et al., *Anti-GBM nephritis in the mouse: severe proteinuria in the heterologous phase.* Virchows Archiv. A, Pathological anatomy and histopathology, 1985. **406**(3): p. 285-99.
38. Ramani, V.C., et al., *The heparanase/syndecan-1 axis in cancer: mechanisms and therapies.* The FEBS journal, 2013. **280**(10): p. 2294-306.
39. Purushothaman, A., et al., *Heparanase-enhanced shedding of syndecan-1 by myeloma cells promotes endothelial invasion and angiogenesis.* Blood, 2010. **115**(12): p. 2449-57.
40. Yang, Y., et al., *Heparanase enhances syndecan-1 shedding: a novel mechanism for stimulation of tumor growth and metastasis.* The Journal of biological chemistry, 2007. **282**(18): p. 13326-33.

41. Asanuma, K., et al., *Synaptopodin regulates the actin-bundling activity of alpha-actinin in an isoform-specific manner.* The Journal of clinical investigation, 2005. **115**(5): p. 1188-98.
42. Sever, S., et al., *Proteolytic processing of dynamin by cytoplasmic cathepsin L is a mechanism for proteinuric kidney disease.* The Journal of clinical investigation, 2007. **117**(8): p. 2095-104.
43. Reiser, J., B. Adair, and T. Reinheckel, *Specialized roles for cysteine cathepsins in health and disease.* The Journal of clinical investigation, 2010. **120**(10): p. 3421-31.
44. Blich, M., et al., *Macrophage activation by heparanase is mediated by TLR-2 and TLR-4 and associates with plaque progression.* Arteriosclerosis, thrombosis, and vascular biology, 2013. **33**(2): p. e56-65.
45. Brunn, G.J., et al., *Conditional signaling by Toll-like receptor 4.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2005. **19**(7): p. 872-4.
46. Goodall, K.J., et al., *Soluble heparan sulfate fragments generated by heparanase trigger the release of pro-inflammatory cytokines through TLR-4.* PloS one, 2014. **9**(10): p. e109596.
47. Ren, J.D., et al., *Involvement of a membrane potassium channel in heparan sulphate-induced activation of macrophages.* Immunology, 2014. **141**(3): p. 345-52.
48. Akbarshahi, H., et al., *TLR4 dependent heparan sulphate-induced pancreatic inflammatory response is IRF3-mediated.* Journal of translational medicine, 2011. **9**: p. 219.
49. Yu, L., L. Wang, and S. Chen, *Endogenous toll-like receptor ligands and their biological significance.* Journal of cellular and molecular medicine, 2010. **14**(11): p. 2592-603.
50. Johnson, G.B., et al., *Receptor-mediated monitoring of tissue well-being via detection of soluble heparan sulfate by Toll-like receptor 4.* Journal of immunology (Baltimore, Md. : 1950), 2002. **168**(10): p. 5233-9.
51. Coleman, D.L., et al., *Lipopolysaccharide induces Egr-1 mRNA and protein in murine peritoneal macrophages.* J Immunol, 1992. **149**(9): p. 3045-51.

Chapter 5

The role of heparanase and the endothelial glycocalyx in the development of proteinuria

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Abstract

Proteinuria is a hallmark of many glomerular diseases and an independent risk factor for the progression of renal failure. Proteinuria results from damage to the glomerular filtration barrier (GFB), which plays a critical role in size- and charge-selective filtration. The GFB consists of three layers, which is the fenestrated endothelium that is covered by the glycocalyx, the podocytes, and the intervening glomerular basement membrane. Defects in one of the three layers in the GFB can lead to the development of proteinuria. Heparan sulfate (HS) is a negatively charged polysaccharide that is abundantly expressed in all layers of the GFB. HS expression in the GFB is reduced in the majority of patients with proteinuria, which is associated with an increased glomerular expression of the HS-degrading enzyme heparanase. The primary role of HS in the development of proteinuria has been challenged after the establishment of several genetically engineered mouse models with an altered HS expression that did not display development of overt proteinuria. However, in a recent study we showed that heparanase is essential for the development of proteinuria in diabetic nephropathy, which suggests that loss of HS contributes to the development of proteinuria. Recent studies also further highlight the importance of the glomerular endothelial glycocalyx in charge-selective filtration and the development of proteinuria. This review aims to summarize our current knowledge on the role of in particular HS and heparanase in the development of proteinuria.

Introduction

The kidney plays an important role in filtering the blood and thereby eliminates waste products. This filtering process takes place in a large number of small functional units within the kidney, called glomeruli that consist of a tuft of microcapillaries. The glomerular capillary wall is semi-permeable, small and positively charged molecules pass the glomerular filtration barrier (GFB) freely, whereas the passage of large and negatively charged molecules is restricted. The GFB is composed of fenestrated glomerular endothelial cells covered by a glycocalyx, the glomerular basement membrane (GBM) and the podocytes with their interdigitated foot processes and slit diaphragms. Damage to the GFB results in proteinuria, which is characterized by proteins with the size of albumin (69 kDa) or larger in the urine. Proteinuria is an independent risk factor for the progression of renal failure [1]. It is therefore critical to understand the mechanisms leading to proteinuria and to identify therapeutic targets for patients with proteinuria. The importance of podocytes and the GBM has been extensively reviewed elsewhere [2-5]. Several proteins in the slit diaphragms of podocytes and in the GBM have been shown to be crucial for a normal GFB function. In this review we provide an update on heparanase and the endothelial glycocalyx, including the heparan sulfate proteoglycans, and their role in the pathogenesis of proteinuria

Glomerular endothelial cells and the glycocalyx

Glomerular endothelial cells are highly specialized cells covering the inner layer of glomerular capillaries. The glomerular endothelium contains numerous fenestrations, transcellular pores of 60-80 nm in diameter. The size of these fenestrations is large compared to albumin. Therefore, it was initially thought that the glomerular endothelium did not play a critical role in providing a barrier to proteins. The formation and maintenance of endothelial cell fenestrations depends on the vascular endothelial growth factor (VEGF) [6]. In the glomerulus VEGF is produced by podocytes. Loss of VEGF in podocytes results in renal disease characterized by proteinuria and endotheliosis. VEGF signals from the podocytes to the glomerular endothelial cells in a paracrine manner, and thereby regulates the structure and function of the adjacent endothelial cell [7]. Glomerular endothelial cells and their fenestrations are covered with a carbohydrate-rich cell-surface layer, the glycocalyx, that might provide a barrier to proteins [6]. The endothelial glycocalyx is a network of glycoproteins anchored to the endothelium through the cell membrane-bound proteoglycans [8]. The cell membrane-bound proteoglycans with their glycosaminoglycan (GAG) chains form the functional and structural backbone and can bind soluble molecules such as albumin, orosomucoid and lumican [9]. These soluble molecules are linked to each other either directly or via soluble proteoglycans or their GAG chains [8]. Heparan sulfate HS is the most common GAG in the endothelial glycocalyx, and accounts for ~50% of the total

amount. Chondroitin sulfate (CS) is typically present in a ratio of 1:4 with HS. However, this expression is variable, since the expression of GAGs in the glycocalyx depends on various stimuli, such as endothelial cell activation or stimulation [8]. The major non-sulfated GAG in the glycocalyx is hyaluronan (HA), which plays an important role in the structural maintenance of vascular integrity [10]. The endothelial glycocalyx plays an important role in many physiological processes, which includes vascular permeability, attenuation of blood cell – vessel wall interactions, mechanotransduction, signaling, and vascular protection [2, 8, 11-14]. Damage to the glycocalyx may lead to disturbances in these aforementioned physiological processes. Therefore, the glycocalyx plays an important role in several vascular pathologies, including the development of proteinuria and inflammation [13, 15-19] (Figure 1).

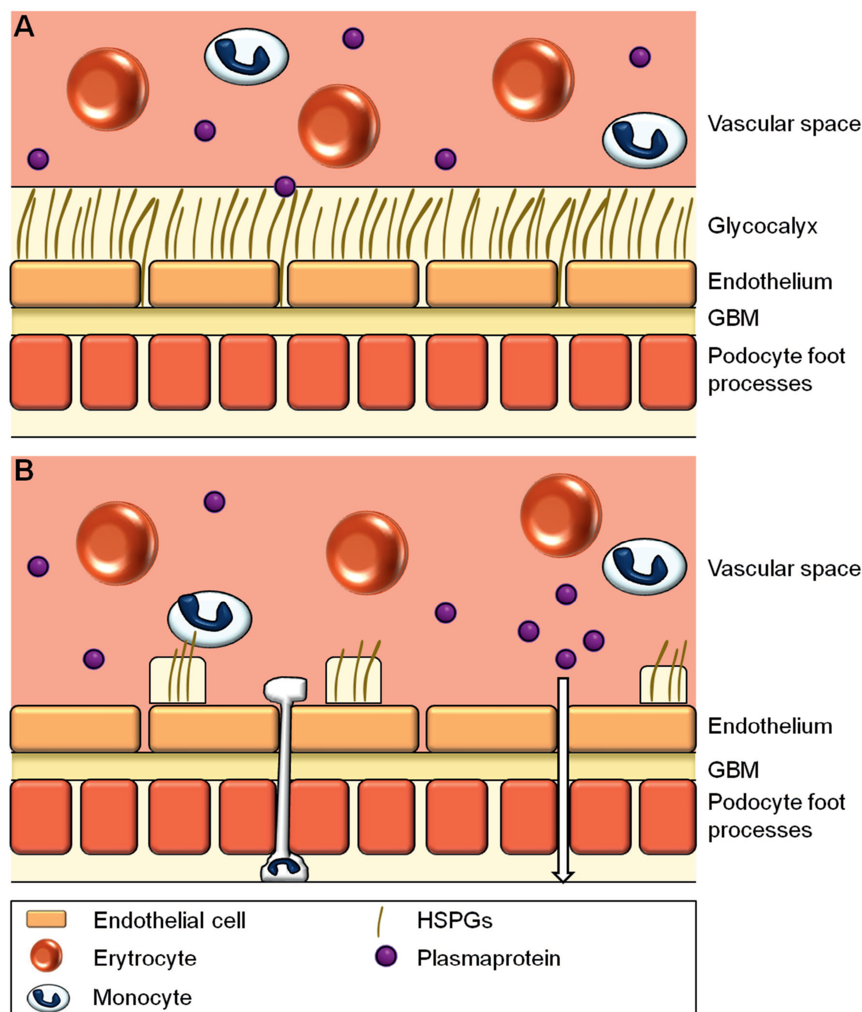

Figure 1: Role of the endothelial glycocalyx in proteinuria and inflammation. (A) Under physiological conditions, the glycocalyx forms a barrier for the passage of proteins and it prevents the adherence of inflammatory cells to the endothelium. (B) When the glycocalyx is damaged, proteins can pass the filtration barrier, resulting in proteinuria, and inflammatory cells can adhere to HSPGs and transmigrate through the endothelium, resulting in glomerular inflammation.

Heparan sulfate proteoglycans

The major functional GAG in the glycocalyx is HS. HS proteoglycans (HSPGs) consist of a core protein, to which one or more HS polysaccharide side chains are covalently attached. These HS chains are highly negatively charged due to the presence of acidic sugar residues and/or sulfate groups. HS belongs to the family of negatively charged GAGs that also include heparin, chondroitin sulfate, dermatan sulfate, keratan sulfate and the non-sulfated hyaluronan. HSPGs are present in extracellular matrices (ECMs) and at the cell surface of all cell types, including endothelial cells and podocytes. Agrin, perlecan and collagen XVIII are the HSPGs present in the GBM and the mesangial matrix. Cell surface HSPGs include glypicans, syndecans, CD44 and betaglycan [15].

HSPGs are involved in a large variety of physiological processes. They participate in cell-cell and cell-ECM interactions via their HS chains. Furthermore, HSPGs function as a receptor or co-receptor for the binding of several soluble ligands, including growth factors, cytokines and chemokines [15]. This indicates that HSPGs play a pivotal role in the control of multiple physiological and pathological processes. The interaction between HS and its ligands is very specific and is dictated by HS sequences of well-defined length and structure. Changes in HS structure will therefore influence the binding of ligands to HS and, subsequently, their physiological activities [15].

HS biosynthesis is a multistep process that occurs in the Golgi apparatus, which is characterized by chain initiation, polymerization and modification. HS synthesis starts with the addition of a pentasaccharide linkage to specific serine residues within the core protein (xylose-xylose-galactose-galactose-glucuronic acid), which is catalyzed by several carbohydrate transferases [20]. The HS copolymerase complex EXT1/EXT2 subsequently adds alternating N-acetyl-glucosamine (GlcNAc) and glucuronic acid (GlcA) residues to the pentasaccharide linker. In addition to HS polymerization, several modification steps take place in the HS biosynthesis, such as N-deacetylation/N-sulfation (catalyzed by 4 N-deacetylases/N-sulfotransferases), C-5 epimerization of glucuronic acid to iduronic acid (catalyzed by a single C5-epimerase), and O-sulfation at 2-O, 3-O and 6-O positions (respectively catalyzed by a single 2-O-sulfotransferase, 7 3-O sulfotransferases and 3 6-O sulfotransferases) (Figure 2). The structure of the HS chain can also be modified by HS-modifying enzymes such as 6-O-endosulfatases and heparanase. 6-O-endosulfatase enzymes (sulf1 and sulf2) edit the final sulfation pattern and function of HS by specifically removing 6-O-sulfate groups [21]. Finally, HS can be degraded by heparanase, a β(1-4)-endoglucuronidase that cleaves HS at specific sites via hydrolysis. The combination of possible modifications in a HS chain gives rise to an enormous structural diversity, which dictates the binding and modulation of a myriad of factors that include growth factors, chemokines, cytokines, enzymes, and other proteins [5, 15] (Figure 2).

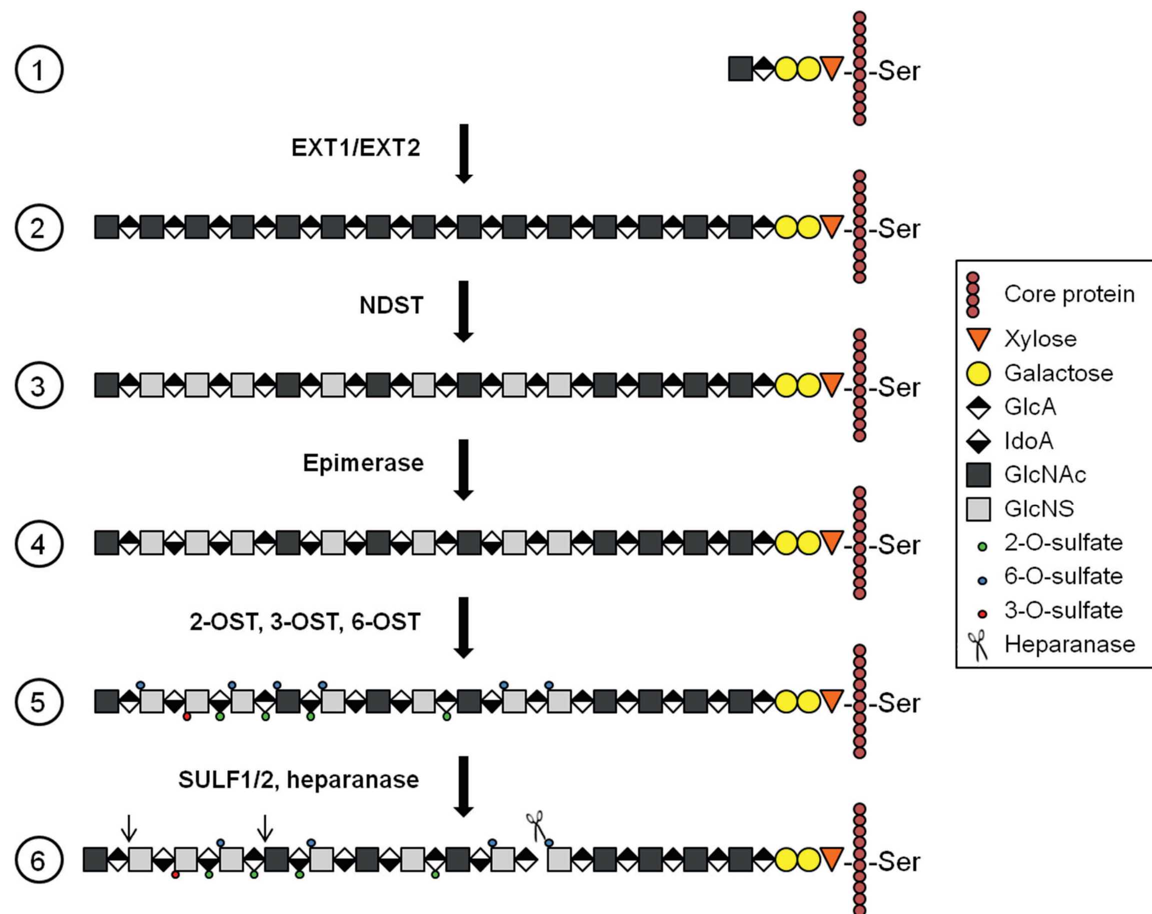

Figure 2: HS biosynthesis, editing and degradation. Schematic overview of the biosynthesis of a HS chain: 1) The EXT1/EXT2 complex recognizes a pentasaccharide linker and generates the initial HS chain, 2) N-deacetylases/N-sulfotransferases (NDSTs) catalyze the N-deacetylation/N-sulfation of glucosamine residues, 3) C5-epimerase catalyzes the epimerization of GlcA to IdoA, 4) 2-O-sulfotransferases (2-OSTs) catalyze the sulfation of GlcA and IdoA and 3-O-sulfotransferases (3-OSTs) and 6-O-sulfotransferases (6-OSTs) catalyze the sulfation of glucosamine residues, and 5) 6-O-endosulfatases (sulf 1 and 2) catalyze the removal of 6-O-sulfate groups and heparanase cleaves the HS chain at specific sites via hydrolysis. Abbreviations: Ser, *serine;* GlcA, *glucuronic acid;* IdoA, *iduronic acid;* GlcNAc, *N-acetyl-glucosamine;* GlcNS, *N-sulfated-glucosamine.*

No primary role of HS in charge-selective filtration

For several decades it was assumed that the presence of negatively charged HS in the GBM is essential for the charge-selective permeability of the GFB. Seminal studies demonstrated the presence of GAGs, including HS, in the GBM. Furthermore, it has been shown that *in situ* removal of GAGs in the GBM by perfusion of bacterial GAG-degrading enzymes led to the passage of ferritin and bovine serum albumin [22, 23]. Moreover, injection of anti-HS(PG) antibodies in rats led to albuminuria [24]. Finally, in many (experimental) glomerular diseases, such as diabetic nephropathy, systemic lupus erythematosus, minimal change disease and membranous glomerulopathy, a decreased expression of HS in the GBM was observed, which in general was inversely correlated with the level of urinary protein excretion [5, 25]. However, the primary role of HS in charge-selective filtration has been challenged

after establishing several genetically engineered mouse models with a disturbed HS(PG) expression in the GFB. Mice with podocytes lacking the predominant core protein agrin did not develop proteinuria and revealed a normal glomerular architecture, despite the fact that they lacked the majority of anionic sites in the GBM [26]. Mice lacking both perlecan and agrin did not develop glomerular abnormalities or proteinuria [27]. Mice deficient for the cell surface HSPG syndecan-1 and mice deficient in endothelial NDST-1 also display no albuminuria [28, 29]. Mice with podocytes lacking the essential HS polymerizing enzyme EXT1, developed glomerular ultrastructural abnormalities such as foot process effacement, but only a mild, not significant, albuminuria [30]. Finally, mice overexpressing heparanase displayed a ~5-fold decrease of GAG-associated anionic sites in the GFB. However, also in these mice no ultrastructural abnormalities or severe albuminuria could be observed [31]. Thus, several mouse models targeting HS expression in the GFB did not develop proteinuria, whereas in proteinuric patients a reduced HS expression is associated with proteinuria (Table 1).Taken the experimental animal data together, the primary role of HS in determining charge-selective properties of the capillary filter can be rejected. However, a role of HS and the HS-degrading enzyme heparanase in the development of proteinuria in situations with glomerular pathology cannot be excluded [5].

Table 1: Involvement of HS in the development of proteinuria.

Disease/ animal model	Species	Glomerular HS expression	Proteinuria	Reference
Diabetic nephropathy	Human	Reduced	+	[25]
Systemic lupus erythematosus	Human	Reduced	+	[25]
Minimal change disease	Human	Reduced	+	[25]
Membranous glomerulonephritis	Human	Reduced	+	[25]
Dense Deposit disease	Human	Reduced	+	[32]
Podocyte-specific agrin knockout	Mouse	Reduced	-	[26]
Perlecan/agrin double knockout	Mouse	Reduced	-	[27]
Endothelial-specific NDST1 knockout	Mouse	Reduced	-	[29]
Podocyte-specific EXT1 knockout	Mouse	Reduced	+/-	[30]
Heparanase overexpression	Mouse	Reduced	+/-	[31]

+, *proteinuria*; +/-, *mild proteinuria;* -, *no proteinuria.*

Heparanase

Heparanase cleaves the glycosidic bond within the HS chain at a few selective sites, yielding HS fragments of 5-7 kDa in size. This cleavage requires N- and 6-O-sulfated moieties in a specific context as exemplified in the trisaccharide sequence GlcNS6OS-α(1-4)-GlcA-β(1-4)-GlcNS6OS [5]. Heparanase is synthesized as a pre-proheparanase of 68 kDa. It is targeted to the endoplasmic reticulum via its signal peptide, and processed into a 65 kDa proheparanase by cleaving off this signal peptide. The proheparanase is then transported to the Golgi apparatus and subsequently packaged into vesicles and secreted. Once secreted, heparanase interacts with cell membrane HSPGs (in particular syndecan), low density lipoprotein receptor-related proteins and mannose 6-phosphate receptors, followed by endocytosis of the heparanase-receptor complex. Proheparanase is then transferred to the late endosomes/lysosomes, where it is processed and activated by cathepsin L through the cleavage of a linker segment [33]. This results in an active heparanase heterodimer consisting of an 8 kDa N-terminal subunit and a 50 kDa C-terminal subunit. Heparanase plays a physiological role in HS turnover, embryo development, hair growth and wound healing. Furthermore, it plays a pathological role in tumor growth, angiogenesis, metastasis, inflammation and glomerular diseases [34].

Heparanase expression in proteinuric diseases

The first study revealing that heparanase plays a role in the development of proteinuria was in rats with puromycin aminonucleoside (PAN)-induced nephrosis. Glomerular heparanase expression was upregulated in these rats and therefore may be involved in the loss of glomerular HS observed in proteinuria [35]. Similar results were found in rats with passive Heymann nephritis (PHN), a model of membranous glomerulopathy. In these rats, glomerular heparanase expression was increased in the proteinuric phase. Administration of a polyclonal antibody against heparanase reduced the level of proteinuria [36]. Furthermore, administration of the heparanase inhibitor PI-88 to rats with PHN reduced proteinuria and was associated with the preservation of glomerular HS expression, suggesting that active heparanase is important in the development of proteinuria [37].

In other experimental glomerular diseases such as streptozotocin (STZ)-induced diabetic nephropathy and adriamycin nephropathy, and human glomerular diseases, such as diabetic nephropathy, IgA nephropathy, minimal change disease, dense deposit diseases and membranous glomerulopathy, glomerular heparanase expression is also increased [5, 32, 38, 39]. In proteinuric diseases, both podocytes and glomerular endothelial cells can show an increased heparanase expression, while tubular cells always express high levels of heparanase, also under healthy conditions. The increased glomerular heparanase expression in patients with glomerular diseases is associated with a decreased expression of

HS in the GBM. Furthermore, as mentioned the reduced HS expression inversely correlates with the degree of proteinuria, suggesting that heparanase is responsible for the glomerular degradation of HS and, most likely, for the development of proteinuria [38].

Heparanase is involved in the pathogenesis of diabetic nephropathy

Recently, we demonstrated that heparanase-knockout mice, unlike their wild type littermates, failed to develop proteinuria and renal damage in response to STZ-induced type1 diabetes [40]. Heparanase mRNA expression was increased in the renal cortex of the diabetic wild type mice. No heparanase was detected in diabetic and non-diabetic heparanase-knockout mice, which failed to develop diabetic nephropathy and proteinuria. Importantly, in this study the expression of 3-O-sulfated HS domains, as recognized by the antibody HS4C3 [41] was reduced in glomeruli of diabetic wild type mice, whereas no change was detected in glomeruli of diabetic heparanase-knockout mice [40]. The crucial role of heparanase in the development of proteinuria was further demonstrated by the treatment of type 1 diabetic wild type mice with the specific heparanase inhibitor SST0001, which resulted in a lower degree of albuminuria and a better renal function compared to vehicle-treated diabetic mice. These results demonstrate that heparanase is causally involved in the pathogenesis of proteinuria and diabetic nephropathy. In addition, inhibition of heparanase expression and activity could be a relevant therapeutic approach in diabetic nephropathy.

As outlined, previous studies demonstrated that loss of anionic sites in the GFB in genetically engineered mice, including transgenic mice overexpressing heparanase, did not result in the development of proteinuria [26, 27, 30, 31]. However, in those previous studies no additional pathogenic signals were applied, in contrast to the study using heparanase-deficient mice where in addition to a manipulated heparanase expression diabetic nephropathy was induced with STZ. However, it is difficult to predict what will happen when diabetic nephropathy will be induced in the transgenic heparanase overexpression mouse. It may be hypothesized that induction of diabetic nephropathy in the heparanase overexpressing mouse will be similar as in wild-type mice, although the disease may be accelerated as well since there is already overexpression of heparanase at the start. Alternatively, it can be hypothesized that induction of diabetic nephropathy in the heparanase overexpressing mouse may not be possible at all, at least when loss of HS is an important event in the chain of events that leads to diabetic nephropathy and proteinuria, since these mice lack already the majority of HS in their GFB at the start. Notably, the heparanase-deficient mice normally have no clear phenotype. We previously suggested several mechanisms due to the action of heparanase and loss of HS that could be involved in the pathogenesis of proteinuria, such as 1) changes in glomerular cell-GBM or cell-cell interactions due to the loss of HS, 2) release of HS-bound growth factors, cytokines, chemokines and bioactive HS fragments and/or 3)

the induction of signaling cascades resulting in changed cell properties [5]. Additional research is needed to proof whether one or more of these mechanisms are required for the development of proteinuria and to which extent heparanase is involved. Nevertheless, it can be concluded that heparanase activity and most likely loss of HS play a crucial role in the pathogenesis of proteinuria in experimental diabetic nephropathy.

The endothelial glycocalyx in proteinuric diseases

As outlined, the endothelial glycocalyx plays an important role in many physiological processes [8]. Damage to the glycocalyx may therefore lead to several vascular pathologies. Mice with adriamycin-induced nephropathy displayed a substantial reduction in glomerular glycocalyx thickness, caused by the impaired synthesis of certain proteoglycans and HS [12, 19]. This resulted in a reduction of charge selectivity and in the development of proteinuria. Similar results were found in diabetic Zucker fatty rats, who displayed a significant reduction in endothelial cell glycocalyx thickness compared to control rats and these rats developed albuminuria at 18 weeks of age [42, 43]. Heparanase expression was increased in these rats, which could be attributed to an increased production of reactive oxygen species (ROS) [42]. Using Munich-Wistar-Fromter (MWF) rats that spontaneously develop albuminuria by aging, Salmon *et al* [44] observed that these rats had a reduction in glycocalyx volume, similar to that of healthy animals with an enzymatically removed glycocalyx. The MWF rats with a reduced glycocalyx developed albuminuria. Modification of the glycocalyx of these MWF rats by adsorption of intravenously injected wheat germ agglutinin (WGA) lectin significantly reduced the glomerular albumin permeability, indicating that loss of glycocalyx increases microvascular permeability. Another study showed that rats exposed to a hypertonic sodium chloride solution to remove non-covalently bound components of the glycocalyx, displayed a 12-fold increase in albumin excretion, without any detectable damage to the GBM or the podocytes [9]. The permeability of Ficoll was unaltered, indicating that damage to the glycocalyx leads to a defect in charge selectivity.

In a recent study we showed that infusion of mice with the hyaluronan-degrading enzyme hyaluronidase leads to degradation of the glycocalyx and leakage of albumin across the endothelium, as demonstrated with correlative light-electron microscopy that allows for complete and integral assessment of glomerular albumin passage. However, no albumin was detected in the urine, and the leaked albumin was found to be associated with podocytes and parietal epithelial cells [45]. Importantly, no albumin passage over the GFB could be observed with an intact glycocalyx. Since hyaluronan is an important structural component of the glycocalyx this recent finding suggests that an intact endothelial glycocalyx is required for a proper GFB function. In future experiments we aim to induce experimental proteinuria in genetically engineered mice targeted for endothelial hyaluronan and HS.

Patients with type 1 and type 2 diabetes show a reduction in systemic glycocalyx volume [16, 19, 46], which correlates with the presence of microalbuminuria [16]. Importantly, urinary heparanase levels in patients with type 1 and type 2 diabetes are associated with albuminuria [47]. Systemic glycocalyx reduction may be an effect of hyperglycemia, as high glucose alters the biosynthesis of sulfated GAG chains, in particular that of HS, as demonstrated in conditionally immortalized human glomerular endothelial cells [43, 48]. Hyperglycemia also increases the production of ROS, aldosterone and angiotensin II, resulting in an increased heparanase expression by both podocytes and glomerular endothelial [39, 42, 49, 50]. Degradation of HS leads to an increased flux of albumin across a monolayer of glomerular endothelial cells *in vitro* [13], also suggesting that the glycocalyx plays an important role in the development of proteinuria.

Conclusion

A causal role for heparanase in the pathogenesis of proteinuria in diabetic nephropathy has now been clearly demonstrated. Previous studies that addressed proteinuria mainly focused on podocytes and the GBM, whereas glomerular endothelial cells were more or less neglected. However, proteinuria can also develop without morphological changes to podocytes and the GBM. A reduction of the glycocalyx on glomerular endothelial cells results in an increased albumin permeability. All layers of the GFB contribute to a normal glomerular filtration, and every layer needs to be intact to maintain a normal filtration barrier. Since there may be several mechanisms involved in the pathogenesis of proteinuria due to the action of heparanase and loss of HS, the exact role of HS in the development of proteinuria has to be determined under pathological conditions. Despite the growing body of evidence, further studies are required to unravel the structure and function of the glomerular endothelial glycocalyx, and its contribution to the development of proteinuria. Ultimately, this will lead to the identification of molecular targets for therapeutic intervention with well-defined GAG-based drugs.

Acknowledgements

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References

1. Tryggvason, K. and E. Pettersson, *Causes and consequences of proteinuria: the kidney filtration barrier and progressive renal failure.* J Intern Med, 2003. **254**(3): p. 216-24.
2. Haraldsson, B., J. Nystrom, and W.M. Deen, *Properties of the glomerular barrier and mechanisms of proteinuria.* Physiol Rev, 2008. **88**(2): p. 451-87.
3. Jarad, G. and J.H. Miner, *Update on the glomerular filtration barrier.* Curr Opin Nephrol Hypertens, 2009. **18**(3): p. 226-32.
4. Patrakka, J. and K. Tryggvason, *Molecular make-up of the glomerular filtration barrier.* Biochem Biophys Res Commun, 2010. **396**(1): p. 164-9.
5. van den Hoven, M.J., et al., *Heparanase in glomerular diseases.* Kidney Int, 2007. **72**(5): p. 543-8.
6. Satchell, S.C. and F. Braet, *Glomerular endothelial cell fenestrations: an integral component of the glomerular filtration barrier.* Am J Physiol Renal Physiol, 2009. **296**(5): p. F947-56.
7. Sison, K., et al., *Glomerular structure and function require paracrine, not autocrine, VEGF-VEGFR-2 signaling.* J Am Soc Nephrol, 2010. **21**(10): p. 1691-701.
8. Reitsma, S., et al., *The endothelial glycocalyx: composition, functions, and visualization.* Pflugers Arch, 2007. **454**(3): p. 345-59.
9. Friden, V., et al., *The glomerular endothelial cell coat is essential for glomerular filtration.* Kidney Int, 2011. **79**(12): p. 1322-30.
10. Lennon, F.E. and P.A. Singleton, *Hyaluronan regulation of vascular integrity.* Am J Cardiovasc Dis, 2011. **1**(3): p. 200-13.
11. Tarbell, J.M. and M.Y. Pahakis, *Mechanotransduction and the glycocalyx.* J Intern Med, 2006. **259**(4): p. 339-50.
12. Jeansson, M., et al., *Adriamycin alters glomerular endothelium to induce proteinuria.* J Am Soc Nephrol, 2009. **20**(1): p. 114-22.
13. Singh, A., et al., *Glomerular endothelial glycocalyx constitutes a barrier to protein permeability.* J Am Soc Nephrol, 2007. **18**(11): p. 2885-93.
14. Vink, H. and B.R. Duling, *Identification of distinct luminal domains for macromolecules, erythrocytes, and leukocytes within mammalian capillaries.* Circ Res, 1996. **79**(3): p. 581-9.
15. Rops, A.L., et al., *Heparan sulfate proteoglycans in glomerular inflammation.* Kidney Int, 2004. **65**(3): p. 768-85.
16. Nieuwdorp, M., et al., *Endothelial glycocalyx damage coincides with microalbuminuria in type 1 diabetes.* Diabetes, 2006. **55**(4): p. 1127-32.
17. Schmidt, E.P., et al., *The pulmonary endothelial glycocalyx regulates neutrophil adhesion and lung injury during experimental sepsis.* Nat Med, 2012.
18. Lipowsky, H.H., *The endothelial glycocalyx as a barrier to leukocyte adhesion and its mediation by extracellular proteases.* Ann Biomed Eng, 2012. **40**(4): p. 840-8.
19. Salmon, A.H. and S.C. Satchell, *Endothelial glycocalyx dysfunction in disease: albuminuria and increased microvascular permeability.* J Pathol, 2012. **226**(4): p. 562-74.
20. Esko, J.D. and S.B. Selleck, *Order out of chaos: assembly of ligand binding sites in heparan sulfate.* Annu Rev Biochem, 2002. **71**: p. 435-71.
21. Morimoto-Tomita, M., et al., *Cloning and characterization of two extracellular heparin-degrading endosulfatases in mice and humans.* J Biol Chem, 2002. **277**(51): p. 49175-85.
22. Kanwar, Y.S., A. Linker, and M.G. Farquhar, *Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion.* J Cell Biol, 1980. **86**(2): p. 688-93.
23. Rosenzweig, L.J. and Y.S. Kanwar, *Removal of sulfated (heparan sulfate) or nonsulfated (hyaluronic acid) glycosaminoglycans results in increased permeability of*

the glomerular basement membrane to 125I-bovine serum albumin. Lab Invest, 1982. **47**(2): p. 177-84.

24. van den Born, J., et al., *A monoclonal antibody against GBM heparan sulfate induces an acute selective proteinuria in rats.* Kidney Int, 1992. **41**(1): p. 115-23.
25. van den Born, J., et al., *Distribution of GBM heparan sulfate proteoglycan core protein and side chains in human glomerular diseases.* Kidney Int, 1993. **43**(2): p. 454-63.
26. Harvey, S.J., et al., *Disruption of glomerular basement membrane charge through podocyte-specific mutation of agrin does not alter glomerular permselectivity.* Am J Pathol, 2007. **171**(1): p. 139-52.
27. Goldberg, S., et al., *Glomerular filtration is normal in the absence of both agrin and perlecan-heparan sulfate from the glomerular basement membrane.* Nephrol Dial Transplant, 2009. **24**(7): p. 2044-51.
28. Rops, A.L., et al., *Syndecan-1 deficiency aggravates anti-glomerular basement membrane nephritis.* Kidney Int, 2007. **72**(10): p. 1204-15.
29. Rops, A.L., et al., *Modulation of heparan sulfate in the glomerular endothelial glycocalyx decreases leukocyte influx during experimental glomerulonephritis.* Kidney Int, 2014. **86**(5): p. 932-42.
30. Chen, S., et al., *Loss of heparan sulfate glycosaminoglycan assembly in podocytes does not lead to proteinuria.* Kidney Int, 2008. **74**(3): p. 289-99.
31. van den Hoven, M.J., et al., *Reduction of anionic sites in the glomerular basement membrane by heparanase does not lead to proteinuria.* Kidney Int, 2008. **73**(3): p. 278-87.
32. Smith, R.J., et al., *New approaches to the treatment of dense deposit disease.* J Am Soc Nephrol, 2007. **18**(9): p. 2447-56.
33. Abboud-Jarrous, G., et al., *Cathepsin L is responsible for processing and activation of proheparanase through multiple cleavages of a linker segment.* J Biol Chem, 2008. **283**(26): p. 18167-76.
34. Nasser, N.J., *Heparanase involvement in physiology and disease.* Cell Mol Life Sci, 2008. **65**(11): p. 1706-15.
35. Levidiotis, V., et al., *Increased expression of heparanase in puromycin aminonucleoside nephrosis.* Kidney Int, 2001. **60**(4): p. 1287-96.
36. Levidiotis, V., et al., *Heparanase is involved in the pathogenesis of proteinuria as a result of glomerulonephritis.* J Am Soc Nephrol, 2004. **15**(1): p. 68-78.
37. Levidiotis, V., et al., *A synthetic heparanase inhibitor reduces proteinuria in passive Heymann nephritis.* J Am Soc Nephrol, 2004. **15**(11): p. 2882-92.
38. van den Hoven, M.J., et al., *Increased expression of heparanase in overt diabetic nephropathy.* Kidney Int, 2006. **70**(12): p. 2100-8.
39. Kramer, A., et al., *Induction of glomerular heparanase expression in rats with adriamycin nephropathy is regulated by reactive oxygen species and the renin-angiotensin system.* J Am Soc Nephrol, 2006. **17**(9): p. 2513-20.
40. Gil, N., et al., *Heparanase is essential for the development of diabetic nephropathy in mice.* Diabetes, 2012. **61**(1): p. 208-16.
41. Ten Dam, G.B., et al., *3-O-sulfated oligosaccharide structures are recognized by anti-heparan sulfate antibody HS4C3.* J Biol Chem, 2006. **281**(8): p. 4654-62.
42. Kuwabara, A., et al., *Deterioration of glomerular endothelial surface layer induced by oxidative stress is implicated in altered permeability of macromolecules in Zucker fatty rats.* Diabetologia, 2010. **53**(9): p. 2056-65.
43. Haraldsson, B. and J. Nystrom, *The glomerular endothelium: new insights on function and structure.* Curr Opin Nephrol Hypertens, 2012. **21**(3): p. 258-63.
44. Salmon, A.H., et al., *Loss of the Endothelial Glycocalyx Links Albuminuria and Vascular Dysfunction.* J Am Soc Nephrol, 2012.
45. Dane, M.J., et al., *Glomerular Endothelial Surface Layer Acts as a Barrier against Albumin Filtration.* Am J Pathol, 2013. **182**(5): p. 1532-40.

46. Broekhuizen, L.N., et al., *Effect of sulodexide on endothelial glycocalyx and vascular permeability in patients with type 2 diabetes mellitus.* Diabetologia, 2010. **53**(12): p. 2646-55.
47. Rops, A.L., et al., *Urinary heparanase activity in patients with Type 1 and Type 2 diabetes.* Nephrol Dial Transplant, 2012. **27**(7): p. 2853-61.
48. Singh, A., et al., *High glucose causes dysfunction of the human glomerular endothelial glycocalyx.* Am J Physiol Renal Physiol, 2011. **300**(1): p. F40-8.
49. Singh, A., et al., *Reactive oxygen species modulate the barrier function of the human glomerular endothelial glycocalyx.* PLoS One, 2013. **8**(2): p. e55852.
50. van den Hoven, M.J., et al., *Regulation of glomerular heparanase expression by aldosterone, angiotensin II and reactive oxygen species.* Nephrol Dial Transplant, 2009. **24**(9): p. 2637-45.

Chapter 6

Endothelin-1 induces proteinuria by heparanase-mediated disruption of the glomerular glycocalyx

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Abstract

Diabetic nephropathy (DN) is the leading cause of chronic kidney disease in the Western world. Recently, endothelin receptor antagonists have emerged as a novel treatment for DN, but the mechanisms underlying their protective effect remain unknown. We previously showed that both heparanase and endothelin-1 are essential for the development of DN. Here, we demonstrate that endothelin-1 activates podocytes to release heparanase. Conditioned podocyte culture medium increases glomerular transendothelial albumin passage in a heparanase-dependent manner. In podocyte-specific endothelin receptor knockout (podETRKO) mice, the increased glomerular heparanase expression and consequently reduced heparan sulfate expression and endothelial glycocalyx thickness during diabetes was prevented, whereas these diabetic podETRKO mice failed to develop proteinuria. Taken together, our data suggest that in diabetes endothelin-1 signaling, as occurs in endothelial activation, induces heparanase expression in the podocyte, damage to the glycocalyx, proteinuria and renal failure. This constitutes a novel mechanism of action of endothelin receptor blockers in DN.

Introduction

Diabetic nephropathy (DN), a major complication of both type I and type II diabetes mellitus, is the leading cause of chronic kidney disease in the Western world. DN is characterized by a progressive increase in proteinuria, glomerular capillary widening, secondary podocyte loss, mesangial matrix expansion, glomerular basement membrane thickening and finally tubulointerstitial fibrosis. Current treatment does not reduce proteinuria sufficiently. Therefore, there is a need to identify new therapeutic targets.

One potent new therapeutic target is the endothelin-1 receptor. Endothelin-1 is a vasoconstrictor that is released upon endothelial activation and activates two G-protein-coupled receptors, endothelin receptor type A (ETRA) and endothelin receptor type B (ETRB). Together, these receptors induce a variety of intracellular signaling cascades, resulting in vasoconstriction, proliferation, inflammation, extracellular matrix production and fibrosis [1-4]. The expression of endothelin-1 is increased in DN [5]. Several studies report a renoprotective effect of endothelin receptor antagonists (ERAs) in both experimental as well as clinical DN [6-8]. Moreover, ERAs reduce residual proteinuria and improve endothelial function in patients with DN, in addition to the standard treatment [6, 9]. In addition to DN, ERAs reduce proteinuria in other forms of nephropathy, such as hypertensive nephropathy and focal segmental glomerulosclerosis [10]. It was shown that podocyte-specific deletion of both ETAR and ETBR protects mice from diabetes-induced glomerulosclerosis and podocyte loss, indicating that endothelin-1 signaling in the podocyte may play a role in the development of DN and the protective effects of endothelin blockers [11]. The mechanism underlying the protective effect of ERAs, however, remains unknown.

We previously showed that heparanase is essential for the development of DN [12]. Heparanase-deficient mice were protected from diabetes-induced proteinuria and renal damage. Heparanase is the only known mammalian enzyme that can cleave the negatively charged heparan sulfate (HS) side chains of HS proteoglycans (HSPGs). Loss of HS expression in the glomerular filtration barrier has been associated with the development of proteinuria [13]. In addition, HS is the major functional glycosaminoglycan in the endothelial glycocalyx, and remodeling of the glycocalyx is associated with the development of proteinuria [14-19].

Based on our previous data, we hypothesize that endothelin-1 signaling modulates the endothelial glycocalyx in DN through heparanase release by the podocyte. In this study we evaluate the effects of endothelin-1 on heparanase expression and function both *in vitro* and *in vivo*.

Materials and methods

Animals

Mice with podocyte-specific deletion of both *Ednar* and *Ednbr* genes (podocin-Cre^{+} *Ednar*$^{lox/lox}$ *Ednbr*$^{lox/lox}$ mice) were generated as described [11]. Mice were on a mixed C57BL/6J/ 129/SV/ FVB/N genetic background. Mice homozygous for floxed *Ednar* and *Ednbr* genes but without Cre were used as control mice.

To induce diabetes, 12-week old male mice were injected intraperitoneally with 100 mg/kg streptozotocin in a citrate buffer (pH 4.5) for 2 consecutive days as described [11]. Control mice were injected with citrate buffer alone. Mice with a fasted blood glucose >16 mmol/l were considered diabetic. Mice were sacrificed 10 weeks after the induction of diabetes. Urinary creatinine concentrations were measured spectrophotometrically using colorimetric methods. Urinary albumin excretion was measured by ELISA (BIOTREND Chemikalien GmbH, Köln, Germany). Glomeruli were isolated from freshly harvested kidneys as described previously [11]. All animal experiments were performed according to French veterinary guidelines and those formulated by the European Commission for experimental animal use (L358-86/609EEC), and approved by the Institute National de la Santé et de la Recherche Médicale (INSERM).

Immunofluorescence staining

Indirect immunofluorescence staining was performed on 2 µM thick cryosections as described [20]. Primary antibodies included heparanase (HPA1, ProsPecTany, Rehovot, Israel) and the VSV-tagged anti-HS antibody HS4C3 (N-, 2-O, 3-O and 6-O sulfation) [21]. Secondary antibodies included goat anti-rabbit IgG Alexa 488 (Invitrogen Life Technologies, Breda, The Netherlands) and rabbit anti-VSV Cy3 (Sigma-Aldrich). At least 50 glomeruli per section were scored for heparanase and HS staining intensities on a scale between 0 and 10 (0= no staining, 5= 50% staining, 10= 100% staining) by two independent investigators on blinded sections using a Leica CTR6000 microscope.

Glycocalyx staining

Small pieces of cortex were fixed in periodate-lysine-paraformaldehyde (PLP; pH 6.2), immersed in 2.3 M sucrose and snap frozen in liquid nitrogen. Frozen sections (20 µm) were stained and fixed with 1% lanthanum hydroxide and 2% glutaraldehyde dissolved in 0.1M sodium cacodylate buffer (pH 7.4). Samples were washed in 0.1 M sodium cacodylate buffer (pH 7.4), postfixed in Palade-buffered 1% O_SO_4 with 1% lanthanum hydroxide, washed in 0.1 M sodium cacodylate buffer (pH 7.4), dehydrated, and embedded in Epon 812, Luft's procedure (EMS, Hatfield, United Kingdom). Endothelial and podocyte glycocalyx thickness

(in nm) was measured in 3 non-diabetic and 4 diabetic mice per group. For each mouse at least 10 randomly selected capillary loops of at least 5 randomly selected glomeruli were analyzed at randomly selected measuring points. The measurements were performed twice by two independent investigators on blinded sections using a Jeol JEM 1400 electron microscope (JEOL, Tokyo, Japan) and Gata microscopy suite.

Cell culture

Conditionally immortalized mouse glomerular endothelial cells (mGEnC-1) and mouse podocytes (MPC-5) were cultured as described previously [22, 23]. Differentiated mGEnC-1 and podocytes were treated with endothelin-1 (100 nM; Sigma-Aldrich, Zwijndrecht, The Netherlands) and/or the combined ERA PD 142893 (0.1 µM; Sigma-Aldrich) or the selective ETRA antagonist BQ-123 (1 µM; Sigma-Aldrich) for 18 hours.

RNA isolation and real-time PCR

Total RNA was extracted from kidney cortex, mGEnC-1 and MPC-5 podocytes. RNA isolation, cDNA synthesis and real-time PCR were performed as described [24].

Heparanase activity assay

Heparanase activity of recombinant heparanase, isolated glomeruli, MPC-5 podocytes, and culture supernatant was determined using a commercially available assay (AMS Biotechnology, Abingdon, UK) according to manufacturer's instructions.

Transendothelial albumin passage and TEER measurement

Differentiated mGEnC-1 seeded on polyester membranes in tissue culture inserts (0.4 µm pore size; Corning Incorporated, NY, USA). Transendothelial electrical resistance (TEER) of untreated cells was measured using a EMD Millipore Millicell-ERS2 Volt-Ohm meter. mGEnC-1 were treated with bacterial heparinase and recombinant heparanase for 1 hour, or with endothelin-1 and/or the combined ERA PD 142893 or the selective ETRA antagonist BQ-123 as outlined. In addition, mGEnC-1 were exposed to culture supernatant of vehicle- or endothelin-1-treated podocytes, and where indicated substituted with the anti-heparanase antibody HPA1 (ProsPecTany) or the isotype control rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA). Transendothelial albumin passage was determined as described [24].

Statistics

Values are expressed as mean ± SEM. Significance was evaluated by a one-way ANOVA and *post hoc* analysis with Tukey's multiple comparison test. Significant differences in

transendothelial albumin passage were evaluated using a two-way repeated measures ANOVA with Bonferroni post-test. Statistical analysis was performed using GraphPad Prism 5.03 (GraphPad Software, Inc., San Diego, CA, USA). A P-value of ≤ 0.05 was considered statistically significant.

Results

First, we evaluated the effects of endothelin-1 on cultured podocytes and glomerular endothelial cells. Endothelin-1 treatment increased heparanase mRNA expression (Figure 1A) and heparanase activity (Figure 1B) in mouse podocytes and culture supernatant of mouse podocytes (Figure 1C), whereas heparanase expression in cultured mGEnC-1 was not affected (Figure 2A). Notably, the endothelin-1-induced increase in heparanase activity was larger in the culture supernatant versus the cell extract, respectively ~100% versus ~20%, suggesting that the majority of endothelin-1 induced heparanase is secreted by the podocytes. Treatment of mGEnC-1 with culture supernatant of endothelin-1-stimulated podocytes increased transendothelial albumin passage (Figure 1D-E). Heparanase expression (Figure 1A), heparanase activity (Figure 1B-C) and transendothelial albumin passage (Figure 1D-E) were reduced by treatment with the combined ERA PD142893 and the ETRA antagonist BQ-123. Addition of the anti-heparanase antibody HPA1 to the conditioned podocyte culture medium also reduced transendothelial albumin passage (Figure 1F), whereas the isotype control had no effect (Figure 1G). As a control we showed that addition of the anti-heparanase antibody to recombinant heparanase reduced heparanase activity (Figure 1H), which suggests that the increased transendothelial albumin passage is mediated by heparanase. Moreover, stimulation of mGEnC-1 with bacterial heparinase or recombinant heparanase to remove HS resulted in an increased transendothelial albumin passage (Figure 3), suggesting that HS in the glycocalyx contributes to barrier function in our *in vitro* transendothelial albumin passage system. Stimulation of mGEnC-1 with endothelin-1 without conditioned podocyte culture medium did not affect transendothelial albumin passage (Figure 2B). The observed differences between mGEnC-1 and podocytes in response to endothelin-1 can be explained by differences in endothelin receptor expression. Podocytes express both ETRA and ETRB, while glomerular endothelial cells only express ETRB [11, 25].

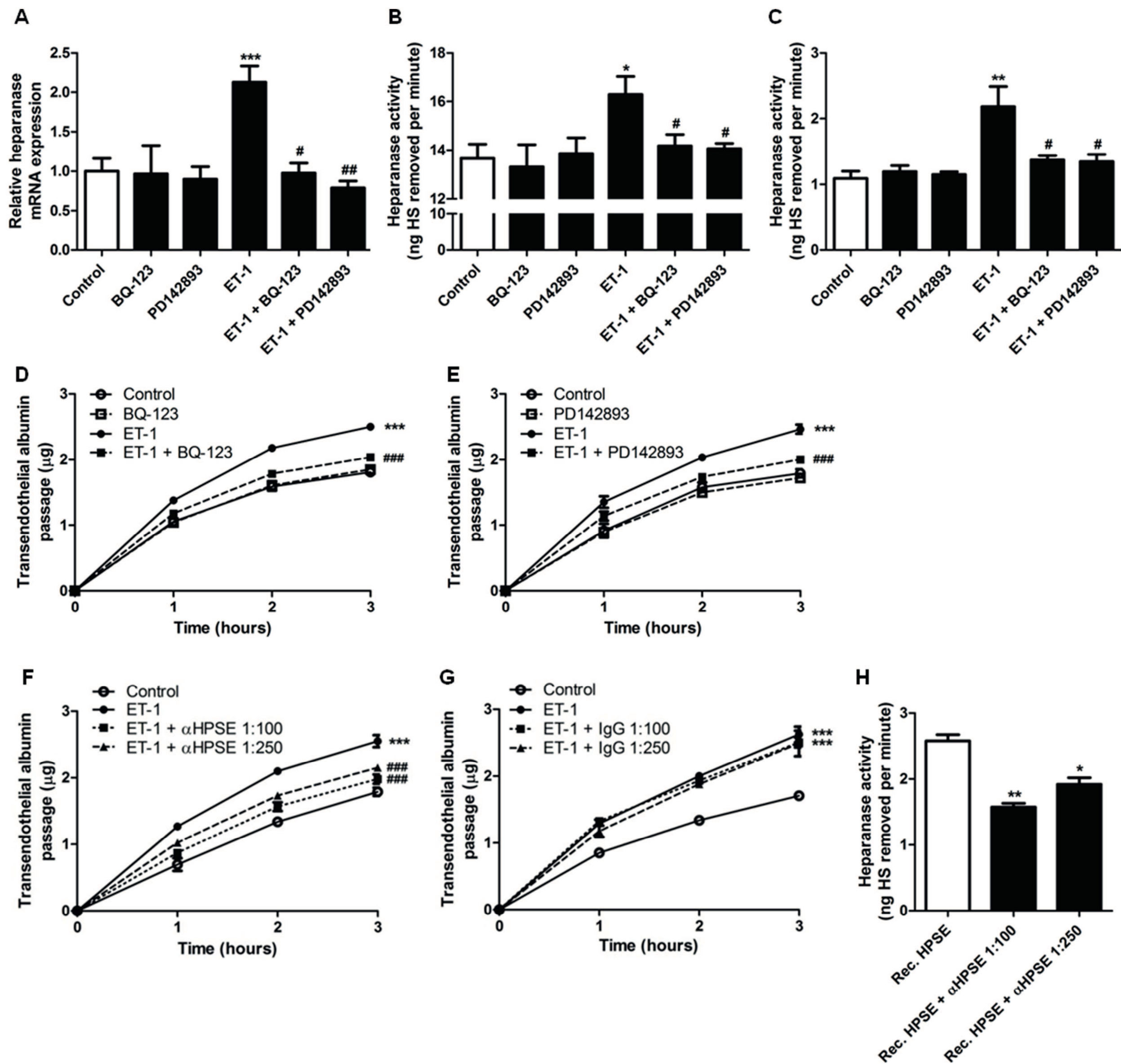

Figure 1: Endothelin-1-induced heparanase expression in the podocyte increases transendothelial albumin passage. (**A**) Heparanase mRNA expression and (**B**,**C**) heparanase activity in (**A**,**B**) mouse podocytes and (**C**) culture supernatant of mouse podocytes were increased by treatment with endothelin-1 for 18 hours, and reduced by treatment with the ETRA antagonist BQ-123 and the combined ERA PD 142893. (**D**,**E**) Cultured mGEnC-1 were treated with culture supernatant of podocytes treated with endothelin-1 in the absence or presence of (**D**) BQ-123 or (**E**) PD 142893. Cumulative passage of FITC-labeled albumin across the mGEnC-1 monolayer (TEER: 28 Ω cm^2) was determined over time. Transendothelial albumin passage was increased by endothelin-1, and reduced by BQ-123 and PD 142893. To evaluate the role of heparanase on endothelin-1-induced transendothelial albumin passage, (**F**) the anti-heparanase antibody HPA1 (1:100 and 1:250) and (**G**) the isotype control rabbit IgG (1:100 and 1:250) were added to the culture supernatant of endothelin-1-treated podocytes. Transendothelial albumin passage was reduced by addition of the anti-heparanase antibody, but not affected by addition of the isotype control. (**H**) To show that the anti-heparanase antibody HPA1 was able to reduce heparanase activity specifically, recombinant heparanase was co-incubated with the antibody (1:100 and 1:250). Heparanase activity was reduced by the anti-heparanase antibody at both tested concentrations. The isotype control had no effect on heparanase activity (not shown). *P<0.05, **P<0.01 and ***P<0.001 versus control, $^{\#}P$<0.05, $^{\#\#}P$<0.01 and $^{\#\#\#}P$<0.001 versus ET-1. ETRA; endothelin receptor type A, ERA; endothelin receptor antagonist, ET-1; endothelin-1, HPSE; heparanase, TEER; transendothelial electrical resistance.

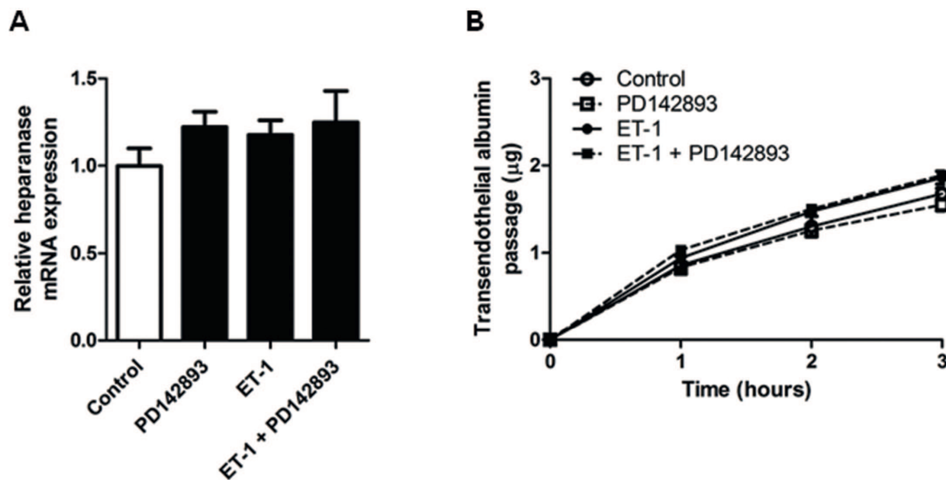

Figure 2: Endothelin-1 did not affect heparanase mRNA expression and transendothelial albumin passage in mouse glomerular endothelial cells. Cultured mGEnC-1 were treated with endothelin-1 in the absence or presence of the combined ERA PD 142893 for 18 hours. Endothelin-1 did not affect (**A**) heparanase mRNA expression and (**B**) transendothelial albumin passage. (**A**) Each bar represents *n*=4; mean ± SEM; analyzed by a one-way ANOVA. (**B**) Each dot represents *n*=3; mean ± SEM; analyzed by a two-way repeated measures ANOVA. ERA; endothelin receptor antagonist, ET-1; endothelin-1, HPSE; heparanase, mGEnC-1; mouse glomerular endothelial cells.

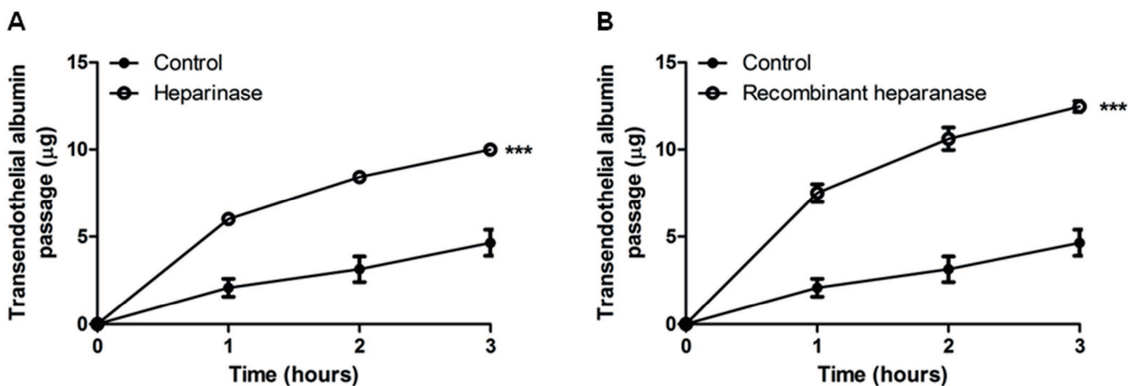

Figure 3: Bacterial heparinase and recombinant heparanase induce transendothelial albumin passage in mouse glomerular endothelial cells. Cultured mGEnC-1 were treated with (**A**) bacterial heparinase or (**B**) recombinant heparanase for 1 hour, which resulted in an increased transendothelial albumin passage. Each dot represents *n*=3; mean ± SEM; analyzed by a two-way repeated measures ANOVA. ****P*<0.001 versus control. mGEnC-1; mouse glomerular endothelial cells.

To translate our *in vitro* data, we used podocyte-specific ETRA/ETRB-deficient (podETRKO) mice, in which we induced diabetes. As we reported previously, diabetic podETRKO mice are, in contrast to diabetic wild type (WT) mice, protected from proteinuria, GBM thickening, mesangial matrix expansion, podocyte loss and glomerulosclerosis during streptozotocin-induced diabetes [11]. Ten weeks after induction of diabetes, WT mice developed a significant albuminuria (Figure 4A), which was significantly reduced in the diabetic podETRKO mice. Diabetic WT mice showed increased cortical heparanase mRNA (Figure 4B), glomerular heparanase protein expression (Figure 4C), and glomerular heparanase

activity (Figure 4D), whereas glomerular HS expression was reduced (Figure 4E). In contrast, heparanase and HS expression were normal in the diabetic podETRKO mice (Figure 4B-E). Next, we analyzed glycocalyx thickness, since increased heparanase expression may reduce glycocalyx thickness in diabetic mice. The glycocalyx was visualized by lanthanum hydroxide staining, which stains the glycocalyx on top of the endothelium and podocytes in black. Indeed, glycocalyx thickness on both the endothelium and podocytes was reduced ~50-60% in diabetic WT mice (Figure 5A-C). Importantly, endothelial glycocalyx thickness was preserved in the diabetic podETRKO mice (Figure 5A-B). Podocyte glycocalyx in diabetic podETRKO mice was ~25% reduced (Figure 5A,C), which possibly could be explained by a reduction in HS synthesis, as glomerular heparanase activity is not increased in the diabetic podETRKO mice. Apparently, the remaining reduction of podocyte glycocalyx thickness in podETRKO mice is not sufficient to induce proteinuria and DN. However, we cannot exclude the possibility that a combined reduction of endothelial and podocyte glycocalyx is required for development of proteinuria and DN in WT mice.

Discussion

The present study is the first to show that endothelin-1 regulates heparanase expression in podocytes. We previously showed that heparanase is essential for the development of proteinuria and renal damage in experimental DN [12]. By induction of diabetes, heparanase-deficient mice showed no mesangial matrix expansion, macrophage infiltration and tubulointerstitial fibrosis, effects that are known to be mediated by signaling through the endothelin receptor [1]. In addition, we previously showed that endothelin receptor deficiency in podocytes protects mice against experimental DN. In the current study we showed that endothelin-1 induced heparanase expression in mouse podocytes both *in vitro* and *in vivo*. Moreover, we showed that podocyte-specific loss of both ETAR and ETBR prevented damage to the endothelial glycocalyx after induction of type 1 diabetes.

Our data suggest that crosstalk between podocytes and the glomerular endothelium may be involved in the development of albuminuria and DN. Most extensively studied is the crosstalk of vascular endothelial growth factor (VEGF) and angiopoietin 1 and 2 between podocytes and glomerular endothelial cells, where the glomerular endothelial cell integrity depends upon signaling from the podocyte [26]. A recent study also reported the crosstalk of endothelin-1 between podocytes and glomerular endothelial cells [26, 27]. We recently showed that loss of podocyte heparanase prevents the increased passage of albumin across the endothelial monolayer induced by adriamycin [24]. In our current study we show that heparanase in culture supernatant of endothelin-1 stimulated podocytes is responsible for

increased transendothelial albumin passage *in vitro,* while endothelin-1 had no direct effect on endothelial cells, suggesting podocyte endothelial crosstalk. Notably, our transendothelial albumin flux model does not intend to mimic albumin flux *in* vivo, but rather is an assay of documenting the effects of endothelin-1 and heparanase on glomerular endothelial cells.

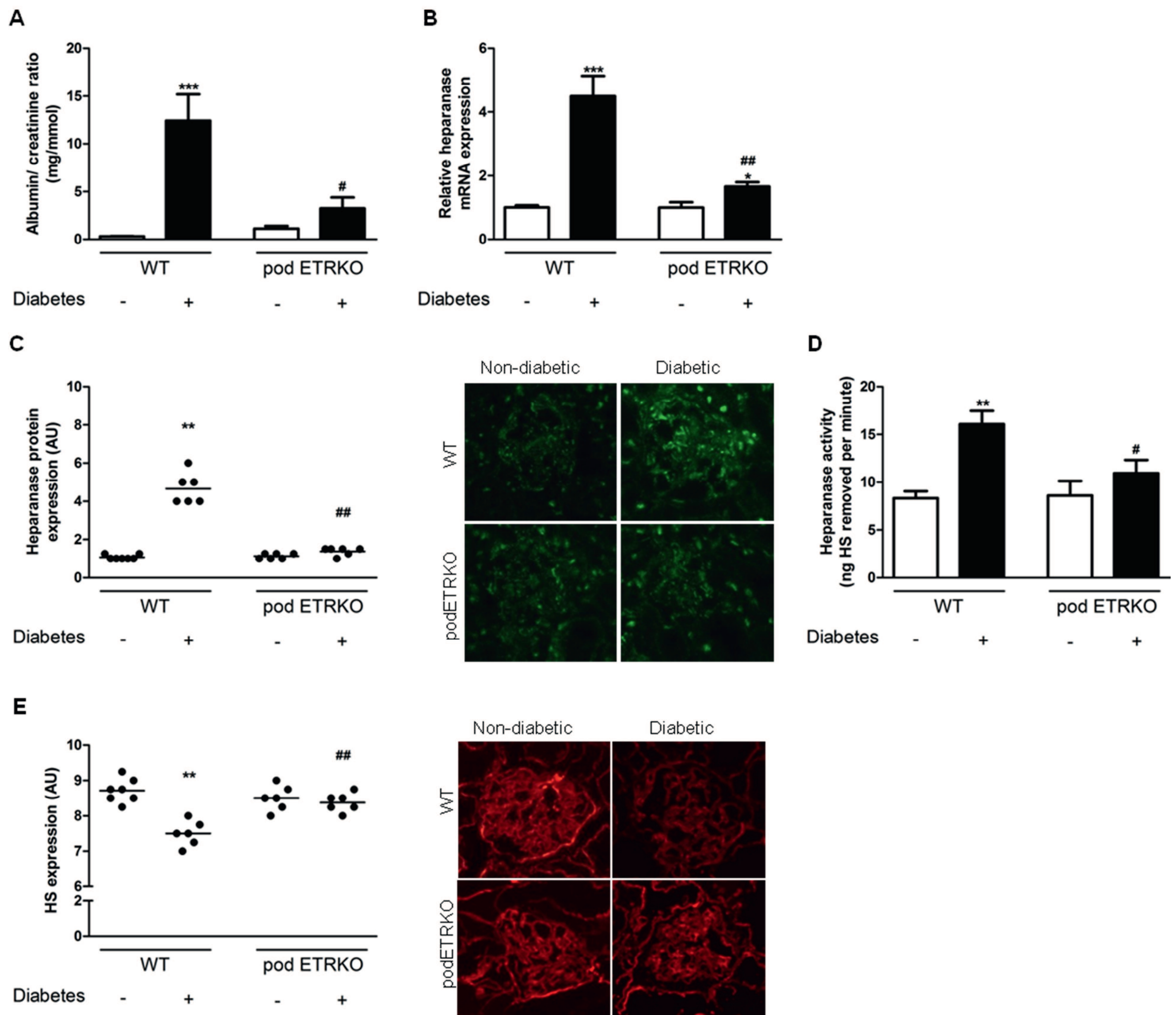

Figure 4: Glomerular heparanase and HS expression are normal in diabetic podETRKO mice. Ten weeks after induction of diabetes by streptozotocin, (**A**) WT mice developed a significant albuminuria, as indicated by an increased albumin/creatinine ratio. Albuminuria was significantly reduced in the diabetic podETRKO mice. (**B**) cortical heparanase mRNA expression, (**C**) glomerular heparanase protein expression, and (**D**) glomerular heparanase activity were increased by induction of diabetes in WT mice, whereas (**E**) glomerular HS expression was reduced. Heparanase mRNA expression was significantly lower in the diabetic podETRKO mice (**B**), whereas heparanase protein expression (**C**), heparanase activity (**D**) and HS expression (**E**) were normal in the diabetic podETRKO mice. 6-7 mice per group were analyzed using a one-way ANOVA. (**C**,**E**) Magnification x 400. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus non-diabetic control, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ versus diabetic WT. HS; heparan sulfate, podETRKO; podocyte-specific endothelin receptor knockout, WT; wild type, AU; arbitrary units.

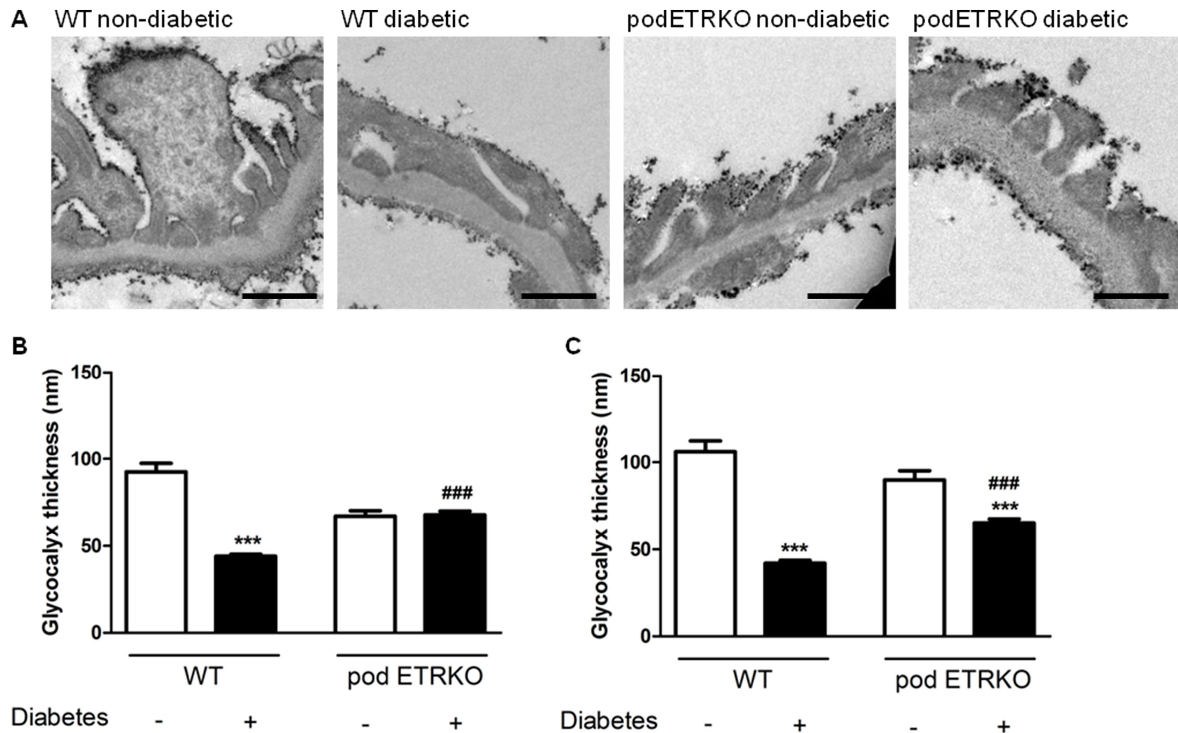

Figure 5: Glomerular endothelial glycocalyx is preserved in diabetic podETRKO mice. (**A**) Representative transmission electron microscopy images of lanthanum hydroxide visualizing the glycocalyx on top of the endothelium and podocytes (in black). Scale bar: 500 nm. (**B**) Quantification of endothelial glycocalyx thickness and (**C**) quantification of podocyte glycocalyx thickness. Glycocalyx thickness on both the endothelium and podocytes was ~50-60% reduced by induction of diabetes in WT mice. Endothelial glycocalyx thickness was preserved in diabetic podETRKO mice, but podocyte glycocalyx was ~25% reduced in diabetic podETRKO mice. Results were analyzed by a one-way ANOVA. $^{***}P<0.001$ versus non-diabetic control, $^{###}P<0.001$ versus diabetic WT. podETRKO; podocyte-specific endothelin receptor knockout, WT; wild type.

In addition to a potential podocyte-endothelial cell crosstalk, there may be various other mechanisms involved in the reduced endothelial glycocalyx thickness in WT mice. We have previously shown that high glucose, angiotensin II, aldosterone, reactive oxygen species, vitamin D and inflammatory cytokines such as TNF-α and IL-1β regulate heparanase expression [24, 28-30]. It could be that in WT mice one or more of these mediators induces endothelial heparanase expression, whereas these mediators are not operative in podETRKO mice or are counteracting each other's activities. Another possibility could be that macrophage influx is altered in podETRKO. Macrophages provide cathepsin L, a protease, that is required to activate endothelial-derived inactive proheparanase. Finally, podocyte-derived cathepsin L may be reduced in podETRKO mice, thereby limiting activation of endothelial-derived proheparanase. The exact molecular mechanisms underlying the observed differences in endothelial glycocalyx thickness in diabetic WT mice and podETRKO remain to be elucidated.

The glomerular endothelial glycocalyx plays an important role in vascular permeability, attenuation of blood cell-vessel wall interactions, mechanotransduction, signaling and vascular protection [31]. The podocyte glycocalyx is essential for maintaining foot process and slit diaphragm structure, and is also suggested to play a role keeping a certain distance between podocytes and parietal epithelial cells, thereby helping to maintain glomerular structure and function [32]. Previous studies have reported variations in endothelial glycocalyx thickness, which may be explained by differences in perfusion-fixed staining versus immersion-fixed staining. Very few studies showed podocyte glycocalyx staining and, to our knowledge, we are the first to report the simultaneous quantitation of podocyte glycocalyx thickness and glomerular endothelial glycocalyx thickness.

Previous studies showed that a reduced endothelial glycocalyx thickness is associated with the development of proteinuria [14-19]. Munich-Wistar-Fromter (MFW) rats showed a reduced endothelial glycocalyx thickness and spontaneously developed albuminuria [14]. Moreover, diabetic Zucker fatty rats showed a reduced endothelial glycocalyx thickness and a significant proteinuria, which was at least in part mediated by an increased glomerular heparanase expression [16]. Here, we showed that endothelin-1 signaling in the podocyte increased heparanase expression, thereby most likely reducing endothelial glycocalyx thickness and inducing proteinuria.

In conclusion, endothelin-1 induces heparanase in the podocyte, which may lead to disruption of the glycocalyx and proteinuria in experimental DN, thereby providing a possible mechanistic basis for the renoprotective effects of ERAs. In addition, targeting of heparanase in DN may serve as an alternative therapeutic option for treatment of DN.

Acknowledgements

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References

1. Barton, M. and M. Yanagisawa, *Endothelin: 20 years from discovery to therapy.* Canadian journal of physiology and pharmacology, 2008. **86**(8): p. 485-98.
2. Clozel, M. and H. Salloukh, *Role of endothelin in fibrosis and anti-fibrotic potential of bosentan.* Annals of medicine, 2005. **37**(1): p. 2-12.
3. Shi-Wen, X., et al., *Fibroblast matrix gene expression and connective tissue remodeling: role of endothelin-1.* The Journal of investigative dermatology, 2001. **116**(3): p. 417-25.
4. Neuhofer, W. and D. Pittrow, *Role of endothelin and endothelin receptor antagonists in renal disease.* European journal of clinical investigation, 2006. **36 Suppl 3**: p. 78-88.
5. Fukui, M., et al., *Gene expression for endothelins and their receptors in glomeruli of diabetic rats.* The Journal of laboratory and clinical medicine, 1993. **122**(2): p. 149-56.
6. Barton, M., *Reversal of proteinuric renal disease and the emerging role of endothelin.* Nature clinical practice. Nephrology, 2008. **4**(9): p. 490-501.
7. Mann, J.F., et al., *Avosentan for overt diabetic nephropathy.* J Am Soc Nephrol, 2010. **21**(3): p. 527-35.
8. Schievink, B., et al., *Prediction of the effect of atrasentan on renal and heart failure outcomes based on short-term changes in multiple risk markers.* Eur J Prev Cardiol, 2015.
9. Gagliardini, E., C. Zoja, and A. Benigni, *Et and diabetic nephropathy: preclinical and clinical studies.* Semin Nephrol, 2015. **35**(2): p. 188-96.
10. Kohan, D.E. and M. Barton, *Endothelin and endothelin antagonists in chronic kidney disease.* Kidney Int, 2014. **86**(5): p. 896-904.
11. Lenoir, O., et al., *Direct action of endothelin-1 on podocytes promotes diabetic glomerulosclerosis.* Journal of the American Society of Nephrology : JASN, 2014. **25**(5): p. 1050-62.
12. Gil, N., et al., *Heparanase is essential for the development of diabetic nephropathy in mice.* Diabetes, 2012. **61**(1): p. 208-16.
13. van den Born, J., et al., *Distribution of GBM heparan sulfate proteoglycan core protein and side chains in human glomerular diseases.* Kidney international, 1993. **43**(2): p. 454-63.
14. Salmon, A.H., et al., *Loss of the endothelial glycocalyx links albuminuria and vascular dysfunction.* Journal of the American Society of Nephrology : JASN, 2012. **23**(8): p. 1339-50.
15. Singh, A., et al., *Glomerular endothelial glycocalyx constitutes a barrier to protein permeability.* Journal of the American Society of Nephrology : JASN, 2007. **18**(11): p. 2885-93.
16. Kuwabara, A., et al., *Deterioration of glomerular endothelial surface layer induced by oxidative stress is implicated in altered permeability of macromolecules in Zucker fatty rats.* Diabetologia, 2010. **53**(9): p. 2056-65.
17. Jeansson, M., et al., *Adriamycin alters glomerular endothelium to induce proteinuria.* Journal of the American Society of Nephrology : JASN, 2009. **20**(1): p. 114-22.
18. Haraldsson, B. and J. Nystrom, *The glomerular endothelium: new insights on function and structure.* Curr Opin Nephrol Hypertens, 2012. **21**(3): p. 258-63.
19. Friden, V., et al., *The glomerular endothelial cell coat is essential for glomerular filtration.* Kidney Int, 2011. **79**(12): p. 1322-30.
20. Rops, A.L., et al., *Syndecan-1 deficiency aggravates anti-glomerular basement membrane nephritis.* Kidney international, 2007. **72**(10): p. 1204-15.
21. van Kuppevelt, T.H., et al., *Generation and application of type-specific anti-heparan sulfate antibodies using phage display technology. Further evidence for heparan sulfate heterogeneity in the kidney.* The Journal of biological chemistry, 1998. **273**(21): p. 12960-6.

22. Rops, A.L., et al., *Isolation and characterization of conditionally immortalized mouse glomerular endothelial cell lines.* Kidney international, 2004. **66**(6): p. 2193-201.
23. Mundel, P., et al., *Rearrangements of the cytoskeleton and cell contacts induce process formation during differentiation of conditionally immortalized mouse podocyte cell lines.* Experimental cell research, 1997. **236**(1): p. 248-58.
24. Garsen, M., et al., *Vitamin D attenuates proteinuria by inhibition of heparanase expression in the podocyte.* J Pathol, 2015. **237**(4): p. 472-81.
25. Wendel, M., et al., *Distribution of endothelin receptor subtypes ETA and ETB in the rat kidney.* The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society, 2006. **54**(11): p. 1193-203.
26. Fu, J., et al., *Glomerular endothelial cell injury and cross talk in diabetic kidney disease.* Am J Physiol Renal Physiol, 2015. **308**(4): p. F287-97.
27. Daehn, I., et al., *Endothelial mitochondrial oxidative stress determines podocyte depletion in segmental glomerulosclerosis.* The Journal of clinical investigation, 2014. **124**(4): p. 1608-21.
28. van den Hoven, M.J., et al., *Heparanase in glomerular diseases.* Kidney Int, 2007. **72**(5): p. 543-8.
29. Rops, A.L., et al., *Heparan sulfate domains on cultured activated glomerular endothelial cells mediate leukocyte trafficking.* Kidney Int, 2008. **73**(1): p. 52-62.
30. van den Hoven, M.J., et al., *Regulation of glomerular heparanase expression by aldosterone, angiotensin II and reactive oxygen species.* Nephrol Dial Transplant, 2009. **24**(9): p. 2637-45.
31. Garsen, M., et al., *The role of heparanase and the endothelial glycocalyx in the development of proteinuria.* Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association, 2014. **29**(1): p. 49-55.
32. Doyonnas, R., et al., *Anuria, omphalocele, and perinatal lethality in mice lacking the CD34-related protein podocalyxin.* J Exp Med, 2001. **194**(1): p. 13-27.

Chapter 7

Endothelial nitric oxide synthase prevents heparanase induction and the development of proteinuria

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Abstract

Endothelial nitric oxide synthase (eNOS) deficiency exacerbates proteinuria and renal injury in several glomerular diseases, but the underlying mechanism is not fully understood. We recently showed that heparanase is essential for the development of experimental diabetic nephropathy and glomerulonephritis, and hypothesize that heparanase expression is regulated by eNOS. Here, we demonstrate that induction of adriamycin nephropathy (AN) in C57BL/6 eNOS-deficient mice leads to an increased glomerular heparanase expression accompanied with overt proteinuria, which was not observed in the AN-resistant wild type counterpart. *In vitro*, the eNOS inhibitor asymmetric dimethylarginine (ADMA) induced heparanase expression in cultured mouse glomerular endothelial cells. Moreover, ADMA enhanced transendothelial albumin passage in a heparanase-dependent manner. We conclude that eNOS prevents heparanase induction and the development of proteinuria.

Introduction

Proteinuria is a key feature of many glomerular diseases and an independent risk factor for the progression to renal failure. Proteinuria is caused by damage to the glomerular filtration barrier, which is composed of glomerular endothelial cells covered by a glycocalyx, the glomerular basement membrane and podocytes. Several studies showed that a reduced glomerular endothelial glycocalyx thickness is associated with the development of proteinuria [1-3]. We recently showed that endothelial dysfunction and damage precedes podocyte damage in adriamycin-induced nephropathy (AN) [4], indicating that a healthy glomerular endothelium is important to prevent the development of proteinuria and renal damage.

Previous studies showed that a decreased nitric oxide (NO) production and availability contributes to endothelial dysfunction [5]. NO is produced by endothelial cells through endothelial NO synthase (eNOS). Multiple factors have been suggested to be involved in the regulation of eNOS, including reactive oxygen species (ROS), angiotensin II, asymmetric dimethylarginine (ADMA), protein kinase C (PKC), advanced glycation end products (AGEs), vitamin D and tumor necrosis factor (TNF)-α [6, 7]. eNOS deficiency exacerbates renal injury in accelerated anti-glomerular basement membrane (GBM) glomerulonephritis [8], experimental focal segmental glomerulosclerosis (FSGS) [9], and diabetic nephropathy (DN) [10]. In addition, eNOS gene delivery prevented the development of proteinuria in a rat model of FSGS [11]. Recently, we showed that induction of AN in C57BL/6 mice, an AN resistant strain, with eNOS deficiency induced overt proteinuria, glomerulosclerosis, tubulointerstitial fibrosis and inflammation [4]. The mechanism how eNOS deficiency exacerbates proteinuria and renal damage remains unknown.

We previously showed that heparanase, a heparan sulfate (HS) specific endoglycosidase, is essential for the development of proteinuria and renal damage in experimental DN and glomerulonephritis [12, 13]. In both aforementioned models heparanase-deficient mice displayed a preserved glomerular HS expression compared to WT mice. Notably, loss of glomerular HS expression is associated with the development of proteinuria in most human and experimental glomerular disease [14]. Heparanase is positively regulated by ROS, angiotensin II, aldosterone, AGEs, PKC, high glucose and TNF-α [13, 15-18], and negatively regulated by vitamin D [19]. Interestingly, the aforementioned factors that regulate heparanase are also involved in regulation of eNOS and endothelial function. Importantly, glomerular endothelial glycocalyx thickness is reduced in diabetic eNOS-deficient mice compared with diabetic wild type (WT) mice [20]. In addition, heparanase has been shown to impair glycocalyx thickness [1, 3]. Therefore, we hypothesize that heparanase expression is controlled by eNOS.

Materials and methods

Animals

WT C57BL/6J mice (Monash Animal Services, Monash University, Australia) and eNOS-deficient mice in a C57BL/6J background (Jackson Laboratories, Ben Harbor, ME, USA) were housed and bred at Monash Animal Services. Animal handling and experiments were approved by the Monash University Animal Ethics Committee and according to the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes". Balb/c mice (Harlan, Horst, The Netherlands) were housed at the "Vrije Universiteit Brussel" and animal experiments were approved by the Animal Care and Use Committee of the "Vrije Universiteit Brussel".

Adriamycin-induced nephropathy

AN was induced in 8-week-old C57BL/6J WT and eNOS-deficient mice by an intravenous injection of 10.5 mg/kg body weight of adriamycin (Sigma-Aldrich, St. Louis, MO, USA), as described previously [4]. Control mice received an equivalent volume of normal saline (NS). Mice were sacrificed 14 days after induction of AN. Five mice were used per group.
In Balb/c mice (8 weeks old), AN was induced by an intravenous injection with 10 mg/kg adriamycin (Pharmacia, Brussels, Belgium) as described [21]. Control mice were injected with NS. Mice, five-six per time point, were sacrificed 10 and 23 days after induction of AN.

Immunofluorescence staining

Glomerular heparanase and HS expression was determined by indirect immunofluorescence staining on cryosections (2 µm) as described [22]. Primary antibodies included heparanase (HPA1, ProsPecTany, Rehovot, Israel) and a mouse monoclonal anti-HS antibody, JM403 [23]. Secondary antibodies included goat anti-rabbit IgG Alexa 488 and goat anti-mouse IgM Alexa 488 (Invitrogen Life Technologies, Breda, The Netherlands). Glomerular heparanase and HS staining intensities were scored in 50 glomeruli per section on a scale between 0 and 10 (0= no staining, 5= 50% staining, 10= 100% staining). Scoring was performed by two independent investigators on blinded sections using a Leica CTR6000 microscope.

Cell culture and transendothelial albumin passage

Conditionally immortalized mouse glomerular endothelial cells (mGEnC-1) were cultured as described previously [24]. Heparanase was stably silenced in mGEnC-1 by transfection of a heparanase shRNA construct (Qiagen, Venlo, The Netherlands) with Lipofectamine 2000 into undifferentiated mGEnC-1 and subsequent selection with G418 (Sigma-Aldrich). Differentiated mGEnC-1 were treated with the eNOS inhibitor ADMA (10 µg/ml; Millipore, Amsterdam, The Netherlands) for 18 hours. For transendothelial albumin passage,

differentiated mGEnC-1 seeded on polyester membranes in tissue culture inserts (0.4 m pore size; Corning Incorporated, NY, USA) were treated with the eNOS inhibitor ADMA as outlined. Transendothelial albumin passage was determined as described previously [19].

RNA isolation, cDNA generation and real-time PCR

Total RNA was extracted from mouse renal cortex and mGEnC-1 using the RNeasy mini kit (Qiagen, Venlo, The Netherlands). 1 µg RNA was reverse-transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). Heparanase mRNA expression levels were measured by real-time PCR using gene-specific primers (Isogen Life Science, de Meern, The Netherlands) and the Fast-start SYBR Green SuperMix (Roche Diagnostics, Mannheim, Germany), and analyzed with the CFX real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Used primers: Heparanase, forward 5'-GAGCGGAGCAAACTCCGAGTGTATC-3', reverse 5'-GATCCAGAATTTGACCGTTCAGTT -3', and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5'-AGAAACCTGCCAAGTATGATGAC-3', reverse 5'-TCATTGTCATACCAGGAAATGAG-3'. Heparanase gene expression levels were quantified with the delta-delta C_T method using GAPDH as the housekeeping gene.

Statistical analysis

Values are expressed as mean ± SEM. Significance was evaluated by a one-way ANOVA and *post hoc* analysis with Tukey's multiple comparison test. A Student's *t*-test was used for comparison of expression between two different groups. A two-way repeated measures ANOVA with Bonferroni post-test was used to determine significant differences in transendothelial albumin passage. Statistical analysis was performed using GraphPad Prism 5.03 (GraphPad Software, Inc., San Diego, CA, USA). A P-value of ≤ 0.05 was considered statistically significant.

Results

eNOS prevents adriamycin-induced heparanase expression and proteinuria

To study the *in vivo* effects of eNOS deficiency on heparanase and HS expression, AN was induced in C57BL/6 WT, normally an AN resistant strain, and C57BL/6 eNOS-deficient mice. Fourteen days after the induction of AN, as expected, WT mice failed to develop proteinuria and had a normal renal function [4]. In contrast, eNOS-deficient mice developed overt proteinuria and had an impaired renal function [4]. Cortical heparanase mRNA expression (Figure 1A) and glomerular heparanase protein expression (Figure 1B,D) were normal in WT AN mice, whereas both heparanase mRNA expression and heparanase protein expression

were significantly increased in the eNOS-deficient AN mice. In addition, glomerular HS expression was normal in the WT mice, but significantly reduced in the eNOS-deficient mice after induction of AN (Figure 1C,E). To evaluate whether AN caused similar effects on heparanase and HS expression in AN-sensitive mice as observed in the eNOS-deficient mice, AN was induced in the AN-sensitive Balb/c mice. We previously showed that eNOS expression was significantly reduced 24 hours after induction of AN in Balb/c mice [4]. By induction of AN, Balb/c mice develop proteinuria and renal damage after 10 and 23 days, as described before [21]. Cortical heparanase mRNA expression (Figure 2A) and glomerular heparanase protein expression (Figure 2B,D) were significantly increased 10 and 23 days after induction of AN, whereas glomerular HS expression was significantly reduced (Figure 2C,E). Together, these data indicate that eNOS prevents adriamycin-induced heparanase expression.

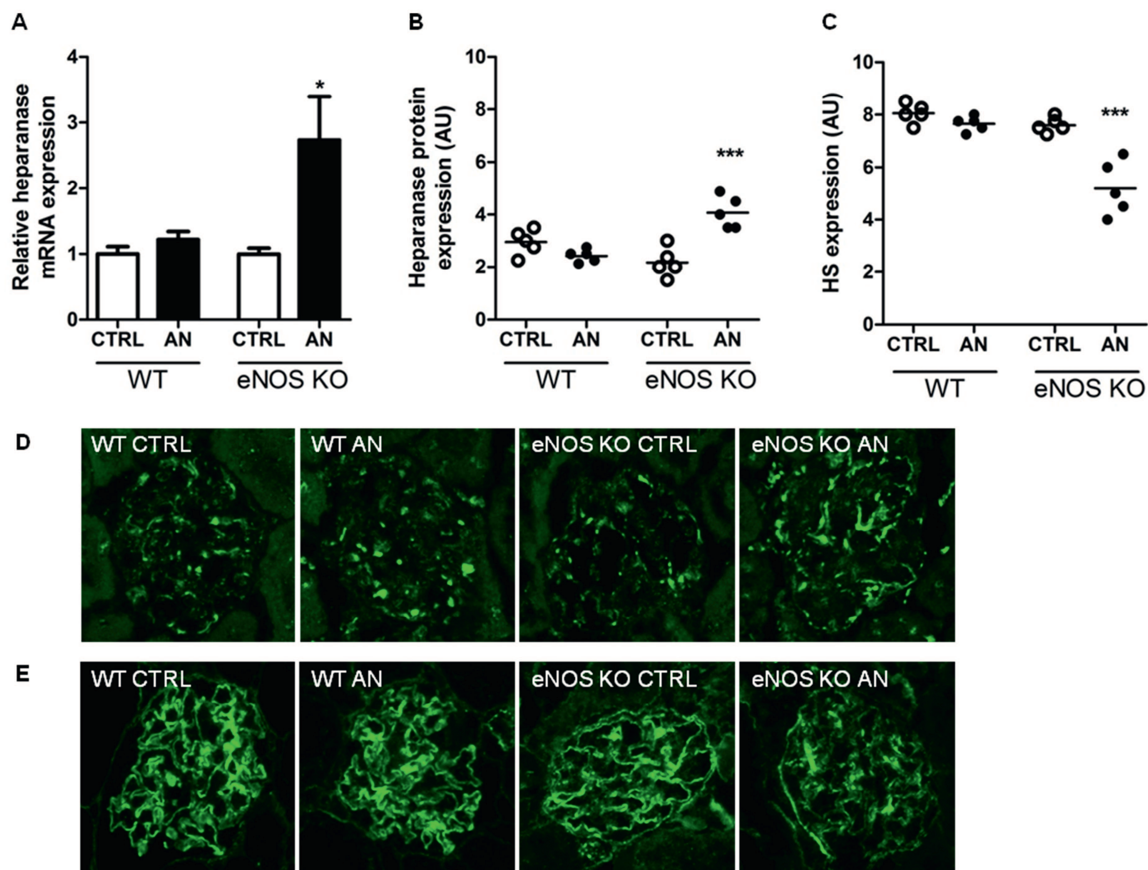

Figure 1: eNOS prevents adriamycin-induced heparanase expression and proteinuria. Adriamycin-nephropathy (AN) was induced in C57BL/6 WT mice, an AN resistant strain, and C57BL/6 eNOS-deficient mice. Mice were sacrificed 14 days after the induction of AN. By induction of AN in WT mice, (**A**) cortical heparanase mRNA expression, (**B**,**D**) glomerular heparanase protein expression and (**C**,**E**) glomerular HS expression were comparable to control. Cortical heparanase mRNA expression and glomerular heparanase protein expression were significantly increased in eNOS-deficient mice after induction of AN, whereas glomerular HS expression was reduced. (**D**) Representative pictures showing glomerular heparanase protein expression and (**E**) glomerular HS expression as determined by immunofluorescence staining (magnification x400). 5 mice per group were used for analysis. *P<0.05 and ***P<0.001 versus CTRL. WT, wild type; eNOS KO, endothelial nitric oxide synthase-deficient; HS, heparan sulfate; CTRL, control; AU, arbitrary units.

The eNOS inhibitor ADMA increases transendothelial albumin passage in a heparanase-dependent manner

To extend the *in vivo* findings of eNOS deficiency on glomerular heparanase expression in AN, we evaluated whether eNOS regulates heparanase expression in glomerular endothelial cells *in vitro*. Treatment of mouse glomerular endothelial cells (mGEnC-1) with the eNOS inhibitor ADMA resulted in a 1.5-fold increased heparanase mRNA expression (Figure 3A). At the functional level, treatment of a monolayer of mGEnC-1 with AMDA increased transendothelial albumin passage 1.4-fold (Figure 3B). To show that the ADMA-induced increase in transendothelial albumin passage could be possibly mediated by heparanase, heparanase expression in mGEnC-1 was silenced with shRNA leading to ~60% reduced heparanase mRNA expression. Upon treatment with ADMA, transendothelial albumin passage was significantly lower in heparanase-silenced endothelial cells compared with endothelial cells transfected with a scrambled shRNA (Figure 3C). Taken together, these data indicate that eNOS inhibition increased heparanase expression and thereby transendothelial albumin passage.

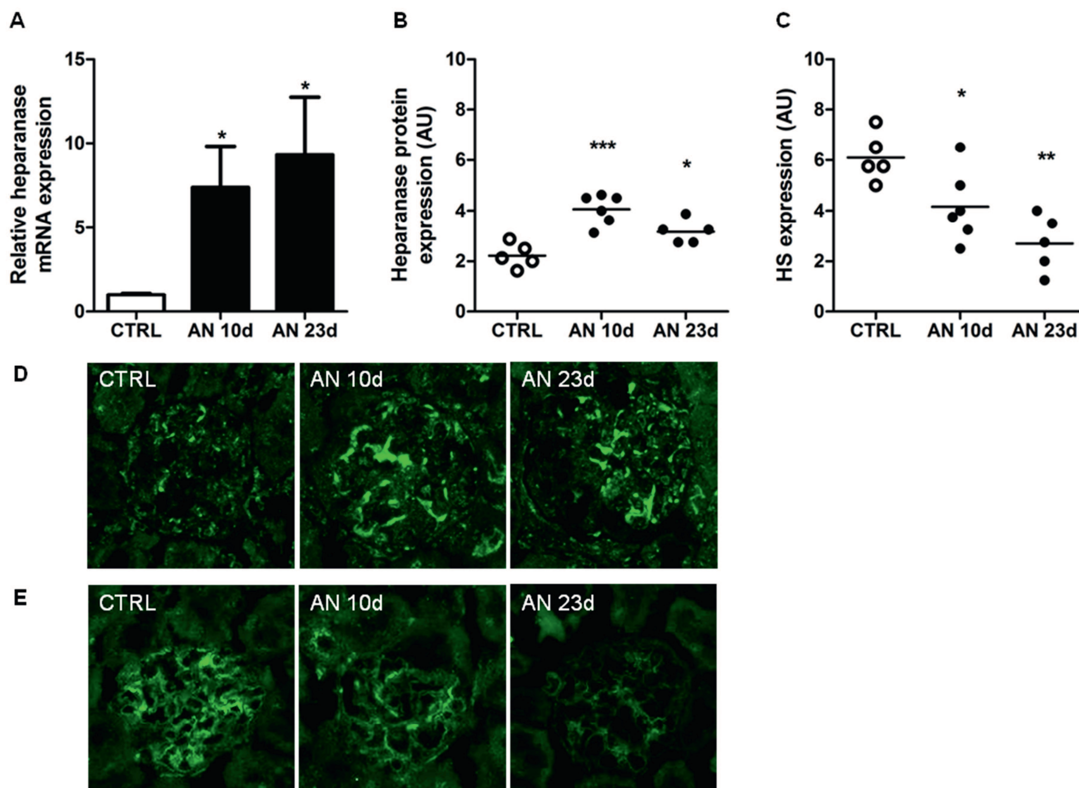

Figure 2: Heparanase expression was increased and HS expression reduced by induction of adriamycin nephropathy (AN) in AN-sensitive Balb/c mice. AN was induced in Balb/c, which are sensitive for AN-induced renal damage. Mice were sacrificed 10 and 23 days after the induction of AN. By induction of AN, (**A**) cortical heparanase mRNA expression and (**B**,**D**) glomerular heparanase protein expression were significantly increased after 10 and 23 days. (**C**,**E**) glomerular HS expression was significantly reduced 10 and 23 days after induction of AN. (**D**) Representative pictures showing glomerular heparanase protein expression and (**E**) glomerular HS expression, as determined by immunofluorescence staining (magnification x400). 5-6 mice were used for analysis. *P<0.05, **P<0.01 and ***P<0.001 versus CTRL. CTRL, control; AU, arbitrary units.

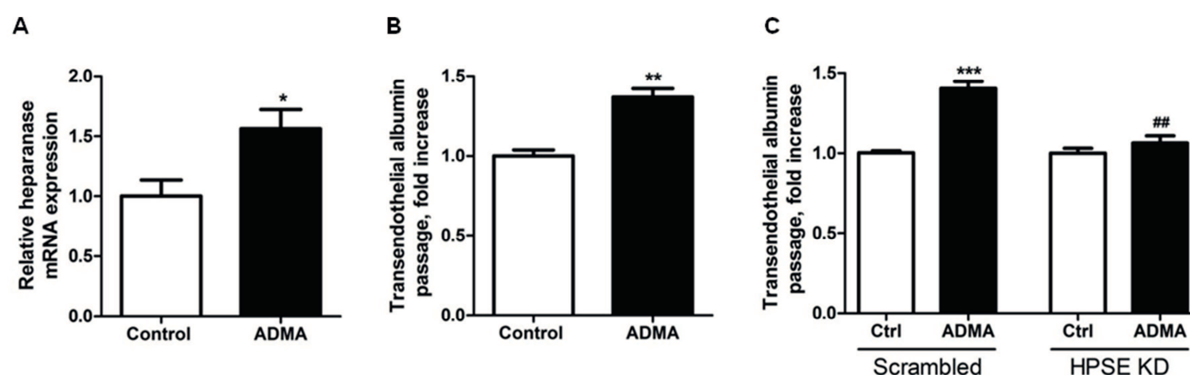

Figure 3: Inhibition of eNOS induces heparanase expression and increases transendothelial albumin passage in a heparanase-dependent manner in cultured mouse glomerular endothelial cells. (**A**) Treatment of mouse glomerular endothelial cells (mGEnC-1) with the eNOS inhibitor ADMA for 18 hours resulted in an increased heparanase mRNA expression. (**B**) Cumulative passage of FITC-labeled albumin across the mGEnC-1 monolayer (TEER: 28 Ω cm^2) was increased 1.4-fold compared to control after treatment with ADMA for 18 hours. (**C**) Treatment of heparanase-silenced mGEnC-1 with ADMA for 18 hours led to lower transendothelial albumin passage compared with scrambled mGEnC-1 treated with ADMA. **P*<0.05, ***P*<0.01 and ****P*<0.001 versus control. ##*P*<0.01 versus ADMA scrambled. eNOS; endothelial nitric oxide synthase, ADMA; asymmetric dimethylarginine.

Discussion

Our data suggest that eNOS prevents the induction of glomerular heparanase expression and the development of proteinuria in AN. In addition, inhibition of eNOS activity in cultured mouse glomerular endothelial cells induces heparanase expression and increases transendothelial albumin passage in a heparanase-dependent manner.

Our study is the first to show that heparanase expression is regulated by eNOS. As outlined, eNOS and heparanase share regulating factors such as ROS, angiotensin II, PKC, AGEs, vitamin D and TNF-α [6, 7, 13, 15-17, 19]. However, not all of these aforementioned regulating factors are activators of eNOS and heparanase, nor solely operative in the endothelium, whereas none of these factors is specific for the glomerular endothelium. Interestingly, vitamin D is a positive regulator of eNOS, but a negative regulator of heparanase [7, 19]. It has also been described that HS present in the endothelial glycocalyx is important for mechanosensing and eNOS-mediated NO production [25], which may suggest that a reduced glycocalyx thickness further hampers NO production, thereby further increasing heparanase expression. This self amplifying loop will ultimately lead to loss of endothelial glycocalyx and loss of NO production, and therefore endothelial dysfunction. We provided clear evidence for the interplay between eNOS and heparanase in AN, which may be operative in other glomerular diseases as well, since in the majority of glomerular diseases heparanase expression is increased, whereas glomerular HS expression is

decreased [14]. Nevertheless, additional research is required to elucidate the complex regulation of both eNOS and heparanase, and their interplay, in glomerular diseases.
The regulation of heparanase expression by eNOS seems important for glomerular diseases, but may have important implications for other vascular beds outside the kidney as well. The vascular involvement in clinical manifestations associated with diabetes, sepsis, ischemia, artherosclerosis, and angiogenesis in cancer [26] may be dictated in part by the interplay between eNOS and heparanase. Future research should address the beneficial effects of a combined targeting of both eNOS and heparanase in experimental models of the aforementioned clinical manifestations and glomerular diseases.
Taken together, we postulate that a reduced glomerular expression/activity of eNOS increases heparanase expression, most likely due to a low NO level. This increased heparanase expression may explain a reduced thickness of the glomerular endothelial glycocalyx in (experimental) glomerular diseases, like diabetic nephropathy and adriamycin nephropathy [2, 3].

Acknowledgements

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References

1. Singh, A., et al., *Glomerular endothelial glycocalyx constitutes a barrier to protein permeability.* J Am Soc Nephrol, 2007. **18**(11): p. 2885-93.
2. Jeansson, M., et al., *Adriamycin alters glomerular endothelium to induce proteinuria.* J Am Soc Nephrol, 2009. **20**(1): p. 114-22.
3. Garsen, M., et al., *Endothelin-1 Induces Proteinuria by Heparanase-Mediated Disruption of the Glomerular Glycocalyx.* J Am Soc Nephrol, 2016.
4. Sun, Y.B., et al., *Glomerular endothelial cell injury and damage precedes that of podocytes in adriamycin-induced nephropathy.* PLoS One, 2013. **8**(1): p. e55027.
5. Tousoulis, D., et al., *The role of nitric oxide on endothelial function.* Curr Vasc Pharmacol, 2012. **10**(1): p. 4-18.
6. Takahashi, T. and R.C. Harris, *Role of endothelial nitric oxide synthase in diabetic nephropathy: lessons from diabetic eNOS knockout mice.* J Diabetes Res, 2014. **2014**: p. 590541.
7. Andrukhova, O., et al., *Vitamin D is a regulator of endothelial nitric oxide synthase and arterial stiffness in mice.* Mol Endocrinol, 2014. **28**(1): p. 53-64.
8. Heeringa, P., et al., *Lack of endothelial nitric oxide synthase aggravates murine accelerated anti-glomerular basement membrane glomerulonephritis.* Am J Pathol, 2000. **156**(3): p. 879-88.
9. Nakayama, T., et al., *Endothelial injury due to eNOS deficiency accelerates the progression of chronic renal disease in the mouse.* Am J Physiol Renal Physiol, 2009. **296**(2): p. F317-27.
10. Nakagawa, T., et al., *Diabetic endothelial nitric oxide synthase knockout mice develop advanced diabetic nephropathy.* J Am Soc Nephrol, 2007. **18**(2): p. 539-50.
11. Savard, S., et al., *eNOS gene delivery prevents hypertension and reduces renal failure and injury in rats with reduced renal mass.* Nephrol Dial Transplant, 2012. **27**(6): p. 2182-90.
12. Gil, N., et al., *Heparanase is essential for the development of diabetic nephropathy in mice.* Diabetes, 2012. **61**(1): p. 208-16.
13. Garsen, M., et al., *Heparanase Is Essential for the Development of Acute Experimental Glomerulonephritis.* Am J Pathol, 2016. **186**(4): p. 805-15.
14. van den Hoven, M.J., et al., *Heparanase in glomerular diseases.* Kidney Int, 2007. **72**(5): p. 543-8.
15. van den Hoven, M.J., et al., *Regulation of glomerular heparanase expression by aldosterone, angiotensin II and reactive oxygen species.* Nephrol Dial Transplant, 2009. **24**(9): p. 2637-45.
16. Masola, V., et al., *Regulation of heparanase by albumin and advanced glycation end products in proximal tubular cells.* Biochim Biophys Acta, 2011. **1813**(8): p. 1475-82.
17. Shafat, I., I. Vlodavsky, and N. Ilan, *Characterization of mechanisms involved in secretion of active heparanase.* J Biol Chem, 2006. **281**(33): p. 23804-11.
18. Maxhimer, J.B., et al., *Heparanase-1 gene expression and regulation by high glucose in renal epithelial cells: a potential role in the pathogenesis of proteinuria in diabetic patients.* Diabetes, 2005. **54**(7): p. 2172-8.
19. Garsen, M., et al., *Vitamin D attenuates proteinuria by inhibition of heparanase expression in the podocyte.* J Pathol, 2015. **237**(4): p. 472-81.
20. Kanetsuna, Y., et al., *Deficiency of endothelial nitric-oxide synthase confers susceptibility to diabetic nephropathy in nephropathy-resistant inbred mice.* Am J Pathol, 2007. **170**(5): p. 1473-84.
21. Van Beneden, K., et al., *Valproic acid attenuates proteinuria and kidney injury.* J Am Soc Nephrol, 2011. **22**(10): p. 1863-75.
22. Rops, A.L., et al., *Syndecan-1 deficiency aggravates anti-glomerular basement membrane nephritis.* Kidney Int, 2007. **72**(10): p. 1204-15.

23. van den Born, J., et al., *A monoclonal antibody against GBM heparan sulfate induces an acute selective proteinuria in rats.* Kidney Int, 1992. **41**(1): p. 115-23.
24. Rops, A.L., et al., *Isolation and characterization of conditionally immortalized mouse glomerular endothelial cell lines.* Kidney Int, 2004. **66**(6): p. 2193-201.
25. Florian, J.A., et al., *Heparan sulfate proteoglycan is a mechanosensor on endothelial cells.* Circ Res, 2003. **93**(10): p. e136-42.
26. Rajendran, P., et al., *The vascular endothelium and human diseases.* Int J Biol Sci, 2013. **9**(10): p. 1057-69.

Chapter 8

Vitamin D attenuates proteinuria by inhibition of heparanase expression in the podocyte

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Abstract

The glomerular filtration barrier consists of podocytes, the glomerular basement membrane, and endothelial cells covered with a glycocalyx. Heparan sulfate (HS) expression in the glomerular filtration barrier is reduced in patients with proteinuria, which is associated with an increased expression of the HS-degrading enzyme heparanase. Previously, we showed that heparanase is essential for the development of proteinuria in experimental diabetic nephropathy. Vitamin D supplementation reduces podocyte loss and proteinuria *in vitro* and *in vivo*. Therefore, we hypothesize that vitamin D reduces proteinuria by reduction of glomerular heparanase expression. Adriamycin-exposed rats developed proteinuria and showed increased heparanase expression, which was reduced by 1,25-dihydroxyvitamin D_3 (1,25-D_3) treatment. *In vitro*, adriamycin treatment increased heparanase mRNA expression in the podocyte, which could be corrected by 1,25-D_3 treatment. In addition, 1,25-D_3 treatment reduced transendothelial albumin passage after adriamycin stimulation. In line with these results, we showed direct binding of the vitamin D receptor to the heparanase promoter, which dose-dependently reduced heparanase promoter activity. Finally, 1,25-D_3-deficient 25-hydroxy-1α-hydroxylase knockout mice developed proteinuria, and showed increased heparanase expression, which was normalized by 1,25-D_3 treatment. Our data suggest that the protective effect of vitamin D on the development of proteinuria is mediated by inhibiting heparanase expression in the podocyte.

Introduction

The glomerular filtration barrier (GFB), composed of glomerular endothelial cells covered with a glycocalyx, the glomerular basement membrane (GBM) and podocytes, is responsible for the charge- and size-selective filtration of the blood. The charge-selective filtration of the GFB has been attributed to the presence of negatively charged molecules in the GFB, of which heparan sulfate (HS) is the most abundant. Heparan sulfate proteoglycans (HSPGs) consist of a core protein to which one or more HS side chains are covalently attached. Loss of HS expression in the GFB is associated with the development of proteinuria [1, 2]. During the last decade, the primary role of HS in the glomerular basement membrane in charge-selective filtration was refuted [2, 3]. However, in patients proteinuria is associated with a reduction in glomerular HS expression, which suggest that the presence of HS in the endothelial glycocalyx is key in dictating the charge-selective properties of the GFB [2].
The decreased glomerular HS expression in patients with proteinuria could be attributed to an increased glomerular expression of the HS-degrading enzyme heparanase, the only known mammalian enzyme that can cleave HS [4]. The expression of heparanase is increased in many experimental and human glomerular diseases [2-6]. Inhibition of heparanase in experimental passive Heymann nephritis by a polyclonal anti-heparanase antibody or the heparanase inhibitor PI-88 reduced proteinuria, suggesting that heparanase is important for the development of proteinuria [7, 8]. Recently, we showed that heparanase is essential for the development of proteinuria in experimental diabetic nephropathy [9]. In response to streptozotocin-induced type 1 diabetes, heparanase knockout mice, unlike their wildtype littermates, failed to develop proteinuria and diabetic nephropathy, and diabetic heparanase knockout mice had had a normal renal function. In addition, treatment with the heparanase inhibitor SST0001 reduced proteinuria and renal function loss [9].
Proteinuria is an independent risk factor for the progression of renal failure. Current treatment of patients with proteinuria is blockade of the renin-angiotensin-aldosterone system (RAAS) by angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs). This treatment only partly reduces proteinuria, and therefore there is a need for additional anti-proteinuric agents. Recent findings suggest anti-proteinuric effects of vitamin D analogues in experimental and human glomerular diseases. Vitamin D is a steroid hormone that is involved in the regulation of calcium and phosphate homeostasis. Vitamin D_3 (cholecalciferol) is taken up from the diet and produced in the skin from 7-dehydrocholesterol by exposure to ultraviolet radiation. Subsequently, vitamin D_3 is converted to 25-hydroxyvitamin D_3 in the liver and to 1,25-dihydroxyvitamin D_3 (1,25-D_3), the active form of vitamin D_3, in the kidney. Vitamin D binds to the vitamin D receptor (VDR). The VDR then interacts with the retinoid X receptor to form a heterodimer that binds to vitamin D responsive

elements in the promoter regions of vitamin D-responsive genes [10]. As a result of renal damage, many patients with chronic kidney disease (CKD) develop a progressive 1,25-D_3 deficiency [11]. Several clinical studies showed that proteinuria in CKD patients, in addition to ACE inhibitors or ARBs, could be further reduced by treatment with 1,25-D_3 [12-18]. In experimental animal models for focal segmental glomerulosclerosis (FSGS) such as adriamycin nephropathy and 5/6 nephrectomy, proteinuria and podocyte loss were decreased by treatment with 1,25-D_3 [19-21].

Since heparanase is a key mediator in the development of proteinuria and vitamin D seems beneficial in reducing proteinuria and kidney injury, we hypothesize that vitamin D regulates glomerular heparanase expression. In the current study, we evaluated the effects of vitamin D on the glomerular heparanase and HS expression in animal models of FSGS or 1,25-D_3 deficiency. In addition, we evaluated whether vitamin D was able to directly regulate heparanase expression in cultured mouse glomerular endothelial cells and mouse podocytes.

Materials and methods

Animals

Animals were housed in a temperature-controlled room with a 12-hour light/dark cycle with *ad libitum* access to food and water. All animal experiments were approved by the Animal Ethical Committee of the Radboud University Nijmegen.

Rats. Adriamycin nephropathy (AN) was induced in 8-week-old Wistar rats (Charles River Laboratories, Wilmington, MA) by an intravenous injection of 5 mg/kg body weight of adriamycin (Sigma-Aldrich, St. Louis, MO), as described previously [21]. Rats were treated with daily i.p. injections of 2.5 µg/kg bodyweight of 1,25-D_3 (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) or vehicle. After 6 weeks, rats were placed in metabolic cages for 24 hours to collect urine, and sacrificed to collect kidneys and blood.

Mice. 1,25-D_3-deficient 25-hydroxy-1α-hydroxylase knockout (KO) mice were generated and genotyped as described [22]. 5-week-old KO mice and their wildtype (WT) littermates received daily i.p. injections with 500 pg/g bodyweight of 1,25-D_3 (Sigma-Aldrich) or vehicle. After 6 weeks, mice were housed in metabolic cages for 24 hours to collect urine. Subsequently, mice were sacrificed and kidneys and blood were collected.

Immunofluorescence staining

Glomerular heparanase and HS expression was determined by immunofluorescence staining as described [23]. Primary antibodies included anti-heparanase (HPA1, ProsPecTany,

Rehovot, Israel) and the VSV-tagged single chain variable fragment (scFv) antibody HS4C3 (N-, 2-O, 3-O and 6-O sulfation) [24]. Bound antibodies were detected with goat anti-rabbit IgG Alexa 488 (Invitrogen Life Technologies, Breda, The Netherlands) for heparanase and anti-VSV-Cy3 (Sigma-Aldrich) for HS4C3. Twenty five glomeruli per section were scored for staining intensities of heparanase and HS on a scale between 0 and 10 (0= no staining, 5= 50% staining, 10= 100% staining), which was performed by two independent investigators on blinded sections.

Heparanase activity assay

Heparanase activity in kidney cortex was determined using a commercially available assay (AMS Biotechnology, Abingdon, UK) according to the manufacturer's instruction.

Cell culture

Conditionally immortalized mouse glomerular endothelial cells (mGEnC-1) and mouse podocytes (MPC-5) were cultured as described previously [25, 26]. Silencing of heparanase in MPC-5 was achieved after transfecting a heparanase shRNA construct (Qiagen, Venlo, The Netherlands) and subsequent selection with G418 (Sigma-Aldrich). Where indicated, differentiated mGEnC-1 and MPC5 were stimulated with vehicle or 0.25 µg/ml adriamycin (Sigma-Aldrich) and treated with different concentrations of 1,25-D_3 (10 nmol/L, 100 nmol/L, and 1 µmol/L; Sigma-Aldrich).

Transendothelial albumin passage

Differentiated mGEnC-1 seeded on polyester membranes (0.4 µm pore size) in tissue culture inserts (Corning Incorporated, NY, USA) were treated with adriamycin and/or 100 nmol/L 1,25-D_3 as outlined. In addition, mGEnC-1 were treated with a 1:1 mix of medium supplemented with adriamycin and/or 100 nmol/L 1,25-D_3 and conditioned culture supernatant of podocytes also treated with adriamycin and/or 100 nmol/L 1,25-D_3. After 16 hours, medium in the insert was replaced by serum free medium (SFM) containing 0.5 mg/ml FITC-labeled BSA (Sigma) and medium in the well was replaced by SFM. After 1, 2 and 3 hours, aliquots were removed from the well and replaced with SFM. Fluorescence of the aliquots was measured on a fluorometer with excitation at 495 nm and emission at 520 and the amount of albumin passing the endothelial cell monolayer was determined by a set of standard dilutions.

RNA isolation and real-time PCR

Total RNA was extracted from kidney cortex, podocytes and mGEnC-1 using the RNeasy mini kit (Qiagen, Venlo, The Netherlands). RNA (1 µg) was reverse transcribed into cDNA

using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). The mRNA levels of heparanase were quantified on the CFX real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) using SYBR Green SuperMix (Roche Diagnostics, Mannheim, Germany) with gene-specific primers (Isogen Life Science, De Meern, The Netherlands). Heparanase (HPSE): forward 5'-GAGCGGAGCAAACTCCGAGTGTATC-3', reverse 5'-GATCCAGAATTTGACCGTTCAGTT-3', and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward 5'-AGAAACCTGCCAAGTATGATGAC-3', reverse 5'-TCATTGTCATACCAGGAAATGAG-3'. Gene expression levels of heparanase were quantified by the delta-delta C_T method using GAPDH as the housekeeping gene.

Determination of HS expression on mGEnC-1 by ELISA

mGEnC-1 were differentiated for 7 days in 96-wells plates and stimulated with vehicle or adriamycin in the absence or presence of 1,25-D_3 for 16 hours. Cells were washed and incubated with the VSV-tagged scFv antibody HS4C3 [24]. Cells were washed and incubated with anti-VSV-G-peroxidase (Sigma-Aldrich). Finally, cells were washed and incubated with tetramethylbenzidine (TMB) substrate (Biolegend, London, UK). Reaction was stopped with 2 M H_2SO_4 and absorption was measured at 450 nm.

Transfection and luciferase activity assay

A pGL3 luciferase reporter vector containing the 3.5 kb promoter region of the human *HPR1* (heparanase) gene was generated previously [27]. Opossum Kidney (OK) cells were cultured in 12-well plates as described previously [28]. Cells were treated with different concentrations of 1,25-D_3 (100 nmol/L and 1 µmol/L; Sigma-Aldrich) and transfected using the following mixture: either pGL3-HPR1 promoter construct or pGL3-basic empty vector with 50 ng of pRL-CMV (Promoga Corp., Fitchburg, WI) as control for transfection efficiency, and 1,25 µL lipofectamin in 120 µL Opti-MEM medium (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection cells were harvested and luciferase activity was determined using the Dual-Luciferase reporter assay (Promega) following manufacturer's instructions.

Chromatin Immunoprecipitation (ChIP)

OK cells were transfected with the HPR1 promoter construct or the empty vector as outlined above and treated with 1 µmol/L 1,25-D_3. Four hours after transfection, the ChIP assay was performed according to manufacturer's protocol (Merck Millipore, Billerica, MA). Briefly, cells were treated with formaldehyde to crosslink proteins to DNA, lysed and sonicated twice on melting ice. Samples were incubated with protein A magnetic beads and 5.0 µg of the rabbit polyclonal anti-VDR antibody (ab3508; Abcam Inc., Cambridge, MA) or the normal rabbit IgG antibody as a negative control. Subsequently, protein-DNA cross-links were reversed and

DNA was isolated. Real-time PCR analysis was performed using specific primers for the *HPR1* promoter region (forward 5'-TGTTCCTCCTTCCTATGTATCC-3', reverse 5'-TTGGCTGAGATCTTGCTCCT-3'). Amplified DNA samples were resolved by 2% agarose gel electrophoresis and visualized by ethidium bromide.

Statistical analysis

Values are expressed as mean ± SEM. Significance was evaluated by an one-way ANOVA and *post hoc* analysis with Tukey's multiple comparison test. Comparison of expression between two different groups was evaluated using the Student's t-test. A 2-way repeated measures ANOVA with Bonferroni post-test was used to evaluate significance for the transendothelial albumin passage experiments. Statistical analysis was performed using GraphPad Prism 5.03 (GraphPad Software, Inc., San Diego, CA, USA). A P-value of ≤ 0.05 was considered statistically significant.

Results

Adriamycin-induced heparanase expression is attenuated by 1,25-D_3 in podocytes

To study the *in vivo* effects of 1,25-D_3 treatment on heparanase and HS expression, AN (an animal model for FSGS) was induced in rats, which were subsequently treated with either 1,25-D_3 or vehicle for 6 weeks. As shown previously, these AN rats develop proteinuria, which could be ameliorated by treatment with 1,25-D_3 [21]. Induction of AN resulted in an increased cortical heparanase mRNA expression (Figure 1A), glomerular heparanase protein expression (Figure 1B,E) and cortical heparanase activity (Figure 1C). Heparanase expression and activity were reduced by treatment with 1,25-D_3, although this was not significant for heparanase protein expression (Figure 1A-C,E). Heparanase mRNA expression was also significantly reduced by 1,25-D_3 treatment in control rats (Figure 1A), suggesting that endogenous heparanase expression is regulated by 1,25-D_3. In addition, glomerular HS expression was reduced in AN rats, but significantly increased by treatment with 1,25-D_3 (Figure 1D,F).

To extend the *in vivo* findings of 1,25-D_3 on glomerular heparanase expression in AN, we evaluated the effects of adriamycin and 1,25-D_3 on heparanase expression in cultured mouse podocytes and mGEnC-1. Stimulation of mouse podocytes with adriamycin resulted in an increased heparanase mRNA expression (Figure 2). Treatment with increasing concentrations of 1,25-D_3 dose-dependently reduced heparanase expression in both adriamycin-injured and uninjured podocytes (Figure 2). Stimulation of mGEnC-1 with adriamycin initially increased heparanase mRNA expression, but reduced heparanase

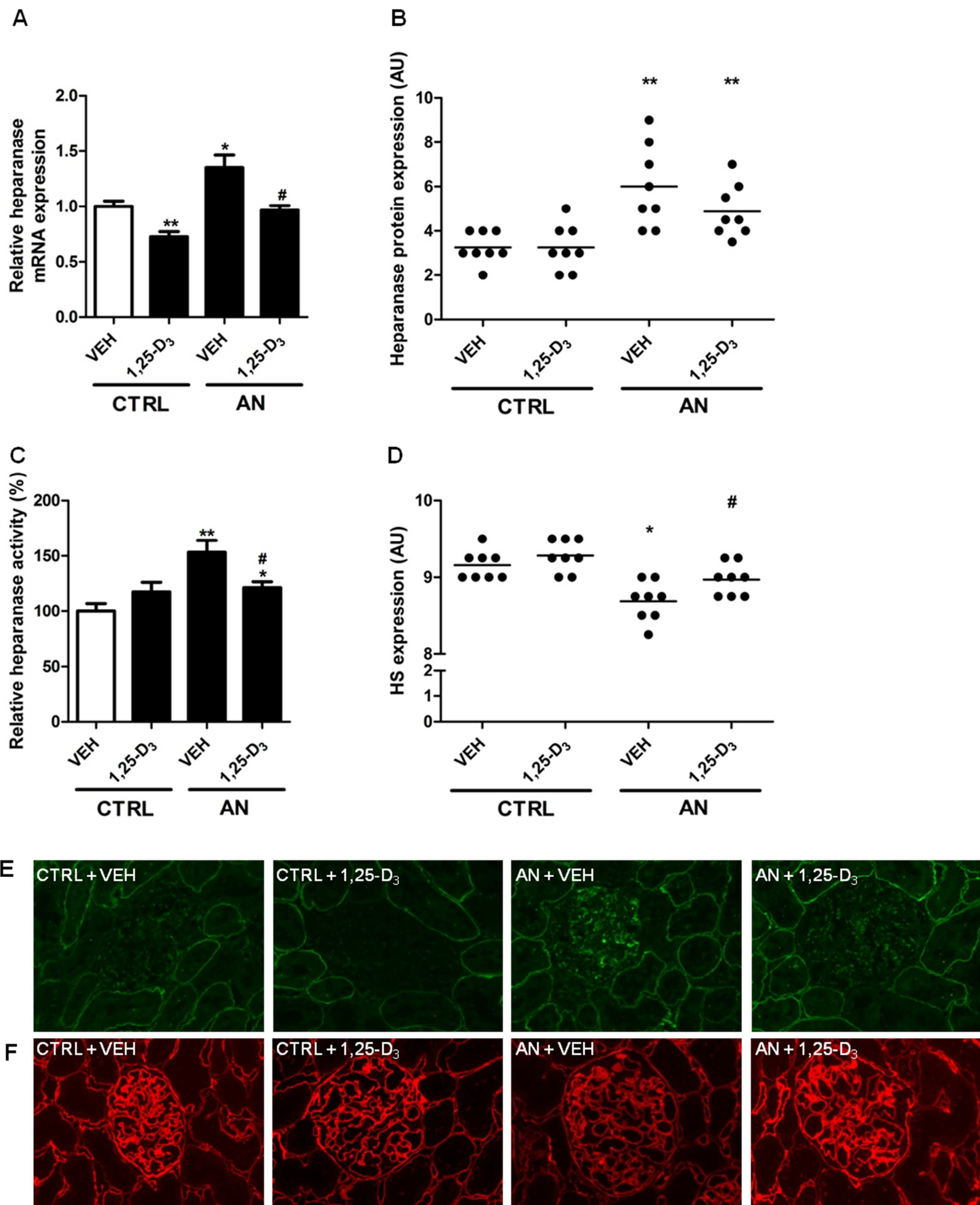

Figure 1: 1,25-D_3 treatment reduces heparanase expression and increases HS expression in rats with AN. By induction of AN in Wistar rats, (**A**) cortical heparanase mRNA expression, (**B**,**E**) glomerular heparanase protein expression, and (**C**) cortical heparanase activity were significantly increased, whereas (**D**,**F**) glomerular HS expression was significantly reduced. Daily treatment with 2.5 µg/kg bodyweight of 1,25-D_3 for 6 weeks reduced heparanase mRNA expression and activity and increased glomerular HS expression. In addition, treatment with 1,25-D_3 alone also reduced heparanase mRNA expression. (**E**) Representative images showing glomerular heparanase protein expression and (**F**) glomerular HS expression as determined by immunofluorescence staining (magnification x 400). 8 rats per group were used for analysis. *P<0.05 and **P<0.01 versus VEH CTRL, $^{\#}P$<0.05 versus VEH AN. VEH, vehicle; CTRL, control; AN, adriamycin nephropathy.

mRNA expression after 16 hours (Figure 3A-B). Importantly, adriamycin decreased HS expression on mGEnC-1 (Figure 3C). 1,25-D_3 treatment did not affect heparanase mRNA expression and HS expression on mGEnC-1 (Figure 3B-C).

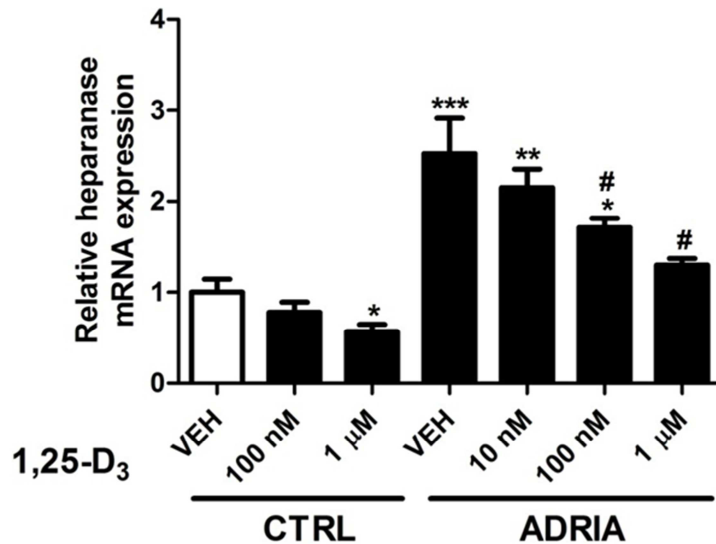

Figure 2: Adriamycin-induced heparanase mRNA expression is attenuated by 1,25-D_3 in podocytes. Cultured mouse podocytes were stimulated with vehicle (VEH) or adriamycin (ADRIA) in the absence or presence of different concentrations of 1,25-D_3 for 24 hours. By stimulation with adriamycin, heparanase mRNA expression was increased. Heparanase mRNA expression was dose-dependently reduced by treatment with 1,25-D_3 in both injured and uninjured podocytes. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus VEH CTRL, $^{\#}P<0.05$ versus VEH ADRIA. CTRL; control.

1,25-D_3 directly regulates heparanase promoter activity

Our *in vivo* and *in vitro* data show that heparanase mRNA expression is reduced by treatment with 1,25-D_3. To determine whether 1,25-D_3 directly regulates heparanase transcription, a luciferase reporter assay was performed using the luciferase HPR1-3.5 promoter construct [27]. OK cells were transfected with the HPR1-3.5 construct or the empty vector, i.e. without heparanase promoter, and treated with vehicle, 100 nM 1,25-D_3 or 1 µM 1,25-D_3 for 24 hours. Treatment with 1,25-D_3 resulted in a decrease in heparanase promoter activity, whereas the luciferase activity in cells with empty vector was very low and not affected by treatment (Figure 4A).

To evaluate whether this effect of 1,25-D_3 on heparanase promoter activity is mediated by direct binding of the VDR to the heparanase promoter, a ChIP assay was performed. By real-time PCR analysis we showed an 8-fold enrichment of the heparanase promoter when precipitated with the anti-VDR antibody compared to the rabbit IgG isotype control (Figure 4B).

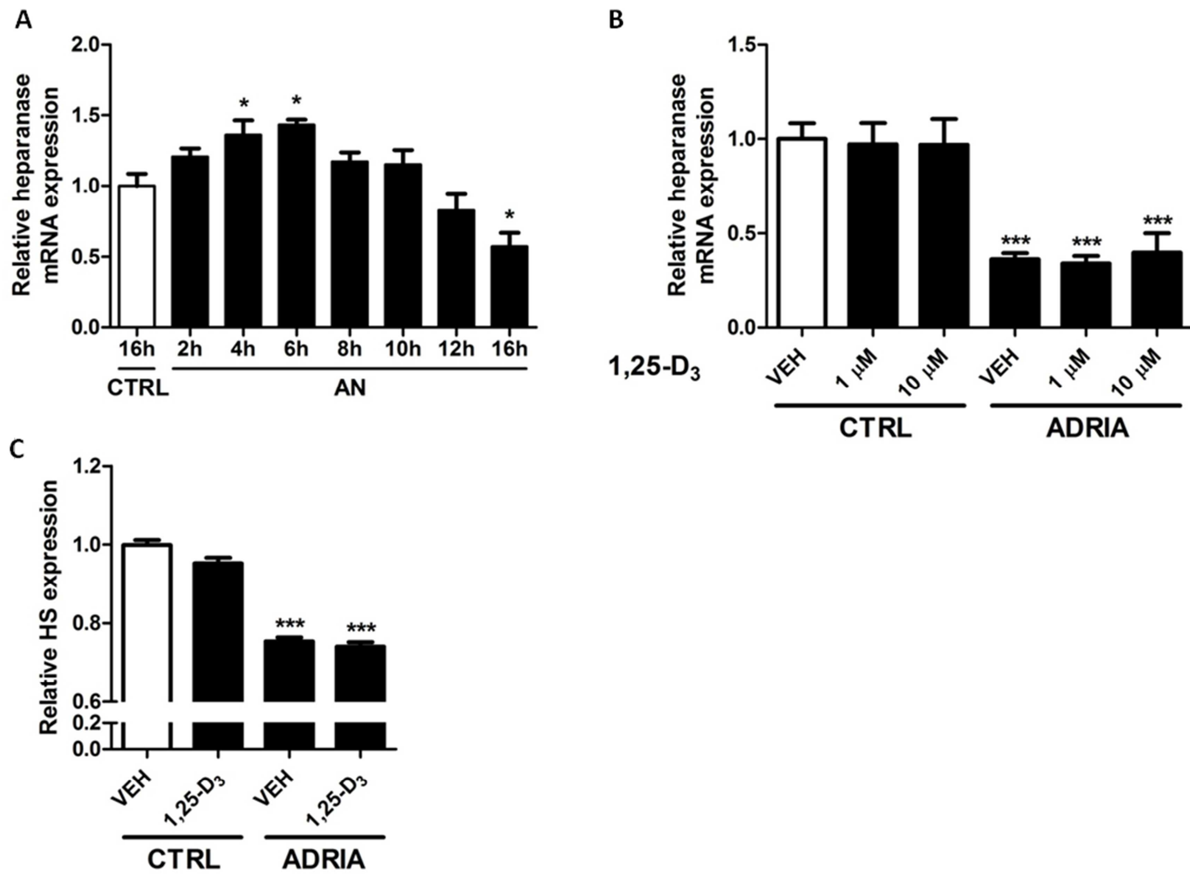

Figure 3: 1,25-D$_3$ treatment did not affect heparanase mRNA expression and HS expression on mouse glomerular endothelial cells. (**A**) Cultured mouse glomerular endothelial cells (mGEnC-1) were stimulated with vehicle (VEH) or adriamycin (ADRIA) for several time points. Stimulation of mGEnC-1 with adriamycin increased heparanase mRNA expression after 4-6 hours, but reduced heparanase mRNA expression after 16 hours. (**B**) mGEnC-1 were stimulated with VEH or ADRIA in the absence or presence of different concentrations of 1,25-D$_3$ for 16 hours. By stimulation with adriamycin, heparanase mRNA expression was reduced. Heparanase mRNA expression was not affected by treatment with 1,25-D$_3$. (**C**) mGEnC-1 were stimulated with VEH or ADRIA in the absence or presence of 1 µM 1,25-D$_3$ for 16 hours. By stimulation with adriamycin, HS expression was reduced. HS expression was not affected by treatment with 1,25-D$_3$. *P<0.05 and ***P<0.001 versus VEH CTRL. CTRL; control.

1,25-D$_3$ treatment reduces transendothelial albumin passage in the presence of podocyte-conditioned culture medium

To study the effects of 1,25-D$_3$ treatment on transendothelial albumin passage, mGEnC-1 were stimulated with vehicle or adriamycin in the presence or absence of 100 nM 1,25-D$_3$. Transendothelial albumin passage was increased by stimulation with adriamycin, but 1,25-D$_3$ treatment did not have an effect on transendothelial albumin passage (Figure 5A). Since 1,25-D$_3$ treatment also did not have an effect on heparanase and HS expression in mGEnC-1, but reduced heparanase mRNA expression in mouse podocytes, we added culture supernatant of mouse podocytes treated with either vehicle or adriamycin, in the presence or absence of 1,25-D$_3$, to the mGEnC-1 monolayer. In the presence of podocyte culture

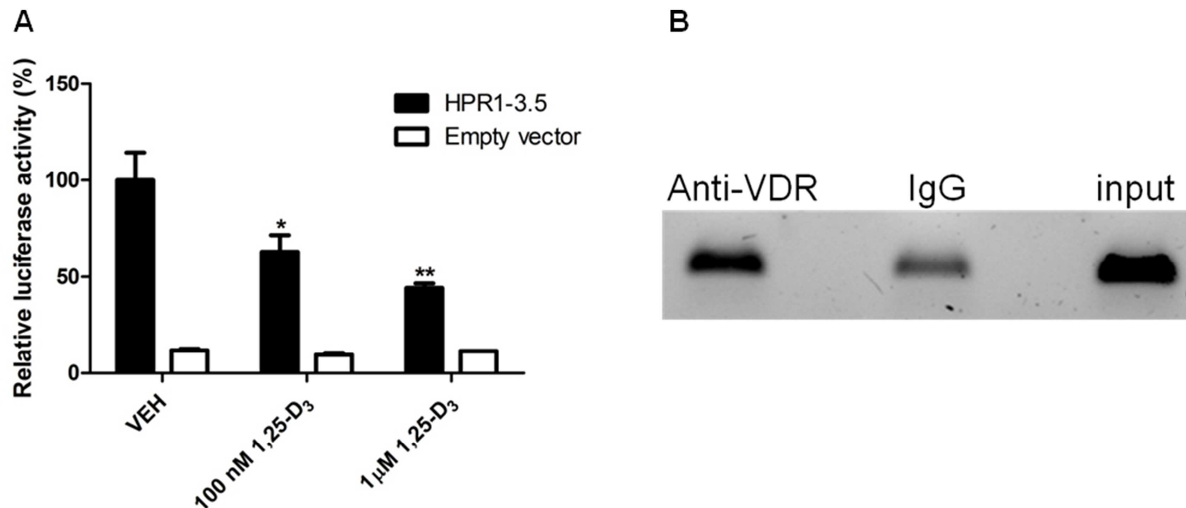

Figure 4: 1,25-D$_3$ directly regulates heparanase promoter activity. (**A**) Heparanase promoter activity determined by a luciferase reporter assay revealed a 40% and 55% decrease in heparanase promoter activity when OK cells transiently transfected with the luciferase HPR1-3.5 promoter construct were treated with 100 nM or 1 µM 1,25-D$_3$, respectively. The luciferase activity of cells with the empty vector, without promoter, was unaltered. (**B**) A ChIP assay was performed to determine whether the VDR binds directly to the heparanase promoter. Real-time PCR analysis showed an 8-fold enrichment of the heparanase promoter using the anti-VDR antibody (anti-VDR lane) compared with the isotype control (IgG lane). As a control, 2% of the chromatin used for the immunoprecipitation is shown (input lane). *P<0.05 and **P<0.01 versus VEH (vehicle). VDR: Vitamin D receptor.

supernatant, transendothelial albumin passage was increased after adriamycin stimulation, and normalized by 1,25-D$_3$ treatment of cultured podocytes (Figure 5B). To show that this effect was really mediated by heparanase, heparanase expression in MPC5 podocytes was completely silenced with shRNA, since no heparanase mRNA expression could be measured (HPSE KD). Stimulation of mGEnC-1 with culture supernatant of adriamycin-stimulated podocytes containing a scrambled shRNA increased transendothelial albumin passage, whereas stimulation of mGEnC-1 with culture supernatant of adriamycin-stimulated HPSE KD podocytes did not have an effect on transendothelial albumin passage (Figure 5C), indicating that the effect of adriamycin on transendothelial albumin passage is completely heparanase-dependent.

1,25-D$_3$ deficiency leads to increased glomerular expression of heparanase that can be corrected by treatment with 1,25-D$_3$

To evaluate the effects of 1,25-D$_3$ deficiency on heparanase expression *in vivo*, 1,25-D$_3$-deficient 25-hydroxy-1α-hydroxylase knockout (KO) mice were used. These mice lack the enzyme 1α-hydroxylase in the kidney, and therefore are not able to produce the active form of vitamin D$_3$. 5-week-old KO mice were daily treated with 1,25-D$_3$ for 6 weeks. Heparanase mRNA expression (Figure 6A), protein expression (Figure 6B-C) and activity (Figure 6D)

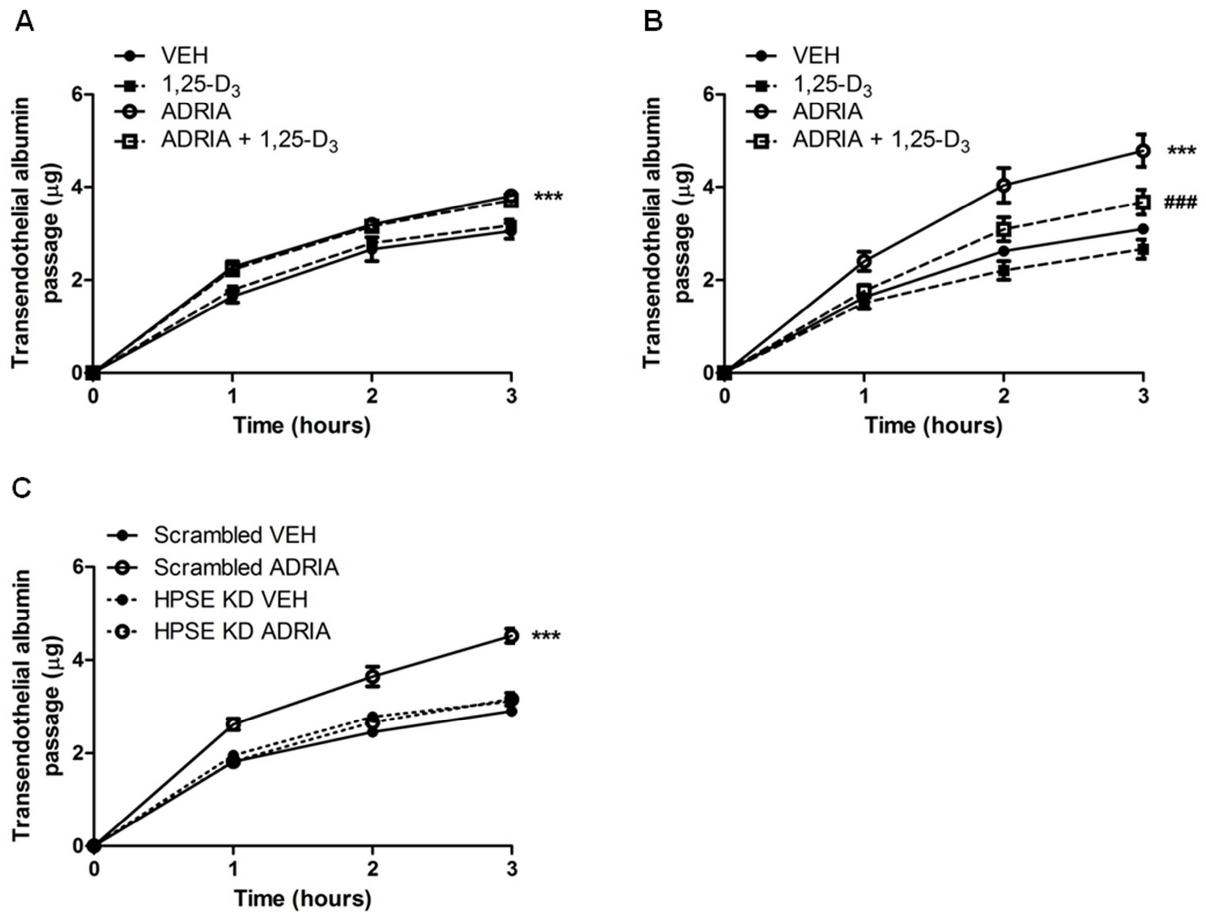

Figure 5: 1,25-D$_3$ treatment reduces transendothelial albumin passage in the presence of podocyte-conditioned culture medium. Cultured mGEnC-1 were stimulated with vehicle (VEH) or adriamycin (ADRIA) in the absence or presence of 100 nM 1,25-D$_3$ for 16 hours. Cumulative passage of FITC-labeled albumin across the mGEnC-1 monolayer was determined over time. (**A**) Stimulation of mGEnC-1 with adriamycin increased the passage of albumin compared to vehicle treated cells. Combined treatment of adriamycin with 1,25-D$_3$ did not reduce transendothelial albumin passage. (**B**) mGEnC-1 were treated with a 1:1 mix of medium supplemented with vehicle or adriamycin in the absence or presence of 100 nM 1,25-D$_3$, and culture supernatant of podocytes also treated with vehicle or adriamycin in the absence or presence of 100 nM 1,25-D$_3$. Stimulation of mGEnC-1 with culture supernatant of adriamycin-stimulated podocytes increased transendothelial albumin passage. Passage of albumin across the mGEnC-1 monolayer was reduced after combined treatment of adriamycin with 1,25-D$_3$ only in the presence of podocyte cell culture supernatant. (**C**) mGEnC-1 were treated with a 1:1 of medium supplemented with vehicle or adriamycin and culture supernatant of scrambled or HPSE KD podocytes treated with vehicle or adriamycin. Stimulation of mGEnC-1 with culture supernatant of adriamycin-stimulated scrambled podocytes increased transendothelial albumin passage, whereas stimulation of mGEnC-1 with culture supernatant of adriamycin-stimulated HPSE KD podocytes did not have an effect on transendothelial albumin passage. $^{***}P<0.001$ versus VEH, $^{###}P<0.001$ versus ADRIA. mGEnC-1; mouse glomerular endothelial cells, CTRL; control.

were increased in the untreated KO mice, which showed a significant proteinuria (Figure 6E). Proteinuria was corrected by daily 1,25-D$_3$ treatment, which was accompanied by a normalized heparanase expression and activity (Figure 6A-E).

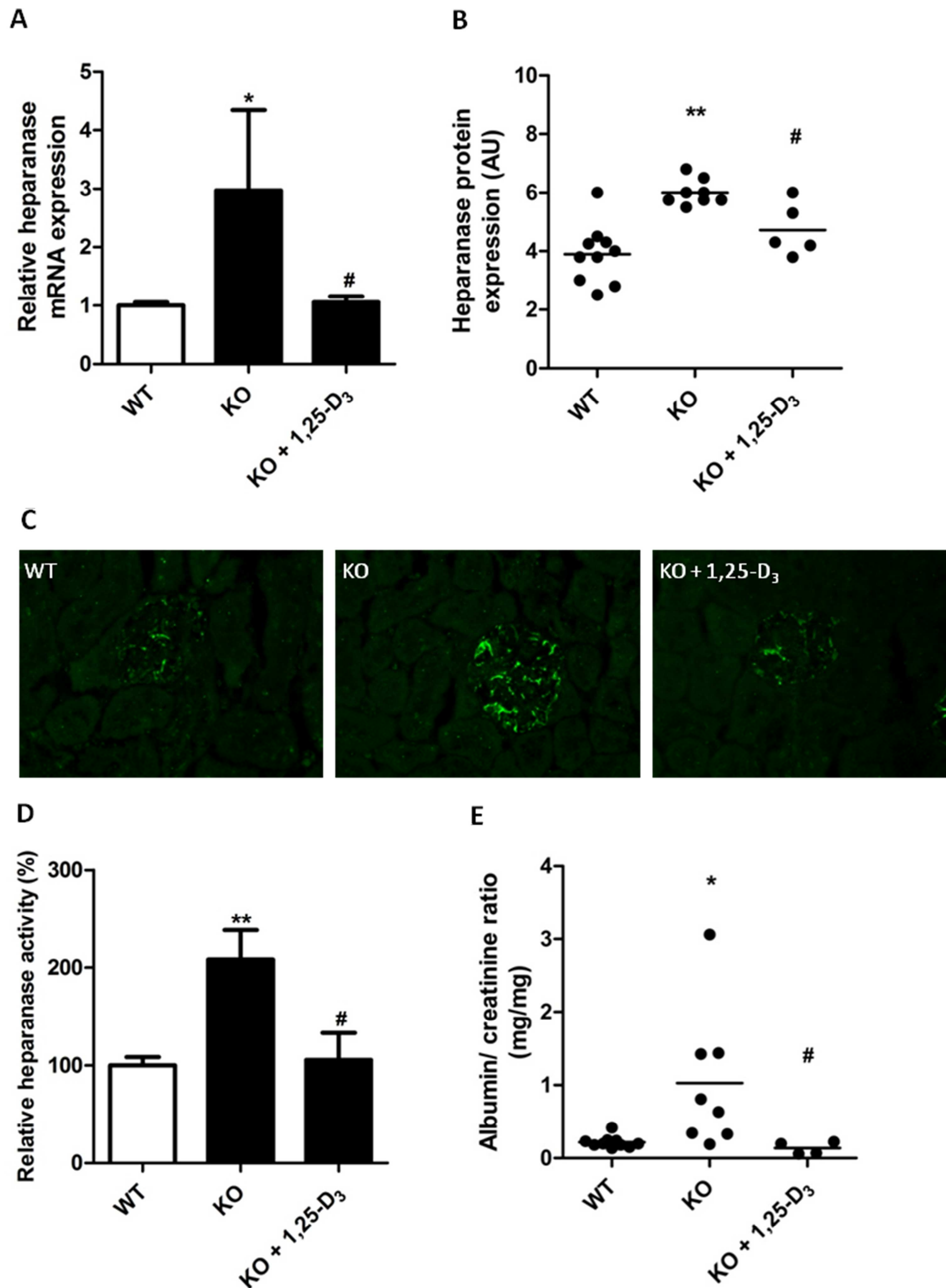

Figure 6: Vitamin D deficiency leads to increased glomerular expression of heparanase that can be corrected by treatment with 1,25-D$_3$. (**A**) Cortical heparanase mRNA expression, (**B**,**C**) glomerular heparanase protein expression, (**D**) cortical heparanase activity and (**E**) proteinuria were significantly increased in 11-week-old 1,25-D$_3$-deficient 25-hydroxy-1α-hydroxylase knockout (KO) mice. Daily treatment with 500 pg/g bodyweight of 1,25-D$_3$ for 6 weeks normalized proteinuria and heparanase expression and activity. (**C**) Representative images showing glomerular heparanase protein expression as determined by immunofluorescence staining (magnification x 400). 10 WT, 8 KO and 5 KO+1,25-D$_3$ mice were used for analysis. *P<0.05 and **P<0.01 versus WT, $^{\#}P$<0.05 versus KO. WT; wildtype.

Discussion

Our *in vivo* and *in vitro* data show that 1,25-D_3 regulates heparanase expression in cultured podocytes and in animal models for FSGS and 1,25-D_3 deficiency. Consistent with our previous studies [6, 29], glomerular heparanase expression was increased and glomerular HS expression reduced in AN *in vivo*. These effects were attenuated by treatment with 1,25-D_3, suggesting that these effects are, at least in part, mediated by 1,25-D_3. This is supported by the *in vitro* data, where we showed that adriamycin induced heparanase expression in podocytes, which could be normalized by treatment with 1,25-D_3. 1,25-D_3 treatment also reduced transendothelial albumin passage, which was dependent on conditioned medium of podocytes. Moreover, we demonstrated that the VDR directly binds to the heparanase promoter region and that 1,25-D_3 treatment inhibits heparanase promoter activity. Finally, 1,25-D_3-deficient mice developed proteinuria and showed increased heparanase expression and activity, which both could be normalized by treatment with 1,25-D_3. Taken together, we showed that vitamin D reduced heparanase expression in *in vitro* and *in vivo* podocyte injury, possibly through a direct effect on heparanase promoter activity.

The present study is the first study that shows that heparanase promoter activity and expression are regulated by the steroid hormone vitamin D. By binding of vitamin D to the VDR, the VDR interacts with the retinoid X receptor to form a heterodimer that binds to vitamin D responsive elements in the promoter regions of vitamin D-responsive genes [10]. In this study we showed that the VDR indeed binds to the heparanase promoter. Most genes regulated by vitamin D are positively regulated. Heparanase is one of the few genes that are negatively regulated by vitamin D, which also include the genes encoding parathyroid hormone, renin, and, as we recently reported, the transient receptor potential cation channel C6 (TRPC6) [21, 30-32].

The importance of heparanase in the development of proteinuria was recently demonstrated in heparanase-deficient mice, which failed to develop proteinuria and had a normal renal function after induction of type 1 diabetes [2, 9]. Moreover, proteinuria and renal function loss were limited when diabetic WT mice were treated with the heparanase inhibitor SST0001. In addition to its essential role in the development of proteinuria in diabetic nephropathy, heparanase has also been suggested to play a role in the development of proteinuria in other glomerular diseases. Treatment with the heparanase inhibitor PI-88 reduced proteinuria in rats with Passive Heymann Nephritis [7] and treatment with an anti-heparanase antibody reduced proteinuria in anti-glomerular basement membrane disease in rats [8]. Moreover, the expression of heparanase is increased in many experimental and human glomerular diseases [2-6], suggesting that heparanase may play an important role in the development of proteinuria in many glomerular diseases.

A recent paper suggests that heparanase may play a nephroprotective role in the development of AN [33]. Transgenic heparanase overexpressing mice developed only mild albuminuria and failed to develop renal damage after induction of AN. We previously showed that transgenic overexpression of heparanase did not result in proteinuria, which we explained by the lack of induced pathology [34]. Results in the paper by Assady *et al.* seem in contrast to previous studies that showed a pathological role of heparanase in models for diabetic nephropathy, minimal change disease, accelerated anti-GBM disease and membranous glomerulopathy [2, 3, 7-9]. Unfortunately, the study by Assady *et al.* does not provide a mechanism for the observed nephroprotective effect of transgenic heparanase overexpression. However, it may be possible that transgenic heparanase overexpression since birth makes the mice more resistant to develop AN. It is known that enzymatically inactive heparanase promotes vascular endothelial growth factor (VEGF) expression, which promotes endothelial health and survival [35, 36]. A recent study showed that endothelial dysfunction precedes podocyte injury in AN [37]. Therefore, heparanase overexpression since birth may increase endothelial health, thereby making these mice more resistant to develop AN. Nevertheless, additional research is needed to evaluate the protective role of heparanase on endothelial cells in AN.
In the present study, we showed that 1,25-D_3 treatment significantly reduced proteinuria and heparanase expression and activity in AN *in vivo*. In addition, we used 1,25-D_3-deficient 25-hydroxy-1α-hydroxylase KO mice to further evaluate the importance of 1,25-D_3. These 1,25-D_3-deficient mice developed proteinuria and showed increased heparanase expression and activity, which both could be normalized by treatment with 1,25-D_3. Although current data demonstrate that heparanase expression and proteinuria are regulated by 1,25-D_3 treatment, and previous studies indicate that heparanase is associated with the development of proteinuria, it does not rule out that simultaneous regulation of other genes by vitamin D also plays a role in the development of proteinuria. We recently reported that the expression of TRPC6, a slit diaphragm protein expressed by podocytes, is regulated by vitamin D [21]. The expression of TRCP6 is increased in several acquired proteinuric diseases, suggesting that TRPC6 also plays a role in the pathogenesis of podocyte injury in proteinuric diseases [38]. In addition to TRPC6, renin is negatively regulated by vitamin D. Renin plays an important role in the biosynthesis of angiotensin II, and the latter can even be produced locally by the podocyte itself. Finally, 1,25-D_3 also negatively regulates the expression of the proinflammatory cytokine tumor necrosis factor-α (TNF-α) [39, 40]. We and others previously showed that both angiotensin II and TNF-α can induce heparanase expression [29, 41, 42], suggesting that the reduction of renin or TNF-α expression may also be involved in the reduced heparanase expression after 1,25-D_3 treatment.

AN in rats has mainly been regarded as a podocyte-injury model for FSGS. However, glomerular endothelial cells also play a role in the pathogenesis of AN, as adriamycin reduces the thickness of the endothelial glycocalyx by 80% in mice [43]. We observed similar effects *in vitro*, where HS expression on mouse glomerular endothelial cells was decreased after stimulation with adriamycin. Our data, however, suggest that 1,25-D_3 treatment only has a direct protective effect on the podocyte. No direct effect of 1,25-D_3 treatment was observed on mouse glomerular endothelial cells. This could be expected, since the VDR is expressed only at very low concentrations by the glomerular endothelium, whereas it is expressed at much higher levels by podocytes [44]. In line with this, 1,25-D_3 treatment did not have an effect on transendothelial albumin passage for adriamycin injured glomerular endothelial cells. Interestingly, when conditioned culture supernatant of mouse podocytes treated with vehicle or adriamycin, in the presence or absence of 1,25-$D_{3,}$ was added to the mouse glomerular endothelial cells, we observed a reduction in transendothelial albumin passage by 1,25-D_3 treatment after adriamycin stimulation. This further demonstrates that 1,25-D_3 treatment mainly affects podocytes and suggests that crosstalk between cells in the glomerulus is involved in the pathogenesis of proteinuria.

We previously showed that heparanase is mainly expressed in the podocyte in AN *in vivo*, as heparanase was expressed on the outside of the GBM and colocalized with synaptopodin [6]. Only minimal heparanase staining was observed inside the capillary loops. This is in line with our current *in vitro* data, where we observed a marked increase in heparanase expression by adriamycin in mouse podocytes, whereas a slight increase followed by a reduced heparanase expression was observed in mouse glomerular endothelial cells. Loss of heparanase expression in the podocyte prevents the increased passage of albumin across the endothelial monolayer induced by adriamycin, suggesting that heparanase is the permeability factor in the conditioned medium of podocytes that is regulated by1,25-D_3. Future research should reveal how podocyte-derived heparanase crosses the GBM *in vivo*. VEGF and endothelin are other examples of podocyte-derived proteins that need to cross the GBM to reach the endothelium.

In conclusion, in this report we provide further mechanistic evidence for the potential benefits of vitamin D treatment in patients with glomerular disease. We add heparanase as a target regulated in experimental glomerular disease. Our current *in vivo* and *in vitro* results show that the protective effect of vitamin D on the development of proteinuria in AN could be mediated by reduction of the increased heparanase expression in the podocyte.

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References

1. van den Born, J., et al., *Distribution of GBM heparan sulfate proteoglycan core protein and side chains in human glomerular diseases.* Kidney international, 1993. **43**(2): p. 454-63.
2. Garsen, M., et al., *The role of heparanase and the endothelial glycocalyx in the development of proteinuria.* Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association, 2014. **29**(1): p. 49-55.
3. van den Hoven, M.J., et al., *Heparanase in glomerular diseases.* Kidney international, 2007. **72**(5): p. 543-8.
4. van den Hoven, M.J., et al., *Increased expression of heparanase in overt diabetic nephropathy.* Kidney international, 2006. **70**(12): p. 2100-8.
5. Smith, R.J., et al., *New approaches to the treatment of dense deposit disease.* Journal of the American Society of Nephrology : JASN, 2007. **18**(9): p. 2447-56.
6. Kramer, A., et al., *Induction of glomerular heparanase expression in rats with adriamycin nephropathy is regulated by reactive oxygen species and the renin-angiotensin system.* Journal of the American Society of Nephrology : JASN, 2006. **17**(9): p. 2513-20.
7. Levidiotis, V., et al., *A synthetic heparanase inhibitor reduces proteinuria in passive Heymann nephritis.* Journal of the American Society of Nephrology : JASN, 2004. **15**(11): p. 2882-92.
8. Levidiotis, V., et al., *Heparanase inhibition reduces proteinuria in a model of accelerated anti-glomerular basement membrane antibody disease.* Nephrology (Carlton, Vic.), 2005. **10**(2): p. 167-73.
9. Gil, N., et al., *Heparanase is essential for the development of diabetic nephropathy in mice.* Diabetes, 2012. **61**(1): p. 208-16.
10. Haussler, M.R., et al., *Vitamin D receptor (VDR)-mediated actions of 1alpha,25(OH)(2)vitamin D(3): genomic and non-genomic mechanisms.* Best practice & research. Clinical endocrinology & metabolism, 2011. **25**(4): p. 543-59.
11. Levin, A., et al., *Prevalence of abnormal serum vitamin D, PTH, calcium, and phosphorus in patients with chronic kidney disease: results of the study to evaluate early kidney disease.* Kidney international, 2007. **71**(1): p. 31-8.
12. de Borst, M.H., et al., *Active vitamin D treatment for reduction of residual proteinuria: a systematic review.* Journal of the American Society of Nephrology : JASN, 2013. **24**(11): p. 1863-71.
13. Agarwal, R., et al., *Antiproteinuric effect of oral paricalcitol in chronic kidney disease.* Kidney international, 2005. **68**(6): p. 2823-8.
14. Krairittichai, U., R. Mahannopkul, and S. Bunnag, *An open label, randomized controlled study of oral calcitriol for the treatment of proteinuria in patients with diabetic kidney disease.* Journal of the Medical Association of Thailand = Chotmaihet thangphaet, 2012. **95 Suppl 3**: p. S41-7.
15. de Zeeuw, D., et al., *Selective vitamin D receptor activation with paricalcitol for reduction of albuminuria in patients with type 2 diabetes (VITAL study): a randomised controlled trial.* Lancet, 2010. **376**(9752): p. 1543-51.
16. Fishbane, S., et al., *Oral paricalcitol in the treatment of patients with CKD and proteinuria: a randomized trial.* American journal of kidney diseases : the official journal of the National Kidney Foundation, 2009. **54**(4): p. 647-52.
17. Liu, L.J., et al., *Oral calcitriol for reduction of proteinuria in patients with IgA nephropathy: a randomized controlled trial.* American journal of kidney diseases : the official journal of the National Kidney Foundation, 2012. **59**(1): p. 67-74.
18. Thadhani, R., et al., *Vitamin D therapy and cardiac structure and function in patients with chronic kidney disease: the PRIMO randomized controlled trial.* JAMA : the journal of the American Medical Association, 2012. **307**(7): p. 674-84.

19. Kuhlmann, A., et al., *1,25-Dihydroxyvitamin D3 decreases podocyte loss and podocyte hypertrophy in the subtotally nephrectomized rat.* American journal of physiology. Renal physiology, 2004. **286**(3): p. F526-33.
20. Zou, M.S., et al., *1, 25-dihydroxyvitamin D3 decreases adriamycin-induced podocyte apoptosis and loss.* International journal of medical sciences, 2010. **7**(5): p. 290-9.
21. Sonneveld, R., et al., *Vitamin D down-regulates TRPC6 expression in podocyte injury and proteinuric glomerular disease.* The American journal of pathology, 2013. **182**(4): p. 1196-204.
22. Hoenderop, J.G., et al., *Modulation of renal Ca2+ transport protein genes by dietary Ca2+ and 1,25-dihydroxyvitamin D3 in 25-hydroxyvitamin D3-1alpha-hydroxylase knockout mice.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2002. **16**(11): p. 1398-406.
23. Rops, A.L., et al., *Syndecan-1 deficiency aggravates anti-glomerular basement membrane nephritis.* Kidney international, 2007. **72**(10): p. 1204-15.
24. van Kuppevelt, T.H., et al., *Generation and application of type-specific anti-heparan sulfate antibodies using phage display technology. Further evidence for heparan sulfate heterogeneity in the kidney.* The Journal of biological chemistry, 1998. **273**(21): p. 12960-6.
25. Rops, A.L., et al., *Isolation and characterization of conditionally immortalized mouse glomerular endothelial cell lines.* Kidney international, 2004. **66**(6): p. 2193-201.
26. Mundel, P., et al., *Rearrangements of the cytoskeleton and cell contacts induce process formation during differentiation of conditionally immortalized mouse podocyte cell lines.* Experimental cell research, 1997. **236**(1): p. 248-58.
27. Jiang, P., et al., *Cloning and characterization of the human heparanase-1 (HPR1) gene promoter: role of GA-binding protein and Sp1 in regulating HPR1 basal promoter activity.* The Journal of biological chemistry, 2002. **277**(11): p. 8989-98.
28. Malstrom, K., G. Stange, and H. Murer, *Identification of proximal tubular transport functions in the established kidney cell line, OK.* Biochimica et biophysica acta, 1987. **902**(2): p. 269-77.
29. van den Hoven, M.J., et al., *Regulation of glomerular heparanase expression by aldosterone, angiotensin II and reactive oxygen species.* Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association, 2009. **24**(9): p. 2637-45.
30. Silver, J., et al., *Regulation by vitamin D metabolites of parathyroid hormone gene transcription in vivo in the rat.* The Journal of clinical investigation, 1986. **78**(5): p. 1296-301.
31. Silver, J., J. Russell, and L.M. Sherwood, *Regulation by vitamin D metabolites of messenger ribonucleic acid for preproparathyroid hormone in isolated bovine parathyroid cells.* Proceedings of the National Academy of Sciences of the United States of America, 1985. **82**(12): p. 4270-3.
32. Zhou, C., et al., *Calcium-independent and 1,25(OH)2D3-dependent regulation of the renin-angiotensin system in 1alpha-hydroxylase knockout mice.* Kidney international, 2008. **74**(2): p. 170-9.
33. Assady, S., et al., *Nephroprotective effect of heparanase in experimental nephrotic syndrome.* PLoS One, 2015. **10**(3): p. e0119610.
34. van den Hoven, M.J., et al., *Reduction of anionic sites in the glomerular basement membrane by heparanase does not lead to proteinuria.* Kidney international, 2008. **73**(3): p. 278-87.
35. Zetser, A., et al., *Heparanase induces vascular endothelial growth factor expression: correlation with p38 phosphorylation levels and Src activation.* Cancer Res, 2006. **66**(3): p. 1455-63.
36. Doi, K., E. Noiri, and T. Fujita, *Role of vascular endothelial growth factor in kidney disease.* Curr Vasc Pharmacol, 2010. **8**(1): p. 122-8.
37. Sun, Y.B., et al., *Glomerular endothelial cell injury and damage precedes that of podocytes in adriamycin-induced nephropathy.* PLoS One, 2013. **8**(1): p. e55027.

38. Moller, C.C., et al., *Induction of TRPC6 channel in acquired forms of proteinuric kidney disease.* Journal of the American Society of Nephrology : JASN, 2007. **18**(1): p. 29-36.
39. Kuo, Y.T., et al., *Effects of vitamin D3 on expression of tumor necrosis factor-alpha and chemokines by monocytes.* J Food Sci, 2010. **75**(6): p. H200-4.
40. Tan, X., X. Wen, and Y. Liu, *Paricalcitol inhibits renal inflammation by promoting vitamin D receptor-mediated sequestration of NF-kappaB signaling.* J Am Soc Nephrol, 2008. **19**(9): p. 1741-52.
41. Rops, A.L., et al., *Heparan sulfate domains on cultured activated glomerular endothelial cells mediate leukocyte trafficking.* Kidney Int, 2008. **73**(1): p. 52-62.
42. Lerner, I., et al., *Heparanase powers a chronic inflammatory circuit that promotes colitis-associated tumorigenesis in mice.* J Clin Invest, 2011. **121**(5): p. 1709-21.
43. Jeansson, M., et al., *Adriamycin alters glomerular endothelium to induce proteinuria.* Journal of the American Society of Nephrology : JASN, 2009. **20**(1): p. 114-22.
44. Wang, Y., M.L. Borchert, and H.F. Deluca, *Identification of the vitamin D receptor in various cells of the mouse kidney.* Kidney international, 2012. **81**(10): p. 993-1001.

Chapter 9

Summary, future perspectives and translational aspects

Summary

Proteinuria is a key feature of many glomerular diseases and an independent risk factor for the progression to renal failure, cardiovascular mortality and all-cause mortality. Proteinuria is caused by damage to the glomerular filtration barrier (GFB), consisting of glomerular endothelial cells covered with a glycocalyx, the glomerular basement membrane (GBM) and podocytes. All layers of the GFB contain negatively charged heparan sulfate proteoglycans (HSPGs). The glomerular expression of heparan sulfate (HS) is reduced in the majority of patients with proteinuria, which is associated with an increased glomerular expression of the HS-degrading enzyme heparanase. Previous studies suggested that heparanase may play an important role in the development of proteinuria, but the exact role remained unknown. In this thesis, the exact role of heparanase in the development of proteinuria in experimental diabetic nephropathy (DN) and experimental glomerulonephritis was evaluated. Moreover, possible mechanisms involved in the regulation of glomerular heparanase expression were evaluated.

In the first part of this thesis the exact role of heparanase in the development of proteinuria in experimental DN and experimental glomerulonephritis was evaluated. To evaluate the role of heparanase in DN, experimental DN was induced by streptozotocin in wild type (WT) and heparanase-deficient mice (**chapter 2**). Heparanase-deficient mice failed to develop proteinuria after induction of diabetes. In contrast, WT mice developed a significant proteinuria accompanied with an increased glomerular heparanase expression and reduced HS expression. The occurrence of proteinuria in diabetic WT mice was paralleled by the development of mesangial matrix expansion, tubulointerstitial fibrosis and macrophage accumulation, characteristics of DN, which was not observed in the diabetic heparanase-deficient mice. To investigate whether inhibition of excessive heparanase activity may prevent the progression of DN, diabetic WT mice were treated with the heparanase inhibitor SST0001. Treatment with SST0001 markedly reduced proteinuria and improved renal function in experimental DN. Finally, by investigating the molecular mechanism underlying the induction of heparanase in DN, it appeared that the transcription factor early growth response 1 (Egr1) is responsible for the activation of the heparanase promoter. In conclusion, heparanase appears essential for the development of proteinuria and renal damage in experimental DN.

Cathepsin L, a lysosomal cysteine protease, can be involved in the development of proteinuria by the activation of heparanase or the degradation of proteins that are essential to maintain a normal podocyte architecture, such as the CD2-assiciated protein, synaptopodin and dynamin. In **chapter 3** the role of cathepsin L in DN was evaluated in

streptozotocin-induced diabetes in WT and cathepsin L-deficient mice. Diabetic cathepsin L-deficient mice, in contrast to their diabetic WT littermates, failed to develop proteinuria, mesangial matrix expansion, tubulointerstitial fibrosis and macrophage accumulation. In addition, diabetic cathepsin L-deficient mice showed a normal renal function. In diabetic WT mice the development of proteinuria correlated with the induction of heparanase activity, loss of glomerular HS expression and macrophage accumulation, whereas a reduced synaptopodin expression accompanied with podocyte damage was observed at a later stage. In conclusion, cathepsin L is causally involved in the pathogenesis of experimental DN. Cathepsin L deficiency prevents the induction of heparanase activity and the subsequent development of proteinuria and renal damage.

The glomerular expression of heparanase is increased in the majority of proteinuric diseases. To find out whether the essential role of heparanase in the development of proteinuria was not restricted to DN, in **chapter 4** the role of heparanase in experimental glomerulonephritis was evaluated. Two models of experimental glomerulonephritis, being anti-GBM and lipopolysaccharide (LPS) glomerulonephritis, were induced in WT and heparanase-deficient mice. Heparanase expression was increased by induction of experimental glomerulonephritis in WT mice, which was associated with a reduced glomerular HS expression and proteinuria. Proteinuria was reduced in the heparanase-deficient mice, which was accompanied by a preserved glomerular HS expression, a better renal function and less renal damage. In addition, glomerular leukocyte influx, macrophage influx and the expression of Th1 and Th2 cytokines were reduced in the heparanase-deficient mice. *In vitro*, tumor necrosis factor (TNF)-α and LPS directly induced heparanase expression in mouse glomerular endothelial cells and mouse podocytes, thereby increasing transendothelial albumin passage. In conclusion, heparanase mediates the development of proteinuria and renal damage in experimental glomerulonephritis. Heparanase deficiency preserves glomerular HS expression, reduces leukocyte and macrophage influx, and affects the local cytokine milieu compared to WT conditions.

In **chapter 5** the current knowledge on the role of heparanase and HS in the development of proteinuria is summarized. In addition, the importance of the glomerular endothelial glycocalyx in the development of proteinuria is discussed.

The second part of this thesis focused on the mechanisms that could play a role in the regulation of glomerular heparanase expression. Previous studies showed that endothelin receptor antagonists (ERAs) reduce proteinuria in several glomerular diseases, but the mechanism underlying the renoprotective effect of ERAs remained unknown. In **chapter 6** the effect of endothelin-1 on heparanase and HS expression was evaluated *in vitro* and *in vivo*. Endothelin-1 failed to induce heparanase expression in cultured mouse glomerular

endothelial cells. In contrast, endothelin-1 induced heparanase expression in cultured mouse podocytes. Transendothelial albumin passage *in vitro* was increased by culture supernatant of endothelin-1-stimulated podocytes in a heparanase-dependent manner. To extend the *in vitro* data, the effect of endothelin-1 signaling in the podocyte on proteinuria and glomerular heparanase expression was studied in podocyte-specific endothelin receptor knockout (podETRKO) mice. By induction of diabetes, WT mice developed proteinuria and renal damage, which was accompanied by an increased glomerular heparanase expression and a reduced glomerular HS expression. Proteinuria and renal damage were reduced in diabetic podETRKO mice, which displayed a normal heparanase and HS expression. Moreover, glycocalyx thickness was reduced by induction of diabetes in WT mice, but preserved in diabetic podETRKO mice. In conclusion, endothelin-1 induces heparanase expression in the podocyte, which cleaves HS in the endothelial glycocalyx, resulting in a reduced glycocalyx thickness and the development of proteinuria.

Loss of endothelial nitric oxide synthase (eNOS) activity exacerbates proteinuria and renal injury in several glomerular diseases, but the underlying mechanism is not completely understood. Previous studies showed that glomerular endothelial glycocalyx thickness is reduced in diabetic eNOS-deficient mice, and indicated that heparanase plays an important role in the impairment of the glomerular endothelial glycocalyx. Therefore, in **chapter 7** the effect of eNOS deficiency on glomerular heparanase expression was studied in adriamycin nephropathy (AN). C57BL/6 WT mice, normally an AN resistant strain, failed to develop proteinuria and had a normal heparanase expression after induction of AN, whereas eNOS-deficient C57BL/6 mice developed overt proteinuria and displayed an increased glomerular heparanase expression. *In vitro*, the eNOS inhibitor asymmetric dimethylarginine (ADMA) induced heparanase expression in mouse glomerular endothelial cells and enhanced transendothelial albumin passage in a heparanase-dependent manner. In conclusion, eNOS controls heparanase expression in the glomerular endothelium, thereby preventing the development of proteinuria.

Vitamin D received recent attention for the treatment of proteinuria. In **chapter 8** the effect of vitamin D on heparanase expression and heparanase promoter activity was studied. By induction of AN, rats developed proteinuria and showed an increased heparanase expression, which was reduced by 1,25-dihydroxyvitamin D_3 (1,25-D_3) treatment. In addition, 1,25-D_3-deficient 25-hydroxy-1α-hydroxylase knockout mice developed proteinuria and showed increased heparanase expression, which was normalized by 1,25-D_3 treatment. *In vitro,* adriamycin increased heparanase expression in cultured mouse podocytes. In addition, medium of combined adriamycin and 1,25-D_3 treated podocytes reduced transendothelial albumin passage compared with medium of adriamycin-treated podocytes. Finally, 1,25-D_3 dose-dependently reduced heparanase promoter activity, and direct binding of the vitamin D

receptor to the heparanase promoter could be demonstrated. In conclusion, the protective effect of vitamin D on the development of proteinuria is mediated by inhibition of heparanase expression in the podocyte.

Taken all data together, it appears that heparanase is essential for the development of proteinuria and renal damage in experimental DN and experimental glomerulonephritis. Induction of heparanase activity resulted in a loss of glomerular HS expression and a reduced endothelial glycocalyx thickness, which causes a loss of negative charge in the GFB. As a result, proteins like albumin can pass the GFB more easily, resulting in the development of proteinuria and renal damage. In addition, it appears that heparanase plays an important role in the renal influx of inflammatory cells such as macrophages, which also play a role in the destruction of the GFB and the development of proteinuria and renal damage. Additional studies are required to evaluate the exact mechanism of heparanase-induced proteinuria. In conclusion, inhibition of heparanase expression/activity represents an potential therapeutic target to reduce proteinuria and renal damage.
In this thesis several factors that are involved in the regulation of heparanase were identified. Heparanase is upregulated by LPS, TNF-α and endothelin-1, whereas heparanase is downregulated by eNOS and vitamin D. Previous studies showed that heparanase is positively regulated by high glucose, angiotensin II, aldosterone and reactive oxygen species (ROS) [1-4]. Interestingly, whereas some of these regulating factors induce heparanase expression in both endothelial cells and podocytes (LPS, high glucose, TNF-α and ROS), other regulating factors are solely operative in the endothelium (eNOS) or the podocyte (angiotensin II, aldosterone, endothelin-1 and vitamin D). Even more interesting, whereas endothelin-1 and vitamin D solely regulate heparanase in the podocyte and fail to regulate heparanase in the endothelium, they are involved in the regulation of endothelial glycocalyx thickness *in vivo* and transendothelial albumin passage *in vitro*, suggesting that there is cross-talk between the podocyte and the endothelium. The effects of endothelin-1 and adriamycin on transendothelial albumin passage were heparanase-dependent, which suggests that heparanase is a key player in the pathogenic cross-talk between podocyte and glomerular endothelium.
As outlined, several factors involved in the direct regulation of heparanase have been described in literature and this thesis. Interestingly, several of these factors seem to regulate each other as well. In figure 1 a schematic representation of interacting factors in the regulation of heparanase expression is depicted. To become biologically active, heparanase needs to be processed by cathepsin L. Previous studies showed that active heparanase may in turn activate macrophages, resulting in an increased production of TNF-α and an enhanced sensitivity to TNF-α, which leads to a further increase of heparanase expression

[5, 6]. Targeting any of the factors involved in the regulation or activation of heparanase may therefore represent an attractive therapy for the reduction of proteinuria and renal damage.

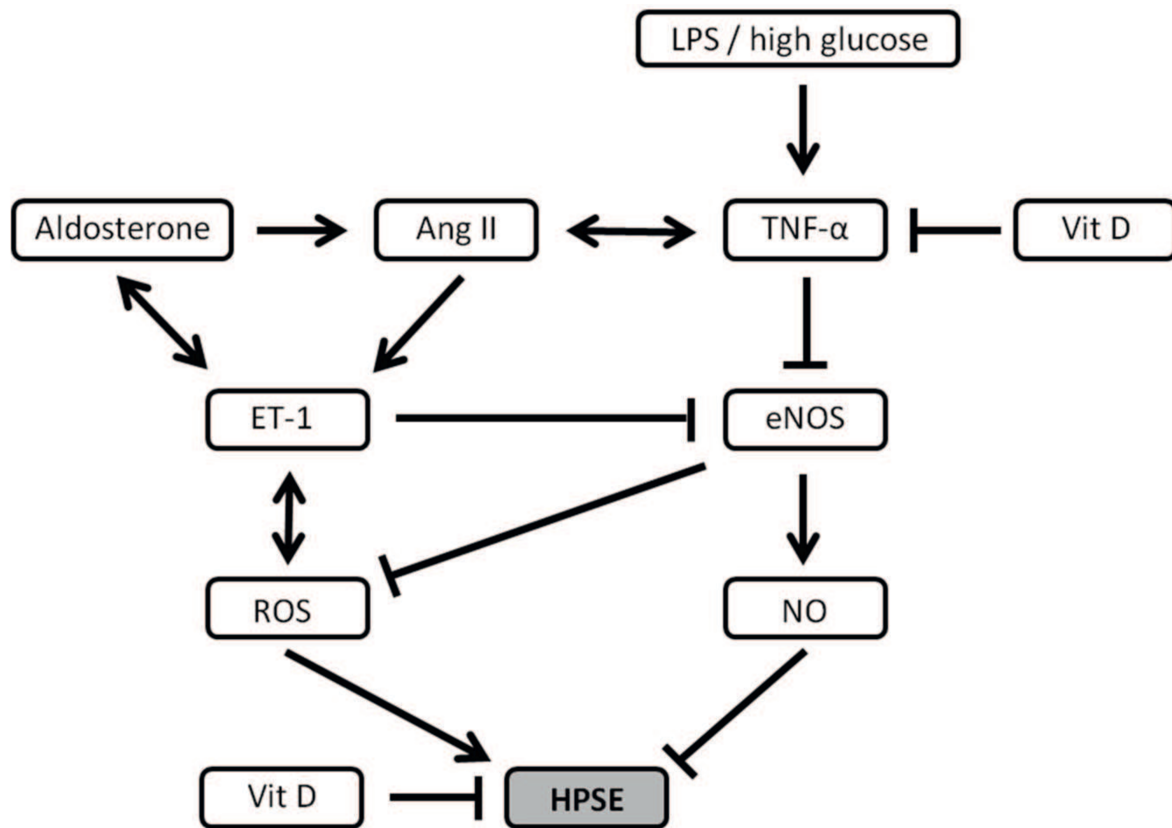


Figure 1: Factors involved in the regulation of heparanase expression. In the presence of LPS or high glucose, TNF-α expression is induced. TNF-α subsequently induces angiotensin II expression and reduces eNOS expression. Angiotensin II induces the expression of endothelin-1, which induces the expression of aldosterone, and thereby further increases angiotensin II expression. Endothelin-1 induces heparanase expression by stimulation of the production of ROS, and reduction of eNOS expression. eNOS controls heparanase expression by a reduced ROS production and an increased production of NO. Vitamin D may reduce heparanase expression either directly, or indirectly by the reduction of TNF-α expression. Abbreviations: LPS, lipopolysaccharide; vit D, vitamin D; TNF-α, tumor necrosis factor-α; Ang II, angiotensin II; ET-1, endothelin-1; ROS, reactive oxygen species; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; HPSE, heparanase.

Future perspectives and translational aspects

In this thesis it is shown that heparanase is essential for the development of proteinuria and renal damage in experimental DN and experimental glomerulonephritis. Previous studies showed that the expression of HS in the GBM is reduced by induction of heparanase expression, which was associated with the development of proteinuria [7]. The primary role of HS in the development of proteinuria was questioned, as genetically engineered mice with a reduced HS expression failed to develop proteinuria [3, 8-10]. However, in these animal models HS expression was only reduced in the GBM, but not in the endothelial glycocalyx. Therefore, an important role for HS in the endothelial glycocalyx in the development of proteinuria seems obvious. Recent studies showed that a reduction in glycocalyx thickness is associated with the development of proteinuria [11-16]. To study the exact role of HS in the endothelial glycocalyx in the development of proteinuria, endothelium-specific EXT1-deficient mice, which most likely lack all HS-associated anionic sites in the glomerular endothelium, should be used. Unfortunately, these mice are not viable (personal communication Jeffrey Esko), which argues to evaluate inducible endothelial-specific EXT1-deficient mice. As the development of proteinuria most likely is not a single-hit mechanism, glomerular pathology should be induced in addition to a manipulated glomerular HS expression. By evaluating the development of proteinuria and renal damage in the aforementioned approach, it can be determined whether loss of HS in the endothelial glycocalyx is important for the development of proteinuria and renal pathology. In addition to the degradation of HS, recent studies, in particular aimed at DN, indicate that heparanase is involved in the development of proteinuria by the activation of macrophages, which also play a role in the destruction of the glomerular capillary wall [5]. Therefore, additional studies are required to evaluate whether proteinuria mediated by heparanase is caused by loss of glomerular HS expression, the activation of macrophages, or both. To this end, bone marrow transplantation experiments, where bone marrow is derived from wild type and heparanase-deficient mice, should be performed in experimental models for DN and glomerulonephritis.

Currently, blockade of the renin-angiotensin-aldosterone system (RAAS) by angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) is the therapy of choice for the treatment of patients with proteinuria. Our group previously showed that the RAAS system is involved in the induction of glomerular heparanase expression [3, 4]. However, RAAS blockade only partially reduces proteinuria, and it is therefore crucial to develop additional anti-proteinuric strategies. In this thesis vitamin D, endothelin-1, and eNOS were identified as regulators of heparanase expression. Vitamin D, endothelin receptor antagonists (ERAs), and tetrahydrobiopeterin (BH4), an eNOS co-factor, have been

approved for clinical use in other diseases, and therefore they are readily available to evaluate their antiproteinuric and renoprotective effect in clinical studies.

Vitamin D has been approved for the prevention and treatment of secondary hyperparathyroidism, which develops in later stages of chronic kidney disease. During the progression of renal diseases, serum levels of active vitamin D decrease progressively, making patients vitamin D-deficient [17]. Vitamin D deficiency may be caused by a reduced vitamin D intake, or a reduction of 1α-hydroxylase activity as a result of loss of renal mass. A recent study showed that vitamin D deficiency itself may also induce proteinuria and renal damage [18]. Several clinical trials in patients with chronic kidney disease showed that vitamin D can reduce residual proteinuria with 16% [19]. Whether this reduction of proteinuria can reduce disease progression should be addressed in future studies.

This thesis also described that endothelin-1 regulates heparanase. ERAs have been approved for the treatment of pulmonary arterial hypertension and for the treatment of digital ulcers in scleroderma [20]. In addition, several phase II and phase III clinical trials have been conducted to test the effect of ERAs in cardiac failure and chronic kidney disease. However, some of these studies were terminated due to increased morbidity and mortality, which was associated with the development of fluid retention [21-23]. The development of fluid retention is dose-dependent, as another study showed that the ERA avosentan caused only modest fluid retention, but was able to reduce proteinuria, at lower doses [24]. Moreover, a recent phase II clinical trial showed that the ERA atrasentan was still able to reduce proteinuria at a dosage where it did not cause significant fluid retention [25]. Therefore, optimal dose finding studies are required to study the antiproteinuric effects of ERAs.

Finally, this thesis described that heparanase is controlled by eNOS. Previous studies identified BH4 as a critical cofactor to maintain a stable eNOS. When the availability of BH4 is reduced, eNOS becomes uncoupled, and produces superoxide instead of NO. BH4 availability is therefore essential to prevent the occurrence of endothelial dysfunction. BH4 is currently approved for the treatment of phenylketonuria (PKU), which is a metabolic disorder. A previous study showed that BH4 supplementation reduced proteinuria and renal damage in a rat model for focal segmental glomerulosclerosis [26]. However, treatment of patients with oral BH4 is limited by oxidation of BH4 to the inactive form BH2, and therefore has no effect on endothelial function [27].

In this thesis we showed that vitamin D supplementation seems to be a promising therapy for reduction of proteinuria in chronic kidney disease, most likely caused by the regulation of heparanase and the regulation of the transient receptor potential cation channel 6 (TRPC6) by vitamin D, as was previously described [28]. The therapeutic effect of ERAs and BH4, on the other hand, seem to be limited due to severe side effects in the case of ERAs, or inactivation of the compound by oxidation in the case of BH4. This thesis describes that both

endothelin-1 and eNOS may affect proteinuria by regulation of heparanase. As heparanase was shown to be essential for the development of proteinuria in several glomerular diseases, heparanase inhibitors represent an attractive alternative treatment to reduce proteinuria.
Currently, there are a few heparin-based heparanase inhibitors described, which have originally been developed as anti-cancer therapy. So far, the heparanase inhibitor PI-88 has been approved for the treatment of post resection liver cancer. In addition, several phase II and phase III clinical trials have been conducted for the heparanase inhibitors PG545 and SST0001 for the treatment of cancer. These heparin-based heparanase inhibitors are very well tolerated and cause only minimal side effects, including mild anticoagulation. Preclinical studies showed that the heparin-based heparanase inhibitors PI-88 and SST0001 reduced proteinuria in experimental diabetic nephropathy and passive Heymann nephritis [29, 30]. However, sulodexide, which is a mixture of glycosaminoglycans composed of low molecular weight heparin and dermatan sulfate, is an effective heparanase inhibitor, but failed to demonstrate renoprotection in a clinical trial aimed at type 2 DN, although preclinical studies with sulodexide were promising [31, 32]. Most likely, the outcome of the clinical trial was affected by the use of sulodexide derived from different sources, i.e. Chinese pigs compared to American pigs for the preclinical studies. A recent study showed that heparin-based heparanase inhibitors mimic soluble HS, and may stimulate macrophage activation independently of its heparanase inhibiting capacity [5]. As macrophages play an important role in the development of proteinuria and renal damage in several glomerular diseases, this may serve as an additional explanation for the failure of sulodexide in the type 2 DN clinical trial. To overcome this problem, there is an unmet need for a better structural characterization of GAG-based therapeutics aimed to inhibit heparanase activity. Preferably, novel heparanase-inhibiting compounds that do not mimic soluble HS and which are not able to activate macrophages should be developed.
Cathepsin L inhibitors may represent such a group of novel, but indirect, heparanase inhibitors. This thesis showed that cathepsin L is causally involved in the development of proteinuria, which is most likely mediated by the activation of heparanase. Previous studies showed that proteinuria can be reduced in accelerated anti-GBM glomerulonephritis by several unspecific cathepsin inhibitors [33, 34]. More specific cathepsin L inhibitors are currently available, but none of these inhibitors have been tested for their ability to reduce proteinuria, nor have they been tested in clinical trials for the treatment of other cathepsin L-mediated diseases such as cancer, inflammatory bowel disease, atherosclerosis, and skin diseases like atopic dermatitis and psoriasis. Preclinical and clinical studies should be performed to test the therapeutic potential of specific cathepsin L inhibitors for the treatment of proteinuria.

Heparanase 2, the structural homolog of heparanase, may represent another novel heparanase-inhibiting compound. Heparanase 2 shares 44% identity and 59% similarity with heparanase, but has no enzymatic activity like heparanase. Interestingly, a recent study showed that heparanase 2 inhibited the activity of heparanase [35]. To become activated, heparanase needs to be re-internalized through binding to cell-associated HS proteoglycans. Heparanase 2 binds with higher affinity to HS than heparanase and stays on the cell surface for a relatively long time. Thereby, heparanase fails to get internalized and remains inactive. In addition, heparanase 2 can physically interact with active heparanase, which interferes with the enzymatic functions of heparanase, such as cleaving the HS chain. A very recent study showed that heparanase 2-deficient mice develop significant albuminuria and die within one month after birth [36]. Heparanase 2 most likely is not able to activate macrophages, and may represent an attractive alternative heparanase inhibitor. Therefore, the potential of heparanase 2 as an inhibitor of heparanase activity in glomerular diseases should be investigated.

References

1. Han, J., et al., *Heparanase upregulation in high glucose-treated endothelial cells is prevented by insulin and heparin.* Exp Biol Med (Maywood), 2007. **232**(7): p. 927-34.
2. Maxhimer, J.B., et al., *Heparanase-1 gene expression and regulation by high glucose in renal epithelial cells: a potential role in the pathogenesis of proteinuria in diabetic patients.* Diabetes, 2005. **54**(7): p. 2172-8.
3. van den Hoven, M.J., et al., *Regulation of glomerular heparanase expression by aldosterone, angiotensin II and reactive oxygen species.* Nephrol Dial Transplant, 2009. **24**(9): p. 2637-45.
4. Kramer, A., et al., *Induction of glomerular heparanase expression in rats with adriamycin nephropathy is regulated by reactive oxygen species and the renin-angiotensin system.* J Am Soc Nephrol, 2006. **17**(9): p. 2513-20.
5. Goldberg, R., et al., *Role of heparanase-driven inflammatory cascade in pathogenesis of diabetic nephropathy.* Diabetes, 2014. **63**(12): p. 4302-13.
6. Lerner, I., et al., *Heparanase powers a chronic inflammatory circuit that promotes colitis-associated tumorigenesis in mice.* J Clin Invest, 2011. **121**(5): p. 1709-21.
7. van den Hoven, M.J., et al., *Heparanase in glomerular diseases.* Kidney Int, 2007. **72**(5): p. 543-8.
8. Harvey, S.J., et al., *Disruption of glomerular basement membrane charge through podocyte-specific mutation of agrin does not alter glomerular permselectivity.* Am J Pathol, 2007. **171**(1): p. 139-52.
9. Goldberg, S., et al., *Glomerular filtration is normal in the absence of both agrin and perlecan-heparan sulfate from the glomerular basement membrane.* Nephrol Dial Transplant, 2009. **24**(7): p. 2044-51.
10. Chen, S., et al., *Loss of heparan sulfate glycosaminoglycan assembly in podocytes does not lead to proteinuria.* Kidney Int, 2008. **74**(3): p. 289-99.
11. Jeansson, M., et al., *Adriamycin alters glomerular endothelium to induce proteinuria.* J Am Soc Nephrol, 2009. **20**(1): p. 114-22.
12. Dane, M.J., et al., *Association of kidney function with changes in the endothelial surface layer.* Clin J Am Soc Nephrol, 2014. **9**(4): p. 698-704.
13. Nieuwdorp, M., et al., *Endothelial glycocalyx damage coincides with microalbuminuria in type 1 diabetes.* Diabetes, 2006. **55**(4): p. 1127-32.
14. Kuwabara, A., et al., *Deterioration of glomerular endothelial surface layer induced by oxidative stress is implicated in altered permeability of macromolecules in Zucker fatty rats.* Diabetologia, 2010. **53**(9): p. 2056-65.
15. Salmon, A.H., et al., *Loss of the endothelial glycocalyx links albuminuria and vascular dysfunction.* J Am Soc Nephrol, 2012. **23**(8): p. 1339-50.
16. Dane, M.J., et al., *Glomerular endothelial surface layer acts as a barrier against albumin filtration.* Am J Pathol, 2013. **182**(5): p. 1532-40.
17. Levin, A., et al., *Prevalence of abnormal serum vitamin D, PTH, calcium, and phosphorus in patients with chronic kidney disease: results of the study to evaluate early kidney disease.* Kidney Int, 2007. **71**(1): p. 31-8.
18. Sonneveld, R., et al., *1,25-Vitamin D Deficiency Induces Albuminuria.* Am J Pathol, 2016.
19. de Borst, M.H., et al., *Active vitamin D treatment for reduction of residual proteinuria: a systematic review.* J Am Soc Nephrol, 2013. **24**(11): p. 1863-71.
20. Kohan, D.E., et al., *Clinical trials with endothelin receptor antagonists: what went wrong and where can we improve?* Life Sci, 2012. **91**(13-14): p. 528-39.
21. Kohan, D.E. and D.M. Pollock, *Endothelin antagonists for diabetic and non-diabetic chronic kidney disease.* Br J Clin Pharmacol, 2013. **76**(4): p. 573-9.
22. Rich, S. and V.V. McLaughlin, *Endothelin receptor blockers in cardiovascular disease.* Circulation, 2003. **108**(18): p. 2184-90.

23. Packer, M., et al., *Clinical effects of endothelin receptor antagonism with bosentan in patients with severe chronic heart failure: results of a pilot study.* J Card Fail, 2005. **11**(1): p. 12-20.
24. Wenzel, R.R., et al., *Avosentan reduces albumin excretion in diabetics with macroalbuminuria.* J Am Soc Nephrol, 2009. **20**(3): p. 655-64.
25. Kohan, D.E., et al., *Addition of atrasentan to renin-angiotensin system blockade reduces albuminuria in diabetic nephropathy.* J Am Soc Nephrol, 2011. **22**(4): p. 763-72.
26. Podjarny, E., et al., *Effect of chronic tetrahydrobiopterin supplementation on blood pressure and proteinuria in 5/6 nephrectomized rats.* Nephrol Dial Transplant, 2004. **19**(9): p. 2223-7.
27. Cunnington, C., et al., *Systemic and vascular oxidation limits the efficacy of oral tetrahydrobiopterin treatment in patients with coronary artery disease.* Circulation, 2012. **125**(11): p. 1356-66.
28. Sonneveld, R., et al., *Vitamin D down-regulates TRPC6 expression in podocyte injury and proteinuric glomerular disease.* Am J Pathol, 2013. **182**(4): p. 1196-204.
29. Gil, N., et al., *Heparanase is essential for the development of diabetic nephropathy in mice.* Diabetes, 2012. **61**(1): p. 208-16.
30. Levidiotis, V., et al., *A synthetic heparanase inhibitor reduces proteinuria in passive Heymann nephritis.* J Am Soc Nephrol, 2004. **15**(11): p. 2882-92.
31. Masola, V., et al., *A new mechanism of action of sulodexide in diabetic nephropathy: inhibits heparanase-1 and prevents FGF-2-induced renal epithelial-mesenchymal transition.* J Transl Med, 2012. **10**: p. 213.
32. Packham, D.K., et al., *Sulodexide fails to demonstrate renoprotection in overt type 2 diabetic nephropathy.* J Am Soc Nephrol, 2012. **23**(1): p. 123-30.
33. Baricos, W.H., et al., *Evidence suggesting a role for cathepsin L in an experimental model of glomerulonephritis.* Arch Biochem Biophys, 1991. **288**(2): p. 468-72.
34. Baricos, W.H., et al., *The cysteine proteinase inhibitor, E-64, reduces proteinuria in an experimental model of glomerulonephritis.* Biochem Biophys Res Commun, 1988. **155**(3): p. 1318-23.
35. Levy-Adam, F., et al., *Heparanase 2 interacts with heparan sulfate with high affinity and inhibits heparanase activity.* J Biol Chem, 2010. **285**(36): p. 28010-9.
36. Guo, C., et al., *A mouse model of urofacial syndrome with dysfunctional urination.* Hum Mol Genet, 2015. **24**(7): p. 1991-9.

Chapter 10

Nederlandse samenvatting

List of publications

Curriculum Vitae

Dankwoord

Nederlandse samenvatting

Proteïnurie is een belangrijk symptoom van veel glomerulaire ziekten en een onafhankelijke risicofactor voor de progressie tot nierfalen en hart- en vaatziekten. Proteïnurie wordt veroorzaakt door schade aan de glomerulaire filtratiebarrière (GFB), die bestaat uit glomerulaire endotheelcellen bedekt met een glycocalyx, de glomerulaire basaalmembraan (GBM) en podocyten. Alle lagen van de GFB bevatten negatief geladen heparansulfaat proteoglycanen (HSPG). De expressie van heparansulfaat (HS) in de glomerulus is gereduceerd in de meeste patiënten met proteïnurie. Deze afname is geassocieerd met een verhoogde expressie van het HS-afbrekend enzym heparanase in de glomerulus. Voorgaande studies hebben gesuggereerd dat heparanase een belangrijke rol speelt in de ontwikkeling van proteïnurie, maar de exacte rol van heparanase in de ontwikkeling van proteïnurie is nog niet bekend. In dit proefschrift wordt de precieze rol van heparanase in de ontwikkeling van proteïnurie in experimentele diabetische nefropatie (DN) en experimentele glomerulonefritis onderzocht. Daarnaast worden mogelijke mechanismen die betrokken zijn bij de regulatie van glomerulaire heparanase-expressie bekeken.

In het eerste deel van dit proefschrift is de exacte rol van heparanase in de ontwikkeling van proteïnurie in experimentele DN en experimentele glomerulonephritis bekeken. Om de rol van heparanase in DN te bepalen, werd experimentele DN geïnduceerd in wildtype (WT) en heparanase-deficiënte muizen door middel van streptozotocine (**hoofdstuk 2**). Heparanase-deficiënte muizen ontwikkelden geen proteïnurie na inductie van diabetes. Daarentegen ontwikkelden WT muizen een significante proteïnurie, welke gepaard ging met een verhoogde glomerulaire heparanase expressie en een gereduceerde glomerulaire HS expressie. Het ontwikkelen van proteïnurie in diabetische WT muizen ging gepaard met de ontwikkeling van mesangiale matrixexpansie, tubuloïnterstitiële fibrose en accumulatie van macrofagen. Heparanase-deficiënte muizen ontwikkelden deze kenmerken van diabetische nefropathie niet. Om te onderzoeken of remming van verhoogde heparanase activiteit de ontwikkeling van DN kan voorkomen, werden diabetische WT muizen behandeld met de heparanase remmer SST0001. Behandeling met SST0001 zorgt voor een verminderde proteïnurie en een verbeterde nierfunctie in experimentele DN. Tenslotte is ook het moleculaire mechanisme achter de inductie van heparanase in DN onderzocht. Het bleek dat de transcriptiefactor early growth response 1 (Egr1) verantwoordelijk is voor de activatie van de heparanase promoter. Concluderend, heparanase is essentieel voor de ontwikkeling van proteïnurie en nierschade in experimentele DN.
Cathepsine L, een lysosomaal cysteïne protease, kan een rol spelen in de ontwikkeling van proteïnurie door de activatie van heparanase of door de afbraak van eiwitten die essentieel

zijn voor een normale podocytarchitectuur, zoals het CD2-geassocieerde eiwit (CD2AP), synaptopodine, en dynamine. In **hoofdstuk 3** is de rol van cathepsine L in streptozotocine-geïnduceerde diabetes bepaald in WT en cathepsine L-deficiënte muizen. Diabetische cathepsine L-deficiënte muizen ontwikkelden, in tegenstelling tot diabetische WT muizen, geen proteïnurie, mesangiale matrixexpansie, tubuloïnterstitiële fibrose en accumulatie van macrofagen. Daarnaast hebben de diabetische cathepsine L-deficiënte muizen een normale nierfunctie. De ontwikkeling van proteïnurie in WT muizen correleerde met de inductie van heparanase-activiteit, een verlaagde glomerulaire HS expressie en de accumulatie van macrofagen, terwijl een verlaagde synaptopodine expressie vergezeld met podocytschade pas werd waargenomen in een later stadium. Concluderend, cathepsine L speelt een causale rol in de pathogenese van experimentele DN. Cathepsine L deficiëntie voorkomt de inductie van heparanase activiteit en de daaropvolgende ontwikkeling van proteïnurie en nierschade.

De glomerulaire expressie van heparanase is verhoogd in de meeste proteïnurische ziekten. Om te bepalen of de essentiële rol van heparanase in de ontwikkeling van proteïnurie niet beperkt was tot DN, werd in **hoofdstuk 4** de rol van heparanase op de ontwikkeling van proteïnurie in experimentele glomerulonefritis bekeken. Anti-GBM en lipopolysaccharide (LPS) glomerulonefritis, twee modellen voor experimentele glomerulonefritis, werden geïnduceerd in WT en heparanase-deficiënte muizen. De glomerulaire expressie van heparanase was verhoogd door inductie van experimentele glomerulonefritis in WT muizen, wat gepaard ging met een verlaagde glomerulaire HS expressie en proteïnurie. De heparanase-deficiënte muizen hadden een lagere proteïnurie, die gepaard ging met een normale HS expressie, een betere nierfunctie en minder nierschade. Daarnaast hadden de heparanase-deficiënte muizen een verlaagde glomerulaire leukocytinflux, macrofaaginflux en een lagere expressie van Th1 en Th2 cytokines in vergelijking met de WT muizen. *In vitro* experimenten toonden aan dat tumor necrose factor (TNF)-α en LPS de expressie van heparanase induceren in gekweekte muis glomerulaire endotheelcellen en muispodocyten, waardoor de transendotheliale albuminepassage toenam. Samenvattend, heparanase medieert de ontwikkeling van proteïnurie en nierschade in experimentele glomerulonefritis. In heparanase-deficiënte muizen is er sprake van een normale glomerulaire HS-expressie, een verminderde influx van leukocyten en macrofagen, en een gunstige beïnvloeding van het lokale cytokinemilieu in vergelijking met WT muizen

In **hoofdstuk 5** werd de huidige kennis over de rol van heparanase en HS in de ontwikkeling van proteïnurie samengevat. Daarnaast werd de rol van de glomerulaire endotheliale glycocalyx op de ontwikkeling van proteïnurie besproken.

Het tweede deel van dit proefschrift richt zich op mechanismen die een rol kunnen spelen in de regulatie van glomerulaire heparanase-expressie. Eerdere studies hebben aangetoond dat endotheline receptor antagonisten (ERA's) proteïnurie kunnen verminderen in verschillende glomerulaire ziekten, maar het onderliggende mechanisme van de nierbeschermende effecten van ERA's was nog niet bekend. In **hoofdstuk 6** werd het effect van endotheline-1 op heparanase en HS expressie zowel *in vitro* als *in vivo* bekeken. Endotheline-1 was niet in staat om heparanase-expressie te induceren in gekweekte muis glomerulaire endotheelcellen. Daarentegen werd heparanase expressie geïnduceerd door endotheline-1 in gekweekte muispodocyten. Transendotheliale albuminepassage *in vitro* werd verhoogd door kweeksupernatant van podocyten die met endotheline-1 waren gestimuleerd, welk effect afhankelijk was van heparanase. Om de *in vitro* resultaten verder uit te breiden, werd het effect van endotheline-1 signalering in de podocyt op proteïnurie en glomerulaire heparanase-expressie onderzocht in podocyt-specifieke endothelinereceptor knockout (podETRKO) muizen. Door het induceren van diabetes ontwikkelden WT muizen proteïnurie en nierschade, welke gepaard ging met een verhoogde glomerulaire heparanase-expressie en een verminderde glomerulaire HS expressie. Proteïnurie en nierschade waren verminderd in diabetische podETRKO muizen, die een normale heparanase en HS expressie hadden. Verder hadden de WT muizen een dunnere glycocalyx dikte, terwijl deze behouden was in de diabetische podETRKO muizen. Concluderend, endotheline-1 induceert de expressie van heparanase in de podocyt. Heparanase knipt vervolgens HS in de endotheliale glycocalyx, hetgeen resulteert in een dunnere glycocalyx en de ontwikkeling van proteïnurie.

Het verlies van endotheliale nitric oxide synthase (eNOS) activiteit verergert proteïnurie en nierschade in verschillende glomerulaire ziekten, maar het onderliggende mechanisme is niet volledig bekend. Eerdere studies hebben aangetoond dat de glomerulaire endotheliale glycocalyx dunner is in diabetische eNOS-deficiënte muizen, en dat heparanase een belangrijke rol speelt in de afbraak van de glomerulaire endotheliale glycocalyx. Daarom werd in **hoofdstuk 7** het effect van eNOS deficiëntie op glomerulaire heparanase expressie onderzocht in adriamycine nefropathie (AN). C57BL/6 WT muizen, die niet gevoelig zijn voor het ontwikkelen van AN, ontwikkelden geen proteïnurie en hadden een normale heparanase-expressie na inductie van AN. eNOS-deficiënte C57BL/6 muizen ontwikkelden proteïnurie en hadden een verhoogde glomerulaire heparanase expressie. In *in vitro* experimenten werd aangetoond dat de eNOS remmer asymmetrisch dimethylarginine (ADMA) heparanase-expressie induceerde in muis glomerulaire endotheelcellen en de transendotheliale albuminepassage verhoogde op een heparanase-afhankelijke wijze. Concluderend, eNOS remt de expressie van heparanase in het glomerulair endotheel, en voorkomt daarmee de ontwikkeling van proteïnurie.

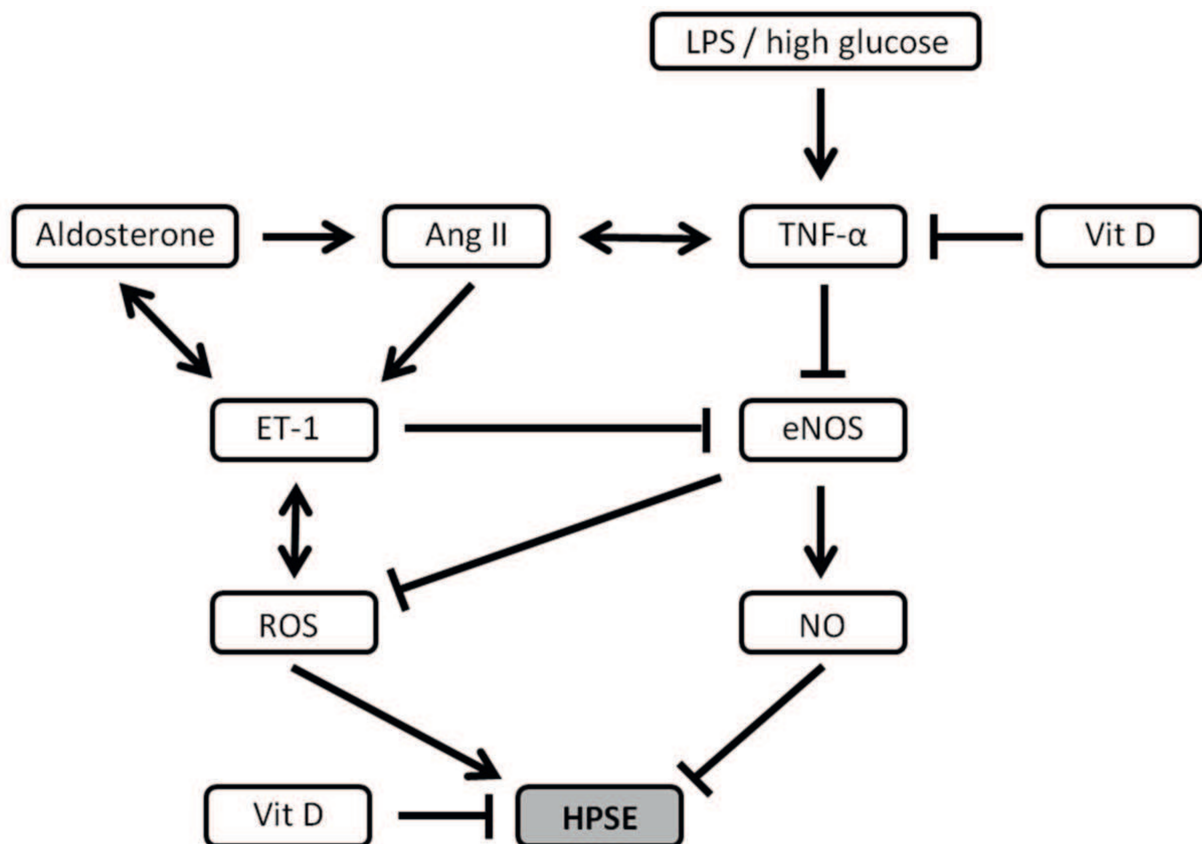
Vitamine D heeft recente aandacht gekregen voor de behandeling van proteïnurie. In **hoofdstuk 8** werd het effect van vitamine D op heparanase-expressie en heparanase promoteractiviteit bestudeerd. Na het induceren van AN ontwikkelden ratten proteïnurie en een verhoogde heparanase-expressie, die beide werden gereduceerd door behandeling met 1,25-dihydroxyvitamine D_3 (1,25-D_3). 1,25-D_3-deficiënte 25-hydroxy-1α-hydroxylase knockout muizen ontwikkelden ook proteïnurie en hadden een verhoogde heparanase-expressie, die beide genormaliseerd werden door behandeling met 1,25-D_3. In *in vitro* experimenten werd aangetoond dat adriamycine de heparanase-expressie verhoogde in gekweekte muispodocyten. Daarnaast was de transendotheliale albuminepassage lager door toevoeging van medium van podocyten behandeld met adriamycine samen met 1,25-D_3 in vergelijking met medium van podocyten behandeld met alleen adriamycine. Tenslotte werd aangetoond dat 1,25-D_3 zorgde voor een dosisafhankelijke afname van de heparanase promoteractiviteit, en dat vitamine D direct bindt aan de heparanase promoter. Concluderend, het beschermende effect van vitamine D op de ontwikkeling van proteïnurie wordt gemedieerd door remming van heparanase-expressie in de podocyt.

Samennemend blijkt dat heparanase essentieel is voor de ontwikkeling van proteïnurie en nierschade in experimentele DN en experimentele glomerulonefritis. Inductie van heparanase-activiteit resulteerde in een verlies van glomerulaire HS expressie en een afname van de glycocalyxdikte, wat zorgt voor een verlies van negatieve lading in de GFB. Hierdoor kunnen eiwitten als albumine makkelijker de GFB passeren, wat leidt tot de ontwikkeling van proteïnurie en nierschade. Daarnaast blijkt dat heparanase een belangrijke rol speelt in de influx van ontstekingscellen zoals macrofagen in de nier. Deze ontstekingscellen spelen ook een belangrijke rol in de afbraak van de GFB en de ontwikkeling van proteïnurie en nierschade. Vervolgstudies zijn nodig om het exacte mechanisme van heparanase-geïnduceerde proteïnurie te evalueren. Concluderend, remming van heparanase-expressie en/of -activiteit lijkt een uitstekende manier om proteïnurie en nierschade te verminderen.

In dit proefschrift werden verschillende factoren die betrokken zijn bij de regulatie van heparanase geïdentificeerd. Heparanase wordt positief gereguleerd door LPS, TNF-α en endotheline-1, terwijl heparanase negatief gereguleerd wordt door eNOS en vitamine D. Eerdere studies hebben al aangetoond dat heparanase positief gereguleerd wordt door hoog glucose, angiontensine II, aldosteron en reactieve zuurstofradicalen (ROS) [1-4]. Interessant is dat sommige van deze regulerende factoren heparanase-expressie induceren in zowel endotheelcellen als podocyten (LPS, hoog glucose, TNF-α en ROS), terwijl andere regulerende factoren uitsluitend werkzaam zijn in het endotheel (eNOS) of de podocyt (angiontensin II, aldosterone, endotheline-1 and vitamine D). Nog interessanter is dat terwijl

endotheline-1 en vitamine D uitsluitend heparanase reguleren in de podocyt en niet in het endotheel, dat ze wel betrokken zijn bij de regulatie van de dikte van de glomerulaire endotheliale glycocalyx *in vivo* en transendotheliale albuminepassage *in vitro*. Dit suggereert dat er sprake is van crosstalk (communicatie) tussen de podocyt en het endotheel. De effecten van endotheline-1 en adriamycine op transendotheliale albuminepassage waren afhankelijk van heparanase, hetgeen suggereert dat heparanase een belangrijke speler is in de pathogene crosstalk (communicatie) tussen de podocyt en het glomerulaire endotheel.

Verschillende factoren die betrokken zijn bij de directe regulatie van heparanase zijn beschreven in de literatuur en dit proefschrift. Het is interessant dat sommige van deze factoren ook elkaar lijken te reguleren. Een schematische weergave van factoren betrokken bij de regulatie van heparanase wordt weergegeven in figuur 1.



Figuur 1: Factoren die betrokken zijn bij de regulatie van heparanase-expressie. De expressie van TNF-α wordt geïnduceerd door LPS of hoog glucose. TNF-α induceert vervolgens de expressie van angiotensine II en vermindert de expressie van eNOS. Angiotensine II induceert de expressie van endotheline-1, die de expressie van aldosteron induceert, wat de expressie van angiotensine II verder verhoogd. Endotheline-1 induceert de expressie van heparanase door stimulatie van de productie van ROS en door een afname van de eNOS expressie. eNOS reguleert de expressie van heparanase door een afgenomen ROS productie en een verhoogde NO productie. Vitamine D verlaagt de expressie van heparanase ofwel direct, of indirect door het verlagen van de TNF-α expressie. Afkortingen: LPS, lipopolysaccharide; vit D, vitamine D; TNF-α, tumor necrose factor-α; Ang II, angiontensine II; ET-1, endotheline-1; ROS, reactieve zuurstofradicalen; eNOS, endotheel stikstofoxide synthase; NO, stikstofoxide; HPSE, heparanase.

Om biologisch actief te worden, moet heparanase gesplitst worden door cathepsine L. Eerdere studies hebben aangetoond dat actief heparanase macrofagen kan activeren, wat resulteert in een verhoogde TNF-α productie en een verhoogde gevoeligheid voor TNF-α, leidend tot een verdere toename van de heparanase expressie [5, 6]. Het reguleren van de expressie van elk van de factoren betrokken bij de regulatie van heparanase is dan ook een aantrekkelijke alternatieve therapie voor de vermindering van proteïnurie en nierschade.

Toekomstperspectieven en translationele aspecten

In dit proefschrift werd aangetoond dat heparanase essentieel is voor de ontwikkeling van proteïnurie en nierschade in experimentele DN en experimentele glomerulonefritis. Eerdere studies hebben aangetoond dat de expressie van HS in de GBM is afgenomen na inductie van heparanase-expressie, dat geassocieerd was met de ontwikkeling van proteïnurie [7]. De primaire rol van HS in de ontwikkeling van proteïnurie werd in twijfel getrokken toen genetisch gemanipuleerde muizen met een verminderde HS expressie geen proteïnurie ontwikkelden [3, 8-10]. Echter, in deze diermodellen was de HS expressie alleen verminderd in de GBM en niet in de endotheliale glycocalyx. Het lijkt dan ook voor de hand liggend dat HS in de endotheliale glycocalyx een belangrijke rol speelt in de ontwikkeling van proteïnurie. Recente studies hebben aangetoond dat een afname van de dikte van de endotheliale glycocalyx geassocieerd is met de ontwikkeling van proteïnurie [11-16]. Om de exacte rol van HS in de endotheliale glycocalyx op de ontwikkeling van proteïnurie te bestuderen, moeten endotheel-specifieke EXT1-deficiënte muizen, die waarschijnlijk geen HS tot expressie brengen op het endotheel, gebruikt worden. Deze muizen zijn helaas niet levensvatbaar (persoonlijke communicatie Jeffrey Esko), wat pleit voor het gebruik van induceerbare endotheel-specifieke EXT1-deficiënte muizen. Aangezien de ontwikkeling van proteïnurie hoogstwaarschijnlijk niet het gevolg is van een single-hit mechanisme is, moet er sprake zijn van glomerulaire schade naast een verminderde glomerulaire HS expressie. Door de ontwikkeling van proteïnurie en nierschade in de bovengenoemde aanpak te evalueren, kan bepaald worden of verlies van HS in de endotheliale glycocalyx belangrijk is voor de ontwikkeling van proteïnurie en nierschade. Naast afbraak van HS, hebben recente studies, die zich met name hebben gericht op DN, aangetoond dat heparanase ook betrokken is bij de ontwikkeling van proteïnurie door het activeren van macrofagen, die ook een rol spelen bij de afbraak van de glomerulaire capillaire wand [5]. Er zijn dan ook extra studies nodig om te bepalen of proteïnurie gemedieerd door heparanase wordt veroorzaakt door een afname van glomerulaire HS expressie, de activatie van macrofagen, of beide. Hiervoor moeten beenmergtransplantaties, waarbij beenmerg wordt verkregen uit WT en heparanase-deficiënte muizen, worden uitgevoerd in experimentele DN en experimentele glomerulonefritis.

Momenteel is remming van het renine-angiotensine-aldosteron systeem (RAAS) door angiotensine converterend enzym (ACE) remmers en angiotensine receptor blokkers (ARB's) de behandeling van keuze voor patiënten met proteïnurie. Onze groep heeft eerder aangetoond dat het RAAS systeem betrokken is bij de inductie van glomerulaire heparanase expressie [3, 4]. Remming van het RAAS systeem zorgt echter slechts voor een gedeeltelijke vermindering van proteïnurie, en het is dan ook belangrijk om additionele antiproteïnurische

middelen te ontwikkelen. In dit proefschrift werden vitamine D, endotheline-1 en eNOS geïdentificeerd als regulatoren van heparanase-expressie. Vitamine D, endotheline receptor antagonisten (ERA's), en tetrahydrobiopterine (BH4), een eNOS cofactor, zijn momenteel goedgekeurd voor klinisch gebruik bij andere ziekten, en zijn daarom beschikbaar voor evaluatie van hun antiproteïnurisch effect.

Vitamine D is goedgekeurd voor de preventie en behandeling van secundaire hyperparathyroïdie, die zich ontwikkelt tijdens de latere stadia van chronische nierziekten. De serumspiegels van actief vitamine D nemen geleidelijk af tijdens de vermindering van de nierfunctie als gevolg van de progressie van nierziekten, met als consequentie dat patiënten vitamine D deficiënt worden [17]. Vitamine D deficiëntie kan veroorzaakt worden door een verminderde inname van vitamine D, of een verlies van 1α-hydroxylase ten gevolge van het verlies van niermassa. Een recente studie heeft aangetoond dat vitamine D deficiëntie zelf ook proteïnurie en nierschade kan veroorzaken [18]. Verschillende klinische studies met patiënten met chronische nierziekten hebben aangetoond dat toevoeging van vitamine D aan de standaard antiproteïnurische therapie de resterende proteïnurie met 16% kan verminderen [19]. Of deze vermindering van proteïnurie ook daadwerkelijk de ziekteprogressie kan verminderen, moet nog aangetoond worden in toekomstige studies.

In dit proefschrift is ook aangetoond dat endotheline-1 heparanase reguleert. ERA's zijn goedgekeurd voor de behandeling van pulmonale arteriële hypertensie en voor de behandeling digitale ulcera by sclerodermie [20]. Daarnaast zijn verschillende fase II en fase III klinische studies uitgevoerd om het effect van ERA's te testen bij hartfalen en chronische nierziekten. Sommige van deze studies werden voortijdig beëindigd door een verhoogde morbiditeit en mortaliteit, die geassocieerd waren met de ontwikkeling van vochtretentie [21-23]. Een andere studie toonde echter aan dat de ontwikkeling van vochtretentie dosisafhankelijk is, aangezien de ERA avosentan slechts milde vochtretentie veroorzaakte, maar nog wel in staat was proteïnurie te verminderen bij lagere doses [24]. Daarnaast heeft een recente fase II klinische studie aangetoond dat de ERA atrasentan nog in staat was proteïnurie te verminderen bij concentraties die geen significante vochtretentie meer veroorzaakte [25]. Daarom zijn er studies nodig om de optimale dosis van ERA's te bepalen, zodat de antiproteïnurische effecten van ERA's beter bepaald kunnen worden.

Tot slot hebben is in dit proefschrift ook aangetoond dat heparanase wordt gereguleerd door eNOS. Eerdere studies hebben BH4 geïdentificeerd als een essentiële cofactor voor een stabiele eNOS expressie. Wanneer de beschikbaarheid van BH4 is verminderd, wordt eNOS ontkoppeld, en produceert het superoxide in plaats van NO. De aanwezigheid van BH4 is daarom essentieel om het ontstaan van endotheeldysfunctie te voorkomen. BH4 is momenteel goedgekeurd voor de behandeling van fenylketonurie (PKU), een stofwisselingsziekte. Een eerdere studie heeft aangetoond dat BH4 suppletie proteïnurie en

nierschade kan verminderen in een ratmodel voor focale segmentale glomerulosclerose [26]. Behandeling van patiënten met orale BH4 is echter beperkt door oxidatie van BH4 naar de inactieve vorm BH2, en heeft dus ook geen invloed op de endotheelfunctie [27].

In dit proefschrift hebben we laten zien dat vitamine D suppletie een veelbelovende therapie lijkt voor de vermindering van proteïnurie bij chronische nierziekten, hetgeen waarschijnlijk wordt veroorzaakt door de regulatie van heparanase en de regulatie van het transient receptor potentiaal kation kanaal 6 (TRPC6) door vitamine D [28]. Het therapeutische effect van ERA's en BH4 lijkt beperkt door ernstige bijwerkingen bij ERA's en inactivatie van het middel door oxidatie bij BH4. Dit proefschrift beschrijft dat zowel endotheline-1 als eNOS invloed hebben op proteïnurie door de regulatie van heparanase. Aangezien heparanase essentieel is voor de ontwikkeling van proteïnurie in verschillende glomerulaire ziekten, vormen heparanase remmers een aantrekkelijke alternatieve behandeling om proteïnurie te verminderen.

Momenteel zijn er enkele heparanaseremmers beschreven die gebaseerd zijn op heparine. Deze heparanase remmers zijn oorspronkelijk ontwikkeld voor de behandeling van kanker. Op dit moment is de heparanaseremmer PI-88 goedgekeurd voor de behandeling van leverkanker nadat een deel van de lever is verwijderd. Daarnaast zijn er verschillende fase II en fase III klinische studies uitgevoerd om de effectiviteit van de heparanaseremmers PG545 en SST0001 voor de behandeling van kanker te testen. Deze heparanaseremmers, gebaseerd op heparine, worden zeer goed verdragen en veroorzaken slechts milde bijwerkingen, waaronder milde antistolling. Preklinische studies hebben aangetoond dat de heparanaseremmers PI-88 en SST0001 proteïnurie verminderen in experimentele DN en passieve Heymann nefritis [29, 30]. Een recente klinische studie heeft echter aangetoond dat sulodexide, een mengsel van glycosaminoglycanen bestaande uit laag moleculair gewicht heparine en dermatan sulfaat, een effectieve heparanaseremmer is, maar geen nierbeschermende effecten had in patiënten met type 2 diabetes, terwijl preklinische studies met sulodexide veelbelovend waren [31, 32]. Waarschijnlijk zijn de resultaten van de klinische studie beïnvloed door het gebruik van sulodexide afkomstig van verschillende bronnen, namelijk Chinese varkens in de klinische studie versus Amerikaanse varkens in de preklinische studies. Een recente studie heeft aangetoond dat heparanase remmers gebaseerd op heparine op oplosbaar HS lijken, en in staat zijn macrofagen te activeren, een effect dat onafhankelijk is van het heparanase remmend vermogen [5]. Aangezien macrofagen een belangrijke rol spelen in de ontwikkeling van proteïnurie en nierschade in verschillende glomerulaire ziekten, kan dit een aanvullende verklaring zijn voor het falen van sulodexide in de type 2 DN klinische studie. Om dit probleem te ondervangen is er behoefte aan een betere structurele karakterisering van GAG-gebaseerde therapieën gericht op de remming van heparanase-activiteit. Bij voorkeur moeten nieuwe heparanase remmende

middelen ontwikkeld worden die niet lijken op oplosbaar HS en die niet in staat zijn macrofagen te activeren.

Cathepsine L-remmers vertegenwoordigen zo'n groep van nieuwe, maar indirecte, heparanaseremmers. Dit proefschrift heeft aangetoond dat cathepsine L een belangrijke causale rol speelt in de ontwikkeling van proteïnurie, wat waarschijnlijk werd gemedieerd door het activeren van heparanase. Eerdere studies hebben aangetoond dat proteïnurie in versnelde anti-GBM glomerulonefritis verminderd kan worden door verschillende aspecifieke cathepsine remmers [33, 34]. Meer specifieke cathepsine L-remmers zijn beschikbaar, maar geen van deze remmers zijn getest op hun vermogen om proteïnurie te verminderen, en evenmin zijn ze getest in klinische studies voor de behandeling van andere cathepsine L-gemedieerde ziekten als kanker, inflammatoire darmziekten, atherosclerose, en huidaandoeningen als atopische dermatitis en psoriasis. Preklinische en klinische studies moeten worden uitgevoerd om het therapeutisch effect van deze specifieke cathepsine L remmers voor de behandeling van proteïnurie te testen.

Heparanase 2, de structurele homoloog van heparanase, kan een ander nieuw heparanase remmend middel vertegenwoordigen. Heparanase 2 komt op aminozuurniveau voor 44% overeen met heparanase en heeft 59% gelijkenis met heparanase, maar heeft geen enzymatische activiteit zoals heparanase. Interessant is dat een recente studie heeft aangetoond dat heparanase 2 de activiteit van heparanase kan remmen [35]. Om geactiveerd te worden, moet heparanase gereïnternaliseerd worden door binding aan celgebonden HS proteoglycanen. Heparanase 2 bindt met hogere affiniteit aan HS dan heparanase, en blijft voor een relatief lange periode gebonden aan het celoppervlak. Hierdoor wordt heparanase niet geïnternaliseerd en blijft het inactief. Daarnaast kan heparanase 2 ook een directe interactie aangaan met heparanase, wat interfereert met de enzymatische functies van heparanase, zoals het knippen van de HS keten. Een recente studie heeft aangetoond dat heparanase-2-deficiënte muizen een significante albuminurie ontwikkelen en binnen één maand na de geboorte sterven [36]. Heparanase 2 is waarschijnlijk niet in staat om macrofagen te activeren, en is mogelijk een zeer aantrekkelijke alternatieve heparanase remmer. Daarom moet de potentie van heparanase 2 als remmer van heparanase activiteit in glomerulaire ziekten onderzocht worden.

Referenties

1. Han, J., et al., *Heparanase upregulation in high glucose-treated endothelial cells is prevented by insulin and heparin.* Exp Biol Med (Maywood), 2007. **232**(7): p. 927-34.
2. Maxhimer, J.B., et al., *Heparanase-1 gene expression and regulation by high glucose in renal epithelial cells: a potential role in the pathogenesis of proteinuria in diabetic patients.* Diabetes, 2005. **54**(7): p. 2172-8.
3. van den Hoven, M.J., et al., *Regulation of glomerular heparanase expression by aldosterone, angiotensin II and reactive oxygen species.* Nephrol Dial Transplant, 2009. **24**(9): p. 2637-45.
4. Kramer, A., et al., *Induction of glomerular heparanase expression in rats with adriamycin nephropathy is regulated by reactive oxygen species and the renin-angiotensin system.* J Am Soc Nephrol, 2006. **17**(9): p. 2513-20.
5. Goldberg, R., et al., *Role of heparanase-driven inflammatory cascade in pathogenesis of diabetic nephropathy.* Diabetes, 2014. **63**(12): p. 4302-13.
6. Lerner, I., et al., *Heparanase powers a chronic inflammatory circuit that promotes colitis-associated tumorigenesis in mice.* J Clin Invest, 2011. **121**(5): p. 1709-21.
7. van den Hoven, M.J., et al., *Heparanase in glomerular diseases.* Kidney Int, 2007. **72**(5): p. 543-8.
8. Harvey, S.J., et al., *Disruption of glomerular basement membrane charge through podocyte-specific mutation of agrin does not alter glomerular permselectivity.* Am J Pathol, 2007. **171**(1): p. 139-52.
9. Goldberg, S., et al., *Glomerular filtration is normal in the absence of both agrin and perlecan-heparan sulfate from the glomerular basement membrane.* Nephrol Dial Transplant, 2009. **24**(7): p. 2044-51.
10. Chen, S., et al., *Loss of heparan sulfate glycosaminoglycan assembly in podocytes does not lead to proteinuria.* Kidney Int, 2008. **74**(3): p. 289-99.
11. Jeansson, M., et al., *Adriamycin alters glomerular endothelium to induce proteinuria.* J Am Soc Nephrol, 2009. **20**(1): p. 114-22.
12. Dane, M.J., et al., *Association of kidney function with changes in the endothelial surface layer.* Clin J Am Soc Nephrol, 2014. **9**(4): p. 698-704.
13. Nieuwdorp, M., et al., *Endothelial glycocalyx damage coincides with microalbuminuria in type 1 diabetes.* Diabetes, 2006. **55**(4): p. 1127-32.
14. Kuwabara, A., et al., *Deterioration of glomerular endothelial surface layer induced by oxidative stress is implicated in altered permeability of macromolecules in Zucker fatty rats.* Diabetologia, 2010. **53**(9): p. 2056-65.
15. Salmon, A.H., et al., *Loss of the endothelial glycocalyx links albuminuria and vascular dysfunction.* J Am Soc Nephrol, 2012. **23**(8): p. 1339-50.
16. Dane, M.J., et al., *Glomerular endothelial surface layer acts as a barrier against albumin filtration.* Am J Pathol, 2013. **182**(5): p. 1532-40.
17. Levin, A., et al., *Prevalence of abnormal serum vitamin D, PTH, calcium, and phosphorus in patients with chronic kidney disease: results of the study to evaluate early kidney disease.* Kidney Int, 2007. **71**(1): p. 31-8.
18. Sonneveld, R., et al., *1,25-Vitamin D Deficiency Induces Albuminuria.* Am J Pathol, 2016.
19. de Borst, M.H., et al., *Active vitamin D treatment for reduction of residual proteinuria: a systematic review.* J Am Soc Nephrol, 2013. **24**(11): p. 1863-71.
20. Kohan, D.E., et al., *Clinical trials with endothelin receptor antagonists: what went wrong and where can we improve?* Life Sci, 2012. **91**(13-14): p. 528-39.
21. Kohan, D.E. and D.M. Pollock, *Endothelin antagonists for diabetic and non-diabetic chronic kidney disease.* Br J Clin Pharmacol, 2013. **76**(4): p. 573-9.
22. Rich, S. and V.V. McLaughlin, *Endothelin receptor blockers in cardiovascular disease.* Circulation, 2003. **108**(18): p. 2184-90.

23. Packer, M., et al., *Clinical effects of endothelin receptor antagonism with bosentan in patients with severe chronic heart failure: results of a pilot study.* J Card Fail, 2005. **11**(1): p. 12-20.
24. Wenzel, R.R., et al., *Avosentan reduces albumin excretion in diabetics with macroalbuminuria.* J Am Soc Nephrol, 2009. **20**(3): p. 655-64.
25. Kohan, D.E., et al., *Addition of atrasentan to renin-angiotensin system blockade reduces albuminuria in diabetic nephropathy.* J Am Soc Nephrol, 2011. **22**(4): p. 763-72.
26. Podjarny, E., et al., *Effect of chronic tetrahydrobiopterin supplementation on blood pressure and proteinuria in 5/6 nephrectomized rats.* Nephrol Dial Transplant, 2004. **19**(9): p. 2223-7.
27. Cunnington, C., et al., *Systemic and vascular oxidation limits the efficacy of oral tetrahydrobiopterin treatment in patients with coronary artery disease.* Circulation, 2012. **125**(11): p. 1356-66.
28. Sonneveld, R., et al., *Vitamin D down-regulates TRPC6 expression in podocyte injury and proteinuric glomerular disease.* Am J Pathol, 2013. **182**(4): p. 1196-204.
29. Gil, N., et al., *Heparanase is essential for the development of diabetic nephropathy in mice.* Diabetes, 2012. **61**(1): p. 208-16.
30. Levidiotis, V., et al., *A synthetic heparanase inhibitor reduces proteinuria in passive Heymann nephritis.* J Am Soc Nephrol, 2004. **15**(11): p. 2882-92.
31. Masola, V., et al., *A new mechanism of action of sulodexide in diabetic nephropathy: inhibits heparanase-1 and prevents FGF-2-induced renal epithelial-mesenchymal transition.* J Transl Med, 2012. **10**: p. 213.
32. Packham, D.K., et al., *Sulodexide fails to demonstrate renoprotection in overt type 2 diabetic nephropathy.* J Am Soc Nephrol, 2012. **23**(1): p. 123-30.
33. Baricos, W.H., et al., *Evidence suggesting a role for cathepsin L in an experimental model of glomerulonephritis.* Arch Biochem Biophys, 1991. **288**(2): p. 468-72.
34. Baricos, W.H., et al., *The cysteine proteinase inhibitor, E-64, reduces proteinuria in an experimental model of glomerulonephritis.* Biochem Biophys Res Commun, 1988. **155**(3): p. 1318-23.
35. Levy-Adam, F., et al., *Heparanase 2 interacts with heparan sulfate with high affinity and inhibits heparanase activity.* J Biol Chem, 2010. **285**(36): p. 28010-9.
36. Guo, C., et al., *A mouse model of urofacial syndrome with dysfunctional urination.* Hum Mol Genet, 2015. **24**(7): p. 1991-9.

List of publications

Garsen M, Rops AL, Dijkman H, Willemsen B, van Kuppevelt TH, Russel FG, Rabelink TJ, Berden JH, Reinheckel T, van der Vlag J. Cathepsin L is crucial for the development of early experimental diabetic nephropathy. *Kidney Int*. 2016; *in press*.

Garsen M, Lenoir O, Rops AL, Dijkman HB, Willemsen B, van Kuppevelt TH, Rabelink TJ, Berden JH, Tharaux PL, van der Vlag J. Endothelin-1 induces proteinuria by heparanase-mediated disruption of the glomerular glycocalyx. *J Am Soc Nephrol.* 2016; *in press.*

Garsen M, Rops AL, Li J, van Beneden K, van den Branden C, Berden JH, Rabelink TJ, van der Vlag J. Endothelial nitric oxide synthase prevents heparanase induction and the development of proteinuria. *PLoS One. 2016; in press.*

Garsen M, Benner M, Dijkman HB, van Kuppevelt TH, Li JP, Rabelink TJ, Vlodavsky I, Berden JH, Rops AL, Elkin M, van der Vlag J. Heparanase is essential for the development of acute experimental glomerulonephritis. *Am J Pathol*. 2016; 186(4): 805-15.

Garsen M, Sonneveld R, Rops AL, Huntink S, van Kuppevelt TH, Rabelink TJ, Hoenderop JG, Berden JH, Nijenhuis T, van der Vlag J. Vitamin D attenuates proteinuria by inhibition of heparanase expression in the podocyte. *J Pathol*. 2015; 237(4): 472-81.

Garsen M, Rops AL, Rabelink TJ, Berden JH, van der Vlag J. The role of heparanase and the endothelial glycocalyx in the development of proteinuria. *Nephrol Dial Transplant*. 2014; 29(1): 49-55.

Gil N*, Goldberg R*, Neuman T, **Garsen M**, Zcharia E, Rubinstein AM, van Kuppevelt TH, Meirovitz A, Pisano C, Li JP, van der Vlag J, Vlodavsky I, Elkin M. Heparanase is essential for the development of diabetic nephropathy in mice. *Diabetes*. 2012; 61(1): 208-16.

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Curriculum Vitae

Marjolein Garsen werd geboren op 22 maart 1986 te Warnsveld. In 2004 behaalde zij haar VWO diploma aan het Baudartius College te Warnsveld. In datzelfde jaar begon zij aan de bachelor Geneeskunde, gevolgd door de master Medische Farmaceutische Wetenschappen aan de Rijksuniversiteit Groningen. Ze heeft onderzoeksstages gedaan op de afdeling Klinische Farmacologie van de Rijksuniversiteit Groningen en de afdeling Kinderoncologie van het Radboudumc. In juni 2010 legde zij het doctoraalexamen met goed gevolg af en is zij aansluitend begonnen als onderzoeker in opleiding op de afdeling Nierziekten van het Radboudumc aan haar promotieonderzoek “Heparanase: an essential factor for the development of proteinuria” zoals beschreven in dit proefschrift onder begeleiding van dr. J. van der Vlag en prof. dr. J.H.M. Berden. Sinds augustus 2015 is zij werkzaam als postdoc op de afdeling Nierziekten van het Radboudumc, waar zij in het kader van een innovatieproject van de Nierstichting Nederland verder werkt aan de heparanase onderzoekslijn.

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