Molecular mechanisms of the kidney in health and disease

Kerstin Ebefors

UNIVERSITY OF GOTHENBURG

Department of Molecular and Clinical Medicine
Institute of Medicine
Sahlgrenska Academy

University of Gothenburg 2011

A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have either already been published or are manuscripts at various stages (in press, submitted or in manuscript).

ISBN 987-91-628-8308-9
Printed by Intellecta Infolog, Göteborg, Sweden 2011
Abstract and summary sections of this thesis are available online:
http://hdl.handle.net/2077/24861

Cover picture: Human mesangial cell stained with anti-perlecan (green) and DAPI (blue)

ABSTRACT

In 2010, 307 Swedish patients received a new kidney through transplantation, and the first of April 2011, 603 patients were on the kidney transplant waiting list. In Sweden over 8000 patients are presently in active uremic care with about half in dialysis and the other half with a functional kidney graft. The numbers of patients in need of active uremic care are escalating and so are the costs for renal health care, in Sweden as in most of the western world. For patients with end stage renal disease active uremic care is the last option for survival since there is no cure or specific treatment for most renal diseases. The lack of treatment options often leaves steroids and chemotherapy as the only available choices. In order to find more specific treatment and to cure or delay the progress of renal disease we need to learn more about the molecular background of these diseases.

To increase our understanding of the molecular mechanisms behind renal disease we have studied the gene expression in both an animal model of the nephrotic syndrome in rat as well as in human material in form of renal biopsies and cell cultures. The most common renal diseases all start in the glomerulus, the capillary tuft in the nephron where the ultrafiltration of blood takes place, and therefore we have focused on gene expression in the glomerulus.

When investigating the gene expression in glomeruli from healthy kidney donors and from mice we found a core cluster of conserved, highly glomerulus-specific genes. Normal function of some of these genes in the glomerulus is already known to be of importance to the filtration barrier and mutations in certain of them are tightly connected to proteinuria. The discovered core cluster also contained genes that so far has not been coupled to renal function and disease, and can therefore be used as a new source of kidney glomerular-specific genes and biomarkers.

By studying gene expression in rats with nephrotic syndrome and in patients with renal disease we found that expression of a special family of extracellular matrix proteins, called proteoglycans, was changed in renal disease compared to healthy controls. Proteoglycans are multifunctional proteins with functions ranging from holding and releasing signal molecules to making up part of the extracellular matrix structure. In patients with IgA nephropathy we found that the proteoglycan perlecan had an increased gene expression compared to control, and that the gene expression correlated to the excretion of protein in the urine and even to the progress rate of the disease. This suggests that perlecan could likely be used as a molecular marker for IgA nephropathy and as such help us to further understand the progression of the disease. In addition we have developed a unique method of culturing cells from patients with renal disease and we believe that this will give us new information about the molecular mechanism of this disorder and help us develop more specific and individualized treatment for patients with kidney failure.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Podocyte proteoglycan synthesis is involved in the development of nephrotic syndrome
Björnson Granqvist A, Ebefors K, Saleem MA, Mathieson PW, Haraldsson B, Nyström JS
Am J Physiol Renal Physiol. 2006 Oct;291(4):F722-30

II. Role of glomerular proteoglycans in IgA nephropathy
Ebefors K, Granqvist A, Ingelsten M, Mölne J, Haraldsson B and Nyström J
PLoS ONE. 2011 6(4):e18575

III. Comparison of human and mouse glomerular transcriptomes by Affymetrix gene array analysis
Sun Y, Ebefors K, Haraldsson B, Katayama M, Lal M, Patrakka J, Pikkarainen T, He L, Tryggvason K, Nyström J and Betsholtz C
manuscript

IV. Mesangial cell matrix production in IgA nephropathy
Ebefors K, Sun Y, Elvin J, Levan K, Fridén V, Tryggvason K, Betsholtz C, Haraldsson B and Nyström J
manuscript

ABBREVIATIONS

ARHGAP28	Rho GTPase activating protein 2
BMP	bone morphogenetic protein
cDNA	complementary DNA
CH4ST	chondroitin4-O-sulfotransferase
CLIC3	chloride intracellular channel 3
COL1A1	collagen, type I, alpha 1
COL4A1, -3, -4, -5	collagen, type IV, alpha 1, 3-5
CS	chondroitin sulfate
DNA	deoxyribonucleic acid
DS	dermatan sulfate
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
EXT1	exostosin 1
FDR	false discovery rate
FGF	fibroblast growth factor
GAG	glycosaminoglycan
GBM	glomerular basement membrane
GFR	glomerular filtration rate
GO	gene ontology
HA	hyaluronic acid
HAA	garden snail (helix aspersa) lectin
HECW2	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2
HPA	human protein atlas
HS	heparan sulfate
HS3ST1	heparan sulfate (glucosamine) 3-O-sulfotransferase 1
IF	interstitial fibrosis
IgA	immunoglobulin A
IgAN	IgA nephropathy
IgG	immunoglobulin G
KEGG	Kyoto encyclopedia of genes and genomes
KS	keratan sulfate
LAMB2	laminin, beta 2
LDA	low density array
MRGPRF	MAS-related GPR, member F
NDST1	N-deacetylase/N-sulfotransferase 1
PAM	partitioning around medoids
PAN	puromycin aminonucleoside
PDGF	platelet derived growth factor
PG	proteoglycan

PLEC1	phospholipase C, epsilon 1
RNA	ribonucleic acid
SAGE	serial analysis of gene expression
SLRPs	small leucine-rich proteoglycans
SVM	support vector machine
TA	tubular atrophy
TGF-β	transforming growth factor beta
Q-PCR	quantitative polymerase chain reaction
uIgA	undergalactosylated immunoglobulin A

CONTENTS

INTRODUCTION

RENAL DISEASE

In December of 2009, 8205 patients in Sweden were under active uremic care. Of these, 4606 had functional transplants while the rest were in dialysis and the numbers are growing[1]. Patients entering active uremic care have only 5-10% of their renal function left and would die without treatment with dialysis or transplantation. Dialysis is a life-saving but time-consuming and life-long treatment. Still, a normal dialysis regime only corresponds to 5-15% of the normal renal function[2]. Renal transplantation, on the other hand, can significantly restore the majority of the many functions performed by the kidneys. When looking at five-year survival, comparing chronic dialysis and transplantation with living or deceased donor, the superior treatment is living-donor transplantation[3]. Renal transplantation is also better than dialysis from an economical viewpoint; the cost of a renal transplantation and short-term follow up is comparable to the cost of six months of dialysis[4]. Although transplantation is an excellent way of regaining renal function, it should be remembered that not all patients in end stage renal failure are eligible for a renal transplant and the waiting lists are long due to shortage of available kidneys. The 1st of April 2011 there were 603 patients on the waiting list for a new kidney while only 370 patients received a new kidney (168 from living donors) in Sweden[5] during 2010.

There are few treatment options for renal disease and this often leaves corticosteroid treatment or chemotherapy as the only available choices. Sometimes the disease goes into remission, but it may also continue to progress into end stage renal disease. At that point dialysis or transplantation are the only alternatives for survival. With more people entering uremic care and the costs escalating it is of great importance to find an early treatment and/or cure of renal disease before patients require active uremic care.

There are many factors that can trigger loss of renal function, including diabetes, chronic heart disease, poisoning, inflammation, infections or drug-induced nephritis. The main cause of uremic care in Sweden is glomerulonephritis, but for new patients entering uremic care today the most common diagnosis is diabetic nephropathy[1]. Although glomerular disease is the leading cause of end stage renal disease, the molecular mechanisms behind most of these diseases are unknown.

The glomerulus is the structure in the kidney where the ultrafiltration of the blood takes place, and damage to any of the glomerular structures often lead to proteinuria. To ensure that patients get a correct diagnosis of their glomerular disease a renal biopsy is required. By investigating morphological changes and staining for disease-specific antibodies most glomerular diseases can be diagnosed but this gives no information about the progression of the disease. To increase the

understanding of the underlying mechanisms and progression of glomerular disease more research on the molecular level is necessary. Hopefully in the near future molecular diagnostics can help us to individualize treatment and reduce the number of patients in need for active uremic care.

THE KIDNEY

The kidneys are highly specialized organs that regulate the volume and composition of the body fluids. Every day 150-180 L of fluids are filtered through the kidneys. There are about one million nephrons in each kidney and the filtration of the blood takes place in the glomerulus, a capillary network enclosed by the Bowmans capsule in the most proximal part of the tubular system. The primary urine formed in the glomerulus is then extensively modified on its way through the tubular system, leaving 1-1.5 L of final urine for excretion.

The Glomerulus

The glomerulus contains three different cell types, fenestrated endothelial cells, specialized epithelial cells named podocytes, and mesangial cells (see figure 1). The filtration barrier is composed of different layers with the fenestrated endothelial cells and their cell surface layer closest to the blood stream. The basement membrane separates the endothelial cells from the podocytes. The podocytes are found on the outside of the glomerular capillaries and make up a zipper-like structure with their foot processes wrapped around the capillaries. Between adjacent foot processes there is a slit diaphragm making up the final part of the barrier to the primary urine[6]. The undamaged barrier filters the contents of the blood based on size, shape and charge, allowing water and small molecules to pass over the barrier but albumin and other large molecules are almost completely retained in the capillary lumen. Regardless which one of the layers that are damaged in renal disease the outcome is proteinuria[7]. The mesangial cells are not a part of the filtration barrier but have structural properties in the glomeruli, as they are situated between the glomerular capillaries. All the cell types in the glomeruli interact with each other and alterations in one cell type can lead to changes in the others. For example, vascular endothelial growth factor is produced by the podocytes and of vital importance for endothelial cell morphology and function[8].

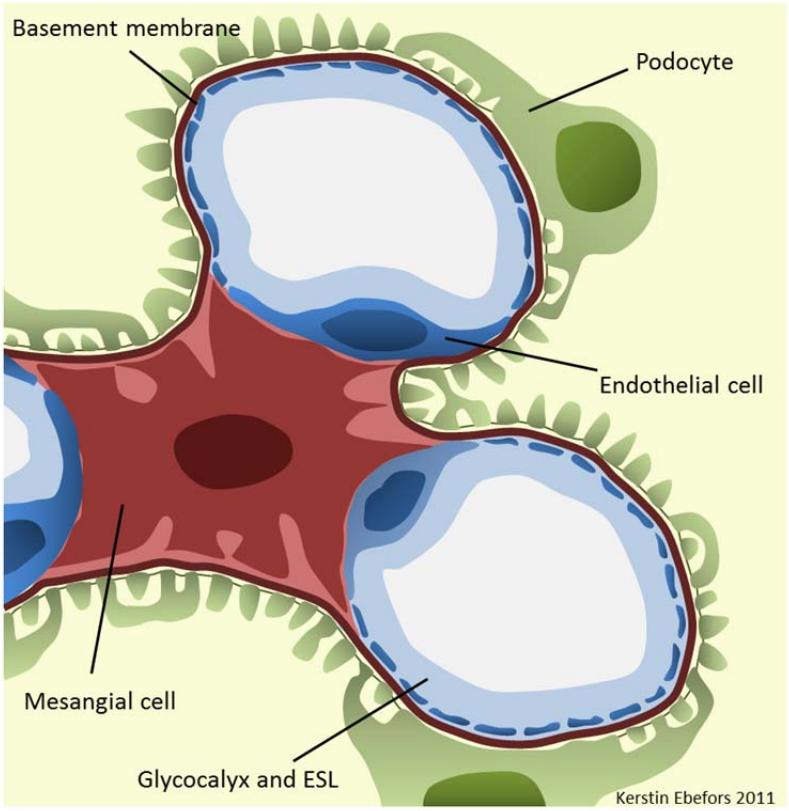

Fig 1. The cells and structure of the glomerulus. Closest to the blood is the endothelial cell surface layer (ESL) and the glycocalyx covering the fenestrated endothelial cells. The podocytes are covering the outside of the glomerular capillaries with their foot processes attached to the basement membrane. The mesangial cells are found in-between the capillaries, surrounded by mesangial matrix.

Endothelial cells

The endothelial cells in the glomeruli are heavily fenestrated and covered with a thick negatively charged cell surface layer. This layer can be divided into the glycocalyx and endothelial cell coat with the latter more loosely attached to the luminal surface of the glycocalyx[6]. This cell coat is suggested to consist of proteoglycans (PGs) and glycoproteins. It covers the surface of most cells, including the fenestrations of the endothelial cells in the glomeruli[9]. Disruption of the components of the endothelial cell surface layer leads to proteinuria[10]. Experiments on cultivated primary glomerular endothelial cells have shown that they produce a diversity of proteoglycans. Also, treatment of the cells with puromycin aminonucleoside (used for induction of nephrotic syndrome and ensuing proteinuria) decreases the overall negative charge from PGs, indicating that they are important for the function of the filtration barrier[11]. Studies in mice with adriamycin-induced nephropathy showed a decreased thickness of the endothelial cell surface layer in nephrotic mice compared to control[12]. Several diseases cause direct or indirect injury to the endothelial cells, for example diabetic nephropathy, obesity and preeclampsia/eclampsia[13].

Basement membrane

Compared to other basement membranes in the body the glomerular basement membrane is unusually thick, probably due to the fusion of basement membranes from the endothelial cells and the podocytes during the development of the nephron[14]. The basement membrane is composed mainly of collagen IV, laminin, nidogen, enactin and proteoglycans[15] and mutations in genes encoding four known proteins in the basement membrane (LAMB2, COL4A3, COL4A4 and COL4A5) cause glomerular disease[16]. The basement membrane molecules form a fibrous network with collagen IV as the backbone, and mutations in the collagen chains give rise to pathological conditions such as Alport's syndrome[17-19]. This is a renal disease characterized by an altered thickness of the basement membrane and with time this leads to chronic kidney disease. Mutations of the LAMB2 gene, coding for the laminin β2 chain, causes the Pierson syndrome in humans[20], and experiments show that mutant mice lacking LAMB2 develop nephrotic syndrome[21]. The basement membrane is rich in anionic charge, mainly from the large PGs agrin[22] and perlecan[23] and has been thought to be of importance for the charge-selective properties of the barrier. This has lately been under debate, since it has been demonstrated that genetically modified mice lacking the perlecan heparan sulfate side chains and podocyte-specific agrin do not develop proteinuria[24].

Podocytes

Podocytes are highly specialized epithelial cells surrounding the glomerular capillaries with their foot processes attached to the basement membrane via $\alpha_3\beta_1$ integrin[25], but PGs has also been proven to be important for podocyte attachment[26]. The actin cytoskeleton maintains the structure of the foot processes and interacts with the slit diaphragm bridging the gap between the foot processes. The slit diaphragms are extracellular structures that have functional pores for water and small solutes but are rather impermeable for larger molecules such as plasma proteins. The importance of the slit diaphragm was originally shown by the discovery that a mutation in the gene nephrin causes congenital nephrotic syndrome of the Finnish type[27]. Nephrin is specific to the podocyte slit diaphragm[28]. Since then other proteins, both intracellular and slit-situated, have been proven crucially important for the maintenance of the podocyte slit diaphragm[29-31]. In glomerular disease there are four major patterns of alterations of podocyte morphology; foot process effacement, apoptosis, arrested development and dedifferentiation[32]. Foot process effacement is found in minimal change nephrotic syndrome, IgA nephropathy (IgAN) with nephrotic range proteinuria[33] and focal segmental glomerulosclerosis[34]. In IgAN the foot process effacement is closely related to proteinuria[35] and podocyte loss can predict the progression of the proteinuria[36]. The depletion of podocytes in progressive glomerulosclerosis may be mediated by transforming growth factor β and smad 7, along with other signaling pathways[37]. Damage to the podocytes can also be mediated by cytokines derived

from mesangial cells exposed to IgA from patients with IgAN[38-43] once again pointing out the importance of communication between the different cells in the glomerulus.

Mesangial cells

The mesangial cells provide structural support for the glomerular capillary loops. They also have contractile properties making it possible for them to fine-tune the glomerular filtration rate of individual nephrons. Mesangial cells are in direct contact with the endothelial cells on the capillary lumen side without an intervening basement membrane, but separated from the podocytes[44]. The mesangial cells are embedded in their own mesangial matrix. It is composed of collagen IV, collagen V, laminin, fibronectin, enactin, nidogen and PGs, i.e. it differs from the composition of the basement membrane[44]. The matrix can serve as a source and target for growth factors and can be altered in disease, for example IgAN[45] and diabetic nephropathy[46]. Expansion of the mesangial matrix and proliferation of mesangial cells, such as that typically seen in IgAN, leads to reduced area available for filtration by affecting the glomerular capillaries. This leads to glomerulosclerosis. In IgAN one of the main findings is deposits in the mesangium of IgA-containing immune complexes. When treating mesangial cells in culture with IgA derived from patients with IgAN in an attempt to mimic the events in IgAN, the cells produced factors that affected podocytes as well as the cells in the tubules[38-43].

The Tubular System

The tubular system is made up of different segments, named from the Bowman's capsule surrounding the glomerulus; the proximal tubule, the loop of Henle, the distal tubule and the collecting duct. There are several renal diseases that concern the tubular system, mostly affecting the reabsorption or excretion of molecules, for example renal tubular acidosis, where the kidney fails to excrete acid into the urine making the blood acidic[47]. There is also acute tubular necrosis where the tubular cells die, either due to exposure to toxins or due to lack of oxygen[48]. Correlation between tubulointerstitial damage and renal function was found as early as 1968[49]. Tubulointerstitial injury may be caused directly by toxic, obstructive or ischemic mechanisms or be a consequence of glomerular damage[50]. In IgAN one often finds tubulointerstitial damage and it has been proposed that this is due to mediators released by the mesangial cells[51]. In a gene expression study by Reich et al, a gene set of 231 genes were found to be albumin-regulated in an in vitro model of tubular epithelial cells. This gene set could then be used to separate patients with IgAN from controls using the gene expression in the tubulointerstitial part of the renal biopsy. 11 of the 231 genes in the gene set correlated to the level of proteinuria and could be used to distinguish all forms of primary glomerulonephritis from controls[52].

THE EXTRACELLULAR MATRIX

The extracellular matrix (ECM) in the body have many different functions such as; support and anchoring of the cells, segregating tissues, regulating intracellular communication and sequestering a wide range of growth factors. The ECM is also important in growth, wound healing and fibrosis[53]. The matrix is secreted from cells and contains a mixture of fibrous proteins and glycosaminoglycans (GAGs), the most abundant protein being collagen. Other proteins often found in ECM include elastin, fibronectin, laminin and different PGs[54]. Collagens can form elongated fibrils and are important for the structure of the matrix, and together with elastin the collagens provide the body with strength and flexibility to help tissues withstand stretching. Collagen IV is found in basement membranes and is a network-forming collagen[54]. COL4A1 encodes its α-chain, and mutations in COL4A1 are known to cause a wide range of abnormalities affecting mainly the brain and the retinal vasculature, the ocular structures and the glomerulus[55]. Laminins are cell adhesion molecules with 18 known isoforms, predominantly found in basement membranes. They are heterotrimers consisting of one α, one β and a γ-chain. Laminin binds to several other matrix proteins as dystroglycan and many members of the PG family (perlecan, syndecans and agrin)[56]. Several renal diseases include changes in the ECM in their phenotype, as diabetic nephropathy and IgAN.

PROTEOGLYCANS

PGs are a family of cell surface proteins that can either be attached to the cell membrane or be secreted (see figure 2). PGs are constructed of a core protein with one or more GAG chains attached. The GAGs are negatively charged and there are five different types of GAG chains, heparan sulfate (HS), dermatan sulfate (DS), chondroitin sulfate (CS), keratan sulfate (KS) and hylaronic acid (HA)[57,58]. HA differs from the other GAGs by lacking attachment to a core protein[59]. PGs are complex molecules, and their properties are determined by their core protein as well as by the GAG chains. Their function ranges from structural roles in the extracellular matrix to involvement in cell signaling, by acting as binding sites, controlling growth factor gradients, or as signaling molecules[60,61]. PGs are found on all levels of the filtration barrier and in the mesangial matrix. They have been suggested to be of importance both for the development of the nephrotic syndrome and normal function of the glomerular filtration barrier[11,12,62-65]. Below follows a further description of the PGs studied in this thesis.

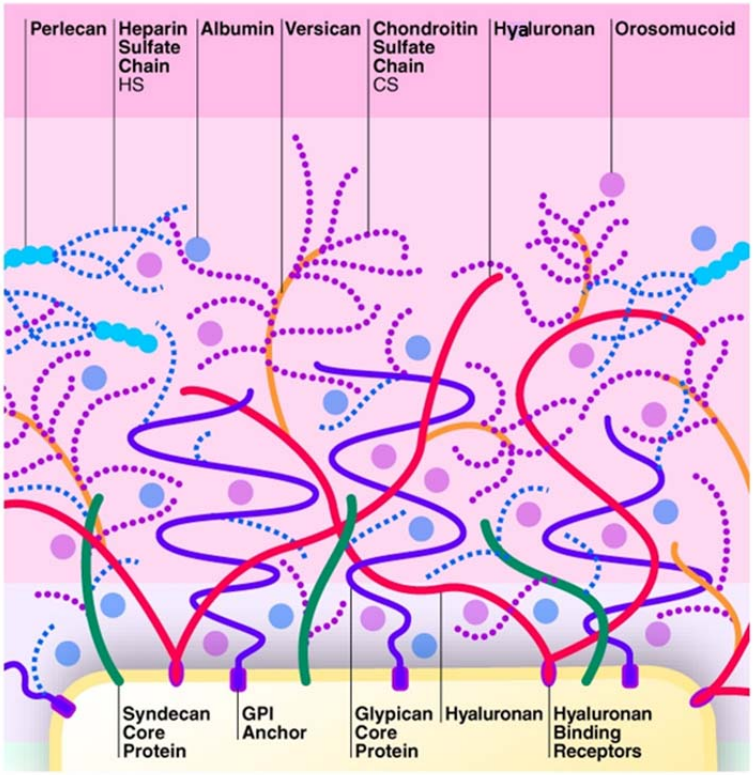

Figure 2. Proteoglycans are found attached to the cell membrane or secreted in the extracellular matrix (ECM). Figure modified from Haraldsson et al.[6]

Cell membrane bound proteoglycans

Syndecans are a family with four members, syndecan -1, -2, -3 and -4 and are type I transmembrane PGs. Most cells express one or more syndecans which is also true for the kidney[11,62,65]. The most commonly expressed is syndecan-4, whereas syndecan-1 primarily is found on epithelial cells, and syndecan-2 on cells of mesenchymal origin while syndecan-3 is expressed in neural tissue. All syndecans have HS GAG chains, but some can have additional CS GAGs. Since syndecans are transmembrane they are able to transduce signals from the extracellular matrix to the inside of the cell. They can bind a wide variety of ligands and are suggested to have roles in cell matrix interactions and matrix assembly. All syndecans are also able to interact with actin-associated proteins[66]. Syndecan-4 is known to interact with the cytoskeleton via alpha-actinin[67]. Syndecan-1 deficiency aggravates anti-glomerular basement nephritis by shifting the Th1/Th2 balance towards Th2 response[68].

Glypicans are a family with six members (glypican-1 to -6). They bind to the plasma membrane via a glycosyl-phosphatidylinositol anchor and they have three HS GAG chains. In vivo evidence indicate that the main function of glypicans is to regulate the signaling of wnts, hedgehogs, fibroblast growth factors and bone morphogenetic proteins[69].

Secreted proteoglycans

Perlecan is a large PG which is ubiquitously expressed and found in basement membranes and extracellular matrixes. It can carry four GAG chains, most commonly HS but can also be substituted with CS. Perlecan mediates cell signaling events, controlling cell migration, -proliferation and -differentiation. It can bind growth factors to the HS chains as well as the core protein. The growth factors binding to perlecan that are most investigated are those from the fibroblast growth factor (FGF) family. In addition, perlecan binds to various other matrix molecules, such as laminin-1, nidogen, fibronectin and collagen IV[57,70]. Perlecan has been suggested to play a role in diabetic nephropathy complications[71].

Versican is found in the extracellular matrix and belongs to a group of hyaluronan-bindings PGs. Versican carry only CS chains, and the largest isoform can carry as many as up to 23 GAGs[72]. Versican is up-regulated in smooth muscle cells treated with PDGF or TGF-β[57]. All cell types in the glomerulus have been shown to produce versican in vitro[11,65,73].

Biglycan, decorin and *lumican* all belong to the family of small leucine-rich PGs (SLRPs). Biglycan and decorin are members of class I SLRPs and have either DS or CS GAGs attached. Lumican with KS chains belong to class II[57]. All SLRPs are ECM organizers, interacting with the other molecules in the matrix. Decorin have antiproliferative effects, first demonstrated by its ability to bind and block transforming growth factor beta (TGF-β)[74] and is involved in diabetic nephropathy[75-77]. Biglycan and lumican can also bind TGF-β[78,79] and in addition biglycan can act as a danger signal by being released from the matrix as a response to tissue stress or injury[61,80]. Lumican interacts with the collagen fibrils and control their assembly, but has functions also in cell proliferation, migration and adhesion[81].

NEPHROTIC SYNDROME

Nephrotic syndrome is defined by nephrotic range proteinuria, hypoalbuminemia (<3.0 g/dL) and peripheral edema. Proteinuria of 3.5 g/24 hours or more is considered to be in the nephrotic range. Proteinuria can develop regardless which structure of the barrier is damaged[7]. Nephrotic syndrome can be caused by primary renal disease, such as membranous nephropathy and minimal change nephropathy, or by secondary causes such as diabetes mellitus[82]. Proteinuria in the nephrotic range can also occur in other renal diseases, for instance IgAN, but patients with IgAN might just as well have mild or no proteinuria[83]. Nephrotic syndrome is treated by trying to lower the intraglomerular pressure using an angiotensin converting enzyme inhibitor or angiotensin II receptor blocker, and by reducing the edema with dietary sodium restrictions and loop diuretics. Corticosteroids are commonly used to treat some of the diseases causing the nephrotic syndrome, such as membranous nephropathy and minimal change disease, but this does not always

lead to any changes in disease state[82]. Some breakthroughs have been achieved in this field during the last years, for example in idiopathic membranous nephropathy where the M-type phospholipase A2 receptor has been found to be a target antigen. This may solve the question about the initiation of the molecular mechanisms causing the disease[84] and improves the possibility to find a more specific treatment for the disease.

IGA NEPHROPATHY

IgAN is defined by the deposition of IgA in the mesangium of the glomerulus leading to matrix expansion and mesangial proliferation, see figure 3. To be able to make a diagnosis, a pathological examination of a renal biopsy is necessary[85]. IgA deposits are found also in Henoch-Schönlein purpura, and the immunofluorescence findings of IgA in biopsies can be indistinguishable between the two diseases[86]. The deposited IgA in IgAN is predominantly J-chain containing polymeric IgA1 and accompanied by C3 and immunoglobulin G (IgG). This particular type of IgA1, aggregated in the glomerulus, has been investigated thoroughly the last years and has been found to be under-galactosylated with reduced galactose and/or sialic acid content, leading to increased exposure of the internal *N*-acetylgalactosamine[87-90]. This under-galactosylated IgA1 (uIgA) tends to self-aggregate and form antigen-antibody complexes with IgG antibodies. IgGs are directed against N-acetylgalactosamine in the IgA1 hinge region and give rise to macromolecular depositions in the mesangium[91,92]. Additional evidence of impaired clearance of IgA and IgA-complexes in IgAN suggests that the hepatic clearance is reduced in patients with IgAN[93]. Interestingly, mesangial deposition of IgA does not always lead to IgAN; in cases of the familiar form of IgAN there are relatives with uIgA in the circulation that do not develop the disease[94,95]. The deposition of IgA seems to be a reversible process. There are cases where the kidney donor has had subclinical IgAN and when engrafted into a patient with chronic kidney disease stage V due to a different renal disorder the immune deposits have been cleared from the allograft within weeks[96]. Another important thing to note is that a Finnish investigation of kidneys from deceased due to suicide or violent death, found IgA depositions in several asymptomatic cases, and so did a Japanese study of healthy kidney donors[97,98] This indicates that the deposition of uIgA is complicated and requires further investigation.

IgAN is considered a mild renal disease, but with time most patients will develop end stage renal disease. Since the population is getting older worldwide, more patients are likely to reach end stage renal disease in the future. Treatment of IgAN is based on the risk for progression and includes blood pressure-controlling drugs and glucocorticoids with or without other immunosuppressive agents to treat the underlying inflammatory disease. If patients proceed to end stage renal disease and receive a transplant there is a risk that the disease develops again in the graft. Histologic recurrence, with or without evidence of clinical disease, is observed in a

large portion of cases[99,100]. Unfortunately there are no prospective studies of IgAN recurrence in the grafts, other than a study of graft loss due to recurrent glomerulonephritis, which showed a number around 10%[101].

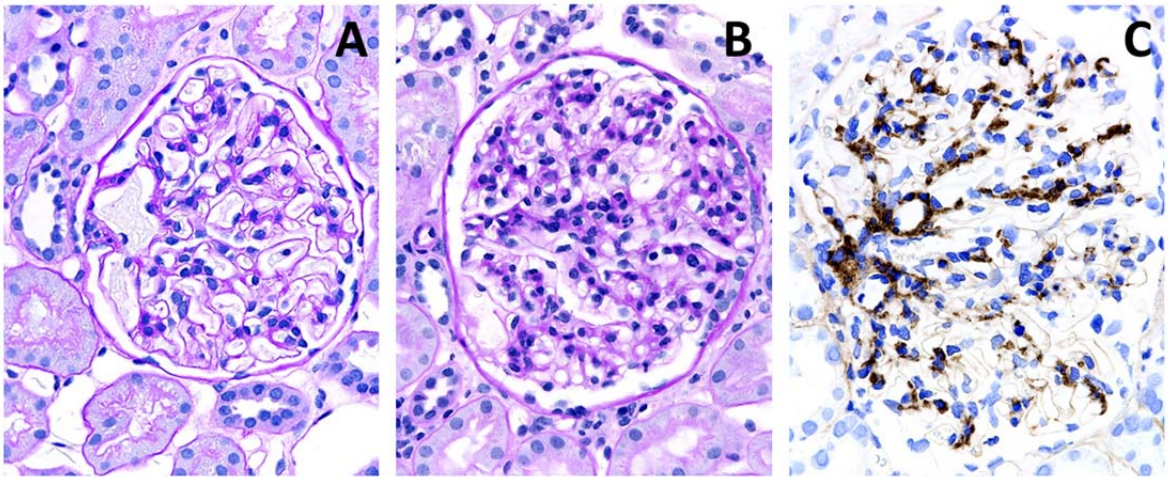

Figure 3. Renal biopsy section showing A) normal glomerulus B) glomerulus with mesangial proliferation C) glomeruli from patient with IgAN stained for IgA deposits (brown).

TRANSCRIPTIONAL PROFILING IN THE KIDNEY

The DNA in the cells is transcribed when needed into a variety of RNA molecules. The sum of all the RNAs transcripts is called the transcriptome, and in comparison to the DNA the transcriptome is always changing and varies between cells and tissues. We believe that investigation of the transcriptome from normal and diseased kidneys will help decipher molecular mechanisms leading to renal disease, and to find molecular markers for disease and progression[102]. When investigating the transcriptome there is concern about significant gene expression alterations before tissue procurement. To avoid this, standardized protocols have been established to process renal biopsies[102,103]. It can also be necessary to microdissect the tissue to investigate the true gene expression in different parts of the nephron. There are a few different approaches such as manual microdissection under a stereomicroscope or laser-capture microdissection. Manual microdissection has the advantage of the possibility to obtain whole glomeruli, and when working with a full renal biopsy 5-20 glomeruli[62] may be retrieved. Laser capture makes it possible to use material already processed for routine diagnostic purpose. This method gives a much smaller RNA yield[103] but makes it possible to choose defined pathologic lesions for expression analysis. To explore the full transcriptome in cells or tissue the microarray is a useful tool. Microarray technology is based on hybridization properties of nucleic acid and uses complementary molecules attached to a solid surface to measure the quantity of specific nucleic acid transcripts of interest that are present in a sample. There are different arrays available; cDNA microarrays, oligonucleotide microarrays and SAGE, and the new upcoming method of deep sequencing[104].

AIMS

The general aim of this thesis was to unravel gene expression patterns involved in kidney function, both in healthy and pathological conditions. We also wanted to find molecular diagnostic markers for different glomerular diseases to be able to improve and individualize treatment of the patients.

The specific aims were to:

Define the role of proteoglycans in a model of nephrotic syndrome and in podocytes.

Expand our knowledge about the role of proteoglycans and proteoglycan-associated genes in IgA nephropathy and find possible molecular markers for IgA nephropathy.

Identify kidney glomerulus-enriched genes in healthy kidneys from humans and mice and compare the two species.

Understand the changes in matrix composition caused by IgA nephropathy.

METHODOLOGICAL CONSIDERATIONS

Detailed descriptions of materials and methods are given in each paper, and only those of particular importance for the results are described below.

ETHICS

All experiments performed in this thesis were approved by the ethical board of west Sweden, except for the mice used in paper III, that were approved by the ethical board of Stockholm north. The following ethical permits concern the human samples used in paper II, III and IV; 414-09, 635-05, R110-98, S552-02, 432-09. All participating patients signed a written informed consent. The ethical permit for the rats treated with puromycin aminonucleoside in paper I is numbered 395-04. The ethical permits concerning the mice used in paper II are numbered N28-06 and N279-04.

ANIMAL STUDIES

It is difficult to study the changes in gene and protein expression in humans when it comes to glomerular disease, especially over time. However, many different rodent models for glomerular disease are available. For example; passive Heymann nefritis, a model for membranous nephropathy, adriamycin nephrosis and puromycin aminonucleoside (PAN) nephrosis (high dose), both models for focal segmental glomerulosclerosis, and PAN nephrosis (low dose) a model for minimal change nephropathy[105]. Focusing on the podocytes, we chose the PAN nephrosis model in the rat to study the changes in the filtration barrier when the kidneys start to leak proteins. This rat model is well-used for studies involving damage to the filtration barrier, with podocyte flattening, and leakage of proteins into the urine. In the study in paper I, the rats were allowed to acclimatize for at least one week before the experiments started. The animals had free access to food and water. PAN was administered in a single dose of 150 mg/kg by intraperitoneal injection. The experiment was performed as a time-point study for 7 days, starting on day one after PAN injection. In paper III we compared the gene expression in mice and humans; data from the mice was reported earlier in a separate study[106].

HUMAN SAMPLES

As valuable as animal studies may be, it is still of importance to study the molecular mechanisms behind glomerular disease in human samples. Patients with glomerular disease are often diagnosed by renal biopsies, and all the material obtained is usually used for diagnostic purposes by the pathologist, thus not leaving any material for molecular analysis. In 2004, we initiated a renal biopsy project at Sahlgrenska university hospital to enable molecular investigations of the biopsies. The project aimed to find molecular markers for different renal diseases and increase the understanding of the molecular mechanisms behind renal disease. The patients that were included in this project were undergoing renal biopsy in order to establish or

confirm the diagnosis of their renal disease. Prior to the biopsy, all patients had received oral information, and those wishing to participate in the study had signed a written consent form to be included. The biopsies were then diagnosed by a pathologist, and patients with IgAN were singled out and used in paper II and IV. The biopsies were put in RNAlater, an RNA-preserving liquid, at bedside and then refrigerated for 24 hours before freezer storage. Biopsies were collected from healthy kidney donors and from the healthy parts of kidneys that had been removed due to tumors. These biopsies were used as control samples and were treated according to the same protocol as the patient's samples. Clinical data from the time of the biopsy were collected and the patients were followed for up to 7 years, but no extra clinical tests were made on the behalf of the study.

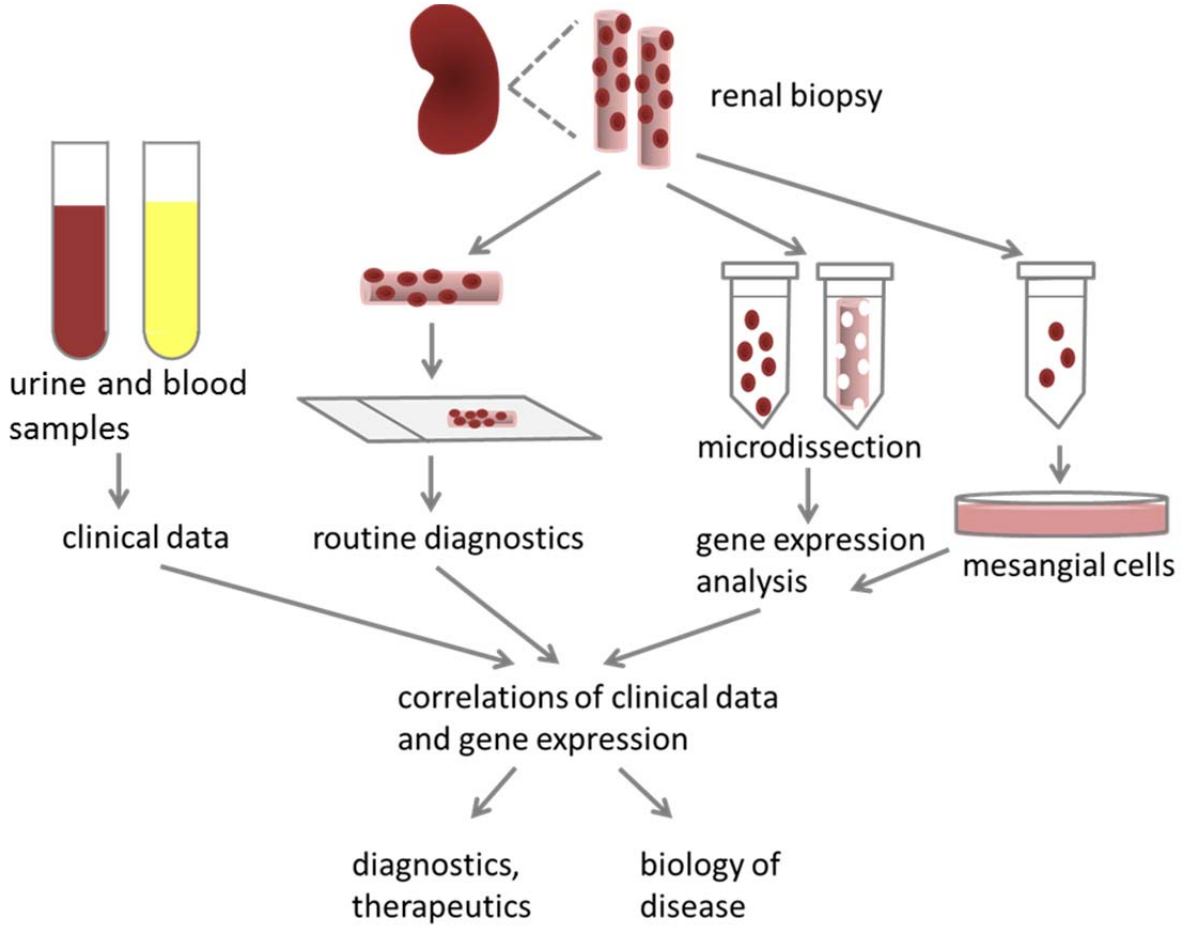

Figure 4. Flowchart of the different steps involved when working with the renal biopsies. Biopsies are taken from the kidney cortex and dipped in saline solution for extraction of loosely attached glomeruli, one biopsy is then transferred to RNAlater and frozen before microdissection and gene expression analysis. The glomeruli in saline solution are used to culture mesangial cells for in vitro studies. The other biopsy is used for microscopy and immuno-staining for diagnostic purposes. Blood and urine samples are taken from the patient for routine tests and other clinical data is recorded as well. All information is then put together to learn more about the molecular mechanisms of the disease.

CELL CULTURE

The glomerulus contains three different cell types; endothelial cells, podocytes and mesangial cells. These cells can all be cultured, but podocytes are difficult to get to differentiate in vitro. The endothelial cells and the podocytes are both a part of the filtration barrier. The mesangial cells are found in-between the capillaries in the glomeruli where they have structural functions. Cell culture is a usable tool that enables studies of the individual cell types and their reaction to different treatments and environmental changes. The primary disadvantage with cell culture is that the cells are removed from their natural surroundings, and it is impossible to co-culture the glomerular cells in an attempt to mimic the filtration barrier and the surrounding cells.

Podocyte culture

To enable culture of human podocytes we used a podocyte cell line (paper I) that was conditionally transformed using a temperature-sensitive mutant of SV-40 T antigen in collaboration with Saleem et al. These cells proliferate at 33°C and at 37°C the SV-40 T antigen is inactivated and the cells differentiate and start to express the same markers as podocytes express in vivo. Podocytes were used for experiments using PAN. PAN is a substance that is well-known for inducing nephrotic syndrome in the rat. In our study, cells were stimulated for 48 hours using 1 μM of PAN administered in the medium.

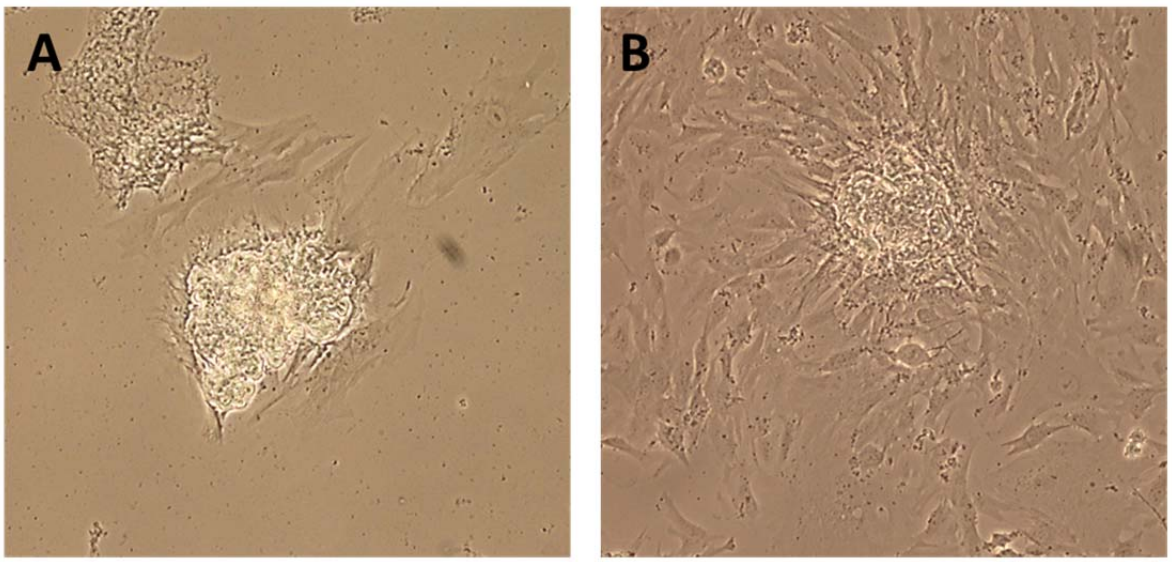

Figure 5. Mesangial cells cultured from a glomerulus. A) Mesangial cells start to spread out from the glomerulus after 15-20 days. B) After a few more days there is a rapid growth of mesangial cells round the glomerulus.

Culture of Healthy and Diseased Mesangial Cells

Culturing glomerular cells from human renal biopsies is a unique and new method for obtaining mesangial cells that makes it possible to culture renal cells from patients with different glomerular diseases. By dipping the renal biopsies in saline solution at bedside before putting them in fix or RNAlater we could collect loosely attached glomeruli that otherwise would have been lost in the fixative solution. The glomeruli that fall off in the saline solution are then moved to cell culture plates coated with attachment factor and with medium substituted with human serum and antibiotics. It takes 15-20 days before mesangial cells start to emerge from the glomeruli (see figure 5). By sub-cloning the cells that emerge we obtained pure cultures of mesangial cells that were characterized by morphology and protein expression of smooth muscle actin. In the experiments performed in paper IV we used mesangial cells cultured from a biopsy from a patient with IgAN and as normal cells primary mesangial cells from Lonza (Lonza, Basel, Switzerland).

GENE EXPRESSION ANALYSIS

One way to look at the molecular mechanisms that take place in the different tissues and cells is to study the gene expression. This gives insight into which genes are switched on in the tissue and gives a lead to which biological functions and processes are turned on. The gene expression can be studied in different ways; one can either choose to look at a few genes using Q-PCR or to do a transcriptional profiling of the whole genome in a tissue or cells. Irrespective of which method one chooses it is of great importance to use RNA of excellent quality. RNA is very easily destroyed by RNases, enzymes that under normal conditions is used to degrade RNA in the cells. It is therefore necessary to work under RNase-free conditions to avoid degradation of the RNA in your samples. To ensure that all our RNA was of excellent quality and not degraded after isolation all samples were run on an Agilent 2100 Bioanalyzer (Agilent Technologies) before any further analysis to verify the quality.

Quantitative Polymerase Chain Reaction

Q-PCR is used to simultaneously amplify and quantify the target gene expression. This method is considered to give a more exact and correct measure of gene expression than transcriptional profiling. To be able to study as many genes as possible using Q-PCR we used Low Density Arrays (LDA) from Applied Biosystems. This method allows quantification even of the tiny amount of RNA obtained from micro-dissected glomeruli from biopsies. We used this method to study the gene expression in rats treated with PAN as well as biopsies from patients with IgAN and healthy controls. Different setups of custom-made cards were used in different studies. Even though Q-PCR is thought to give a good picture of the gene expression and can be used to understand the molecular mechanisms in the cell it is still necessary to confirm the results with protein expression analysis.

Transcriptional Profiling

Transcriptional profiling gives a molecular signature of the given tissue or cell type being studied and generates large amounts of data. This can be useful as a fingerprint of the cell type or tissue and can be used for classification or correlation of samples. The profile can be beneficial for a more accurate disease staging or individualized treatment. Furthermore, this type of analysis generates a map of the global transcriptome that can be used to increase the understanding of the molecular functions in the tissue or cells.

Using oligonucleotide microarrays we studied the gene expression both in glomeruli and in the tubulointerstitium from humans and mice. This was done in order to find similarities and differences between the species as well as to find glomerulus-enriched genes. The gene chip used for the human studies was the GeneChip® Human Genome U122 Plus 2.0 array, and for mice the Affymetrix Mouse Genome 430 2.0 array. First we compared the gene expression in microdissected biopsies from healthy kidney donors (n=15) and glomeruli from mice (n=4), paper III. There were 14 717 genes that were present on both the human and mouse arrays and these genes were used for further analyzed. To study the glomerular gene expression in patients with IgAN, we used microdissected glomeruli from patients with IgAN (n=13) and compared these to glomeruli from healthy controls (n=23, including the 15 controls used in paper III) using the human gene chip. We then investigated the differences in gene expression between the two groups, focusing on matrix-associated genes.

PROTEIN ANALYSIS

There are many different ways to study protein expression, from immunohistochemistry to mass spectometry. The most common techniques all include the use of anti-bodies.

Immunohistochemistry

Immunohistochemistry is an excellent method used for identifying the expression of a protein on tissue sections or cells using anti-bodies against that specific protein in a visual manner. Immunohistochemistry was performed to characterize the primary mesangial cells cultured from renal biopsies and to study the expression of different proteins in the glomeruli. Both paraffin-embedded and frozen tissue sections were used. To measure up- or down-regulation of the proteins investigated we used two different methods to measure the intensity in the pictures taken of glomeruli in the sections, for TGF-β we used a classification scale from 1-10 in a blinded fashion and for perlecan, the Biopix software (Biopix AB).

Western blot

This is one of the most powerful techniques for investigating the expression of a specific protein in tissue lysates and it gives the size and relative amount of the

protein. Western blot was used to compare the protein expression in different treatment groups in the PAN-treated rats and podocyte studies. To ensure that equal amounts of proteins are loaded on the gels the amount of β-actin was measured on each gel as an internal control.

RESULTS AND DISCUSSION

This thesis is based on four papers that examine the gene expression in the kidney. One paper is focused on gene expression in the healthy kidney of mouse and man. The other three are focused on the expression of extracellular matrix components in glomerular disease.

Study I firstly describes changes in the production of proteoglycans in rats treated with PAN, a model of nephrotic syndrome, and secondly the in vitro production of PGs of podocytes treated with PAN. In the second study we investigated the expression of PGs in microdissected biopsies, separating glomeruli from the tubulointerstitium, from patients with IgAN compared to healthy control subjects. In paper III we compared the glomerular and tubulointerstitial global gene expression in mice and humans in order to identify glomerular-enriched genes and, in addition, to find similarities and differences between mice and humans. This can help us find genes of interest for glomerular function in health and disease. In paper IV we compared the gene expression in glomeruli and tubulointerstitium in renal biopsies, both from healthy controls and patients with IgAN, with focus on molecular changes in the ECM in IgAN. In addition, a novel method to culture mesangial cells from patient biopsies is described. Gene expression data from these experiments are reported in paper IV.

Working with gene expression in the kidney is a challenge since the kidneys are composed of many different structures with different tasks and therefore expressing different sets of genes and proteins. Glomeruli are known to make up about 5% of a human renal biopsy while the rest is made up of tubular structures and blood vessels (in this discussion called the tubulointerstitial compartment). Having these numbers in mind it is of great importance to investigate the gene expression in the glomeruli separately from the expression in the tubulointerstitial compartment in order to explore the specific gene expression of the glomeruli. In the first paper rat glomeruli was extracted from the kidney by a method of gradual sieving. In paper II, III and IV, dealing with human renal biopsies, the glomeruli were microdissected by hand from the tubulointerstitial compartment using a stereomicroscope and tweezers. Each biopsy contained 5-20 glomeruli, giving only a small amount of material to work with. In paper III glomeruli from mice were obtained using an extraction technique with magnetic Dynabeads[106]. All these methods allow near-quantitative isolation of the glomeruli present in the tissue with minimal contamination of non-glomerular cells. Results from paper II highlight the importance of tissue separation since the gene expression of perlecan was up-regulated in the glomeruli, and down-regulated in the tubulointerstitial compartment. The glomerular effect would have been completely masked by the opposite change in the much larger tubulointerstitial compartment without microdissection.

THE HEALTHY KIDNEY

To understand the molecular mechanisms operating in the diseased kidney it is important to investigate the transcriptome of the healthy kidney. Even if our understanding of the structure and mechanisms involved in ultrafiltration and reabsorption in healthy kidneys has increased dramatically there are still many question-marks left. There have been previous reports on mouse and human glomerular transcriptomes[107-114] but none of these have compared human and mouse glomerular expression. This comparison is of vital importance since many mouse models are used to investigate glomerular disease and conclusions are being drawn from these studies and extrapolated to the human situation. By microdissecting or using other methods to separate the glomerular structures from the tubulointerstitial compartment we could investigate the glomerular transcriptome separately from the tubulointerstitial one in mice and humans and then compare expression between the two species.

The transcriptome data from mouse was obtained in a previous study[115] and has been used here for comparison with the human expression. To investigate the transcriptome of humans we microdissected renal biopsies from healthy living donors into glomerular (n=15) and tubulointerstitial compartments (n=6). Gene expression profiles in the respective compartment were then obtained using Affymetrix Human Genome U133 plus 2.0 array. The raw data was first normalized and the expression signals for each probe set and RNA sample was obtained. Clustering of results by hierarchical clustering methods showed that glomerular and tubulointerstitial samples clustered into two separated groups. This demonstrates that the microdissection of the tissues is valid and that the small amount of sample used (5-20 glomeruli) is sufficient. Furthermore, the glomerular and tubulointerstitial compartments display significantly different gene expression patterns, and the data obtained from different human subjects have good concordance. It is known that small parts of tubular segments may accompany the glomerulus (where the efferent and afferent arteriole attaches to the glomerulus and the distal tubule connects in the Macula densa region). However, our data show that known tubular genes are expressed at a minimum in our glomerular preparations.

The obtained gene expression profiles from human glomerular and tubulointerstitial compartments were compared to the previously obtained data from mice. The probe set presented on the Affymetrix Mouse Genome 430 2.0 array were mapped to 21239 Entrez mouse genes using the NCBI HomoloGene database. These genes were further mapped to 15218 human orthologs. The probe set from the human array was mapped to 20231 Entrez genes and 14717 of these overlapped with the human orthologs from the mouse arrays. This gene set of commonly expressed genes was then used for further analysis.

By setting all possible combinations of fold-changes (for humans 2^0-2^7, for mice 2^0-2^8) of the gene expression between glomeruli and the tubulointerstitial compartment we could find glomerular overexpressed genes. This gave us two top lists of highly expressed genes in glomeruli from both human and mouse. Five of the top 10 glomerulus overexpressed genes in mouse were found among the 1017 human glomeruli overexpressed genes found within the 2^2 fold cut-off for the human experiments. Eight of the top 21 genes in humans appeared in the top 1489 mouse glomeruli overexpressed genes found within the 2^2 fold cutoff. The differences in glomerular overexpressed genes in the two species indicate surprisingly large differences between the most glomerular-specific genes in human and mouse. This could be partly explained by the fact that the genetic variation is larger within the human samples than the inbred mice. The genes that were strongly and significantly glomerulus-enriched in just one of the species need further investigation in order to understand the underlying causes for these differences. One example is CLIC3 (chloride intracellular channel 3) that was glomerulus-enriched in the mouse transcriptome but not in the human counterpart. In mouse CLIC3 has been shown to be highly expressed in the podocytes[108], but in human kidney only in lower levels, as shown by northern blot[116]. We as well as others studying the human renal transcriptome[107,110,112], have failed to show CLIC3 human glomerular overexpression. This demonstrates that there are true differences between the species, something that one has to have in mind when performing animal studies of human diseases.

Clustering is one of the most useful ways of discovering groups and identifying interesting patterns for microarray data based on similarities or differences. So to find out more about the glomerulus-enriched genes the fold change cut-off was set to 2^0 for both the human and mouse data. This generated a list of 3119 genes that overlapped between the species and represents approximately 60% of the genes that were more than one-fold up-regulated in either human or mouse. In this list we found 58 known glomerular genes expressed in either of the glomerular cell types[108,111,117]. These known glomerular genes were then divided into six clusters using the unsupervised PAM clustering method. Using these 58 genes as a training set, the rest of the genes from the 3119 gene list were classified into the different clusters, using the supervised SVM algorithm.

Table 1. Number of genes assigned to each cluster.

Cluster NO.	1	2	3	4	5	6
number of genes in cluster	425	251	1936	265	160	24

Cluster NO.6 contains the most highly glomerular overexpressed genes, and in this cluster well-known podocyte-specific genes are found, which are associated to or can cause renal disease in humans. Examples are NPHS1 (nephrin) a gene mutated in the congenital nephrotic syndrome of the Finnish type[27] and PLEC1 (also

known as NPHS3) that has been reported to be associated to early onset nephrotic syndrome[118,119]. Genes that have not previously been associated with glomerular function were also found in cluster NO.6, for example HECW2, MRGPRF and ARHGAP28. We need to investigate these genes further to understand their role in the glomerulus. For example HECW2 plays an important role in ubiquitination and is involved in male infertility[120]. By applying gene ontology (GO) classification into different biological groups to the clusters we obtained further information on the functional properties of the genes in the clusters. Cluster NO. 6 gathered the highest proportion of glomerulus-specific genes and in line with that the GO biological process categories *kidney development* and *glomerulus development* were overrepresented in this cluster.

To validate the glomerulus-enriched genes we used the human protein atlas data base (HPA) and we mapped the 3119 overlapping genes of human and mouse glomerulus-enriched genes and found that 1410 gene products were represented (not all proteins are at this time point represented in the HPA) and of these 149 (approx. 10%) showed a stronger protein staining in the glomerulus than in the tubulointerstitial compartment.

One of the requirements for investigating the glomerular expression in diseased kidneys is that we are familiar with the normal transcriptome in the healthy kidney. We have revealed that there are similarities as well as large differences in the transcriptome between the glomerular samples from healthy individuals from both human and mouse. The glomerular and tubulointerstitial parts of the biopsies from humans clustered separately and proved to us that the microdissection of the tissues is valid and that this material can be used as healthy controls in other studies.

THE DISEASED KIDNEY

PGs are secreted or cell membrane-bound molecules found at all levels of the glomerular barrier and also in the mesangial matrix. PGs have been shown to be involved in glomerular disease and to be important for the charge selectivity and morphology of the glomerular barrier[11,12,62-65]. We wanted to further investigate the role of proteoglycans in the diseased kidney glomeruli. We therefore treated rats with puromycin aminonucleoside (PAN). The rats then developed a nephrotic syndrome that resembles minimal change and focal segmental glomerulosclerosis in humans, and we studied the changes in gene expression compared to healthy controls (paper I). In addition we investigated the effects of PAN on podocytes in vitro. In paper II and IV, we worked with human material in form of renal biopsies from patients with IgAN. This gave us the opportunity to investigate glomerular gene expression of PGs and other ECM molecules in patients and compare it to that in healthy controls.

Nephrotic syndrome

Nephrotic syndrome is characterized by proteinuria and can develop regardless of which part of the glomerular filtration barrier is damaged[6]. In the PAN-induced model of nephrotic syndrome in rats one of the hallmarks of the disease is podocyte flattening and marked proteinuria, with onset 4-6 days after PAN administration[105]. In concordance with this, our rats showed an increased fractional albumin clearance at day five while glomerular filtration rate started to decrease already at day four. In the control rats fractional clearance and GFR were unaffected.

We could see an altered mRNA expression in the glomeruli over time for several of the PGs analyzed and one of the PG-associated enzymes, NDST1, compared to healthy controls. The enzyme NDST1 had an unchanged gene expression for the first 3 days and then, at the same time as we could see changes in the GFR and fractional clearance of albumin, the gene expression of NDST1 decreased. NDST1 is an enzyme important for adding sulphate (and thereby negative charge) to the HS GAG chains[121]. There is a previous report of down-regulation of NDST1 in rat tissue and rat podocytes after PAN treatment[122]. In addition it is interesting to note that Syndecan-1 was the only PG to be increased by PAN treatment and this increase was seen day 5-7, the same time as the decrease in GFR and increase in fractional clearance. The other syndecan studied, syndecan-4, as well as the large PG versican, were down-regulated all seven days. Studying the protein expression of syndecan-1 and -4 confirmed the down-regulation of syndecan-4 (day 4-7), and an increase over time (day 5-7) for syndecan-1. Syndecan-4 is known to have direct effects on cell attachment, spreading and cytoskeletal organization[123,124].

It is interesting to note that some of the alterations seen in the glomerular gene expression in the PAN-treated rat, such as the down-regulation of perlecan and versican, occur before any physiological changes are detected. Since PGs are important for cell signaling, function and morphology these early changes in PG gene expression can be the initiating step for further changes in the extracellular matrix. Patients with nephrotic syndrome are usually treated with steroids, and we therefore treated one group of rats with both PAN and the steroid solumidrol. The physiological parameters, GFR and proteinuria, were not affected by the steroid treatment. On the gene expression level, treatment with solumidrol increased the effects of PAN rather than giving any beneficial effects. Only two PGs and one enzyme had a reversed effect on the gene expression back to normal state with the steroid treatment.

The in vitro study of the podocytes showed that the proteoglycans expressed include syndecan-1 and -4, versican, glypican, perlecan, decorin and biglycan. Treatment with PAN significantly decreased the expression of versican and perlecan as well as several of the enzymes involved in GAG biosynthesis, EXT1, HS3ST1 and CH4ST, compared to control. Down-regulation of perlecan and

versican was confirmed on the protein level using western blot and was in concordance with the results seen in the rats. Treatment with PAN and dexamethasone further decreased the expression of perlecan and two of the enzymes and in addition down-regulated the gene expression of syndecan-1. Protein analysis with western blot of the decorin expression in podocytes treated with both PAN and dexamethasone showed an increase in expression.

In addition we used metabolic co-labeling with [D-6-^{3}H]-glucose amine and sulfate-35 to measure the length and sulfate content of the GAG chains. The experiments showed that GAG chains of large molecules (>73 Å, ~50%), were shortened in comparison to control, implicating the large PGs perlecan or versican or both. For molecules size 55-73 Å there were a ~10 % decrease in radioactivity, for the smaller molecules increased amounts of radioactivity were incorporated and treatment with dexamethasone increased the inclusion of isotopes even further, implicating the small PG decorin, as seen on western blot. Our results confirm and extend previous data demonstrating a reduction of sulfate incorporation in cell cultures of podocytes treated with PAN[125,126]. A reduced concentration of anionic sites has been reported both in experimental[127] and human glomerular disease[128-131].

The changes in gene and protein expression of PGs and enzymes involved in the biosynthesis of GAGs in the glomeruli from PAN-treated rats and podocytes have both similarities and differences. The down-regulation of versican and the enzymes NDST1 and HS3ST are seen in both models. In contrast the gene expression of syndecan-1 is up-regulated in PAN-treated rats but down-regulated in PAN-treated podocytes. One must note, however, that the increase in syndecan-1 in the PAN-treated rats was seen first at day five while the podocytes were treated only for 48 hours. After stimulation with PAN for 2 days we could see a down-regulation of versican and perlecan both in rat glomeruli and podocytes. Both perlecan and versican are large PGs found in the extracellular matrix. It has earlier been shown that versican is expressed by glomerular endothelial cells and that its synthesis is sensitive to PAN treatment[11]. Perlecan binds cytokines as well as growth factors, both to its HS chains and core protein[57]. It has recently been shown that podocytes use HS chains to attach and migrate and that podocytes lacking HS chains (by knocking out EXT1) have diminished adhesion efficiency[26]. Perlecan is also known to mediate cell attachment for podocytes to the glomerular basement membrane via $\alpha_3\beta_1$-integrin and dystroglycan[132].

We have established that the PG and GAG enzyme synthesis is sensitive to PAN treatment, resulting in a decreased amount of PGs and negative charge. The similarities in the gene expression in the PAN-treated rats and podocytes suggest that some of the changes in gene expression of PGs and associated enzymes can be due to changes in podocyte morphology and function.

IgA nephropathy

Mesangial matrix expansion is a prominent feature of IgAN, and deposition of IgA-immune complexes in the mesangium is believed to be the onset of the disease in the kidney. The mesangial matrix contains various matrix molecules including PGs but it is not known how the glomerular expression of PGs is affected in IgAN and what impact this has on the development and progress of the disease.

To investigate the role of glomerular PGs in IgAN we collected renal biopsies from patients with IgAN and from healthy controls. The renal biopsies were microdissected in order to investigate the glomerular and tubulointerstitial gene expression separately. Additionally, we continuously collected clinical data starting at the time of the biopsy and following the patients for up till 7 years after the biopsy (range 1.9-7.1 years). This made it possible to calculate the progress of the disease over time. All patients had a well maintained blood pressure during the follow-up period and there was no correlation between the progress of the disease and the mean arterial pressure, indicating that the changes seen in gene expression are not due to blood pressure.

Low density arrays gave us the opportunity to explore the gene expression of several genes within one Q-PCR experiment. This was a necessity because of the limited amount of RNA obtained from glomeruli. The expression levels of PGs and PG-associated genes were affected to a higher degree in the glomerular than in the tubulointerstitial compartment. The glomerular portion displayed an increased gene expression of the SLRPs biglycan, decorin and the SLRP-associated transforming growth factor beta (TGF-β). The up-regulation of TGF-β was confirmed on the protein level using paraffin-embedded kidney sections and comparing the protein staining of TGF-β in glomeruli from patients with IgAN and controls. Further we investigated the protein expression of decorin and found that there was almost no staining for decorin in the non-sclerotic glomeruli, but when the glomerulus or parts of the glomerulus were sclerotic there was an abundant expression of decorin in the sclerotized part. TGF-β is known to increase the synthesis of small extracellular CS/DS PGs[133], and both decorin and biglycan can bind TGF-β[78] and decorin acts as a natural inhibitor of TGF-β[134]. TGF-β is involved in tissue remodeling after injury and is found in three isoforms, and in humans the most common isoform is TGF-β1. TGF-β is secreted from the cells in a large latent complex and stored in the ECM. Cleavage of this larger precursor molecule is needed for activation. Excessive or prolonged production of TGF-β is the key mediator to fibrosis[135]. The increase in decorin production in the glomerulus seen in the sclerotized parts may be an attempt of the glomerulus to stop the fibrotic process. In a model of anti-Thy-1-initiated glomerulonephritis, injections with decorin suppressed the TGF-β activity[136]. Decorin is also thought to protect against progression of diabetic kidney disease[77].

In the glomeruli from patients with IgAN there was also an up-regulation of the basement membrane PG perlecan and the enzyme NDST1. The increase in perlecan gene expression was confirmed on the protein level as well. Perlecan is produced by all cell types in the glomerulus[11,65,137]. In IgAN it has been shown that there is an increased expression of perlecan in the mesangial area but not in the basement membrane[138]. NDST1 is, as mentioned before, essential for adding sulphate and thereby negative charge to the GAG chains[121]. One PG was down-regulated, syndecan-1. In anti-glomerular basement nephritis deficiency of syndecan-1 aggravates the disease[68].

In the tubulointerstitial part there was an unchanged gene expression of all investigated genes except perlecan and vascular endothelial growth factor (VEGF). In comparison to the increased gene expression of perlecan in glomeruli, the expression in the tubulointerstitial compartment was down-regulated. To further confirm that the gene expression in the glomeruli and the tubulointerstitial compartment are independent of each other we plotted the glomerular and the tubular gene expression against each other and found, as expected, no correlation.

To increase the understanding of the molecular impact of the PGs on clinical manifestations of the disease, the albumin excretion in urine and the calculated progress of the disease was correlated to the gene expression in the tubulointerstitial compartment and the glomeruli. This analysis revealed that the glomerular perlecan expression correlated to both the albumin excretion in urine and progress of the disease (increased expression of perlecan gave a lower excretion and slower progress of the disease). These findings suggest that perlecan may have what is required for a molecular marker of IgAN, even if further investigations may be needed.

Another protein that correlates with the disease progression was nephrin. Nephrin is mutated in the congenital nephrotic syndrome of the Finnish type and is of key importance to the integrity of the slit diaphragm between the podocyte foot processes[27]. The fact that glomerular gene expression of nephrin is not significantly changed proves that even small changes in nephrin expression may be important for disease progression of IgAN. The nephrin effect might be due to podocyte loss or flattening that is sometimes seen in IgAN[33,35,36].

The patients with IgAN were further classified using the Oxford classification system[139,140]. This system was developed as a classification system to reliably predict the risk of disease progression. The classification divides the patients based on mesangial hypercellularity, endocapillary hypercellularity, segmental glomerulosclerosis and tubular atrophy/interstitial fibrosis (TA/IF). We correlated our clinical data and gene expression data from the glomerular and tubulointerstitial compartment to the results from the Oxford classification. In our patient group the TA/IF stood out as the most powerful parameter with tubular gene expression for

biglycan, decorin, perlecan, glypican-1, NDST1 and TGF-β all correlating to the TA/IF. When looking at the clinical data, GFR and albumin excretion in urine, but not the progression of the disease, correlated to the TA/IF scores. Patients with a higher TA/IF had reduced GFR and excreted more albumin into the urine. Correlations between tubulointerstitial damage and renal function were found as early as 1968[49], showing that not only changes seen in the glomeruli but also alterations in the tubular parts of the nephron are important for development of proteinuria. IgAN is a disease that starts in the glomeruli but with matrix expansion and sclerosis ultimately leads to tubular damage.

To expand and increase our understanding of the changes seen in the expression of glomerular PGs in IgAN we performed a global gene expression analysis, comparing glomerular gene expression of patients with IgAN (n=13) to healthy controls (n=23).

The analysis was made at two different time points, generating two sets, with the first analysis run with five samples from patients with IgAN and 20 controls, and the second analysis included eight IgAN samples and three controls. Raw data was converted to expression data using the GCRMA algorithm. Differently expressed genes were identified using significance analysis of microarray (SAM) and nearest shrunken centroid algorithm. Analyzing the first set generated a list of 102 unique genes that could be used to separate the IgAN samples from the controls. To see if the 102 unique genes could be used as biomarkers for classification of an unknown sample of IgAN or control we then applied the principle component analysis (PCA) to classify the two sets together. A correct classification was obtained for all IgAN and controls, indicating that these biomarkers can indeed be used to separate IgAN from controls. Whether this probe set can be used to separate IgAN from other diseases requires further investigation, but would be of great interest as it then could be used as a tool for giving patients a correct diagnosis without the need for pathological assessment.

We then analyzed the whole material of samples from 13 patients with IgAN and 23 controls. Genes that were differently expressed between the groups were filtered for low variation across all the samples, thus reducing the list. We searched in the gene list for genes associated with perlecan and other matrix molecules since we found in paper II that perlecan gene expression correlated with progression and proteinuria. Genes with a fold-change above 1 or under -1 with a false discovery rate (FDR) < 0.05 were considered interesting. The generated gene list was further subjected to pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG). Two lists were generated, one with significantly up-regulated pathways and one with significantly down-regulated pathways. The top two up-regulated pathways for IgAN were ECM-receptor interacting pathway and focal adhesion. The top down-regulated pathways are all involved in the metabolism, the top two being; metabolic pathways and glycine, serine and threonine metabolism.

Perlecan gene expression was increased in this study as well as in study II, and the fact that this protein plays an important role in the ECM-receptor interaction KEGG pathway, further strengthens the prospect of perlecan to be of importance in IgAN. Perlecan can bind and interact with many other ECM proteins, both with its core protein and its HS chains, for example; laminin-1, collagen type IV, fibronectin, platelet derived growth factor (PDGF), heparanase, bone morphogenetic protein (BMP) 2, nidogen, β1 integrin, α-dystroglycan and many others[70]. Other PGs were found in the generated gene list as lumican and syndecan-1. Lumican belongs to the family of SLRPs and had an increased expression compared to control samples. Lumican is important for collagen fibril synthesis and the increase in lumican expression could be coupled to the increased gene expression of collagens[81]. Syndecan-1 was down-regulated, giving the same result as in paper II.

To our surprise we could not detect an up-regulation above the cut of level of a fold change of 1 of TGF-β, a growth factor, which was shown in Paper II and by others[141] to be increased in IgAN (on both gene and protein expression level). One reason for not seeing the expected up-regulation of TGF-β in the global gene expression can be poor binding of the RNA for TGF-β to the TGF-β probe on the Affymetrix gene chip. Q-PCR as performed in paper II is a more robust way of investigating gene expression and often used to confirm global gene expression results.

Other ECM proteins with a changed gene expression in IgAN samples and of interest due to association with perlecan were; BMP 2, fibronectin, fibrillin 1, collagen I and collagen IV. We investigated the expression of BMPs in the generated gene list and found that BMP 2 and BMP 4 were up-regulated in glomeruli from IgAN patients. BMP 2 have been shown to play a protective role against cellular fibrosis by affecting the TGF-β receptor 1 in rat fibroblasts[142]. Furthermore, in lung fibroblasts BMP 4 reduced TGF-β-induced extracellular matrix protein production[143]. BMPs are part of the TGF-β superfamily and known to be important in the developing kidney with BMP 7 as the BMP of main importance[144]. In our study, we could establish a down-regulation of BMP-7 just above the cut of level of a fold change of -1. Fibronectin, as well as many of the collagens (I, III, IV, V, VI, XII, XIII, XV and XXI), was highly up-regulated in IgAN and TGF-β is known to stimulate the expression and incorporation into the ECM of both fibronectin and collagens in various cell types[145].

Mesangial cells are known to be the starting point for IgAN with the deposition of uIgA-immune complexes. We have developed a novel method for culturing primary mesangial cells from renal biopsies by dipping the biopsy in a saline solution at the time of the biopsy being taken. Loosely attached glomeruli that otherwise would be lost detach and are used for culturing of mesangial cells from patients. We isolated mesangial cells from glomeruli from a patient with IgAN and

compared the gene expression of these diseased mesangial cells to that in healthy mesangial cells. Interestingly, we found that the mesangial cells from a patient with IgAN had a different gene expression when it comes to ECM proteins, with an increased gene expression of collagen I (COL1A1), decorin and a decreased gene expression of syndecan-1 compared to mesangial cells from healthy tissue. Treatment of the mesangial cells with purified IgA from patients with IgAN and healthy controls seems to give a stronger response in the mesangial cells cultured from a patient with IgAN than the control cells, but these results are very preliminary and the study is ongoing.

We have established that glomeruli from patients with IgAN have a changed gene expression of PGs as well as several other ECM molecules compared to healthy controls. One PG showed to be of special interest, perlecan, with its gene expression correlating to disease progression and albumin excretion in urine, and could possibly be used as a molecular marker of IgAN. We also found a gene set of 102 unique genes that can be used to separate glomeruli from patients with IgAN from controls. In addition we have established a method to culture mesangial cells from patients with IgAN, giving us a new tool to study the onset of IgAN.

CONCLUDING REMARKS

We found that healthy kidneys from humans and mice have important similarities, but also significant differences, in their glomerular transcriptomes. We classified common glomerular overexpressed genes into six clusters according to their relative level of overexpression compared to the tubulointerstitial compartment and to biological properties. The identification of novel, conserved genes that are highly overexpressed in glomerular tissue can help us pinpoint genes and proteins relevant for normal glomerular function, as well increase our understanding of glomerular disease.

Studying the diseased kidney we found that there are changes in the expression of glomerular PGs in the PAN nephrosis model in rats and podocytes, as well as in patients with IgAN compared to healthy controls.

We show that the glomerular expression of PGs and the biosynthesis of their GAG chains are sensitive to PAN treatment, leading to a decrease in glomerular negative charge, and destabilizing structures in the glomerular filtration barrier. In biopsies from patients with IgAN we found a markedly changed gene expression of PGs in patients with IgAN compared to healthy controls. In the glomerular portion of the biopsies there was an increased expression of several of the PGs investigated, compared to the tubulointerstitial compartment where the only PG affected was perlecan which was down-regulated in contrast to the increase seen in the glomeruli. These changes in perlecan gene expression correlated to proteinuria and progress of the disease suggesting that perlecan can be used as a molecular marker for IgAN. However, further studies are required to shed full light on the role of perlecan in IgAN.

Investigation of the glomerular transcriptome from patients with IgAN gave us a probe core set of 102 unique genes that could be used to separate IgAN from healthy controls. We found that the ECM production is drastically up-regulated in IgAN, probably due to mesangial matrix expansion. In addition, KEGG pathway analysis demonstrated that the ECM-receptor pathway was the strongest up-regulated pathway. This pathway includes perlecan.

It is interesting to note that perlecan was down-regulated in the disease model of nephrotic syndrome, both in the PAN-treated rat and the PAN-treated podocytes, but up-regulated in IgAN. Syndecan-1, another PG, was the only PG to exhibit increased gene expression in the PAN nephrosis model, and was the only of the investigated PGs to have a down-regulated expression in IgAN. The PAN nephrosis model is a non-inflammatory disease model with major proteinuria and IgAN is an inflammatory disease with major proteinuria in only a few cases, suggesting that PGs have different roles in inflammatory and non-inflammatory renal disease.

FUTURE PERSPECTIVES

The prospect that gene expression in mesangial cells from patients with IgAN differs from that in mesangial cells from controls fits well with the dual hit hypothesis of IgAN. The dual hit hypothesis is based on the thought that the mesangial cells in the kidney need to be sensitive to the glomerular deposition of uIgA-containing immune-complexes for the disease to develop. Since IgAN sometimes, but not always, reoccurs in the engrafted kidneys, we hypothesize that that part of the population in the western world has kidneys with mesangial cells sensitive to uIgA. By increasing the understanding of the underlying mechanisms we hope that in the future there will be a way to test donor kidneys prior to transplantation to see if the kidney is uIgA-sensitive or not.

We have developed a unique system for culturing mesangial cells from renal biopsies. By culturing cells from biopsies from patients with IgAN and biopsies from renal living donor transplants and treating the cells with IgA purified from either patients with IgAN or healthy controls we hope to shed more light on the sensitivity of mesangial cells to uIgA. This system will also allow us to investigate the initial changes in the gene and protein expression that occur in the mesangial cells and lead to the morphological changes seen in IgAN, increasing our knowledge of the molecular mechanisms behind IgAN.

We are continuously collecting renal biopsies as part of the biopsy project at Sahlgrenska University hospital. In collaboration with Christer Betsholtz and Karl Tryggvasons groups at the Karolinska institute we are already investigating the transcriptome of glomeruli and the tubulointerstitial compartment from microdissected biopsies from patients with membranous nephropathy. We are also beginning to collect renal biopsies from patients with diabetes. By investigating the transcriptome in various renal diseases we will increase our knowledge of the changes in the transcriptome and molecular mechanisms that lie behind these diseases. In addition, by searching for gene sets that can be used to separate the different diseases from each other, we hope to enable methods for molecular diagnostics of renal biopsies.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Förra året genomgick 307 patienter njurtransplantation i vårt land och i år, den 1 april 2011 stod 603 patienter på väntelistan för en ny njure. I Sverige finns det idag över 8000 patienter i aktiv njursjukvård, ungefär hälften i dialys och hälften transplanterade. Antalet ökar hela tiden, liksom kostnaderna för njursjukvården i Sverige.

Ett stort problem är att man idag saknar specifik behandling för de flesta njursjukdomar och därmed sällan botar utan i bästa fall bara fördröjer sjukdomsförloppet. När njurfunktionen väl sjunker under 5-10% är aktiv njursjukvård i form av dialys eller transplantation den enda utvägen. Avsaknaden av specifik behandling beror till stor del på att vi idag inte har den kunskap vi behöver om de mekanismer på molekylär nivå som leder till njursjukdom, en kunskap som är viktig för att få fram specifika läkemedel. Vi saknar dessutom diagnostiska verktyg för att förutse hur sjukdomen kommer utvecklas och därmed identifiera de patienter som skulle ha nytta av medicinering. Det skulle därför vara till stor hjälp om man kunde ställa en detaljerad diagnos och ge prognos redan tidigt i sjukdomsförloppet.

Genom att studera och jämföra genuttrycket i glomeruli från friska njurdonatorer och möss fick vi fram ett antal gener som är höguttryckta i glomeruli och mycket väl bevarade oavsett art. Vissa av dessa gener är redan kända för att vara av stor vikt i njuren och felaktigheter i dem leder till njursjukdom. Men vi fann också gener som tidigare inte har varit kända i njursammanhang och som kan fungera som ny källa för biomarkörer vid specifika njursjukdomar. Vi har också utvecklat en unik metod för att kunna odla celler från njursjuka patienters glomerulinystan. Genom att studera dessa sjuka celler hoppas vi kunna få ytterligare information kring de förändringar som sker på molekylär nivå vid sjukdomar i njuren. Detta kan i sin tur underlätta framtagandet av mer specifik behandling av patienter med njursjukdom.

Vidare har vi genom att studera genuttrycket vid njursjukdom sett att en speciell sorts proteiner, proteoglykaner, som finns på och utanför cellerna har fått förändrat genuttryck jämfört med friska kontroller. De har flera olika uppgifter som att fungera som signalmolekyler, binda till sig tillväxtfaktorer och bidra till ett skyddande ytskikt på celler. Hos patienter med IgA nefrit fann vi att en speciell sorts proteoglykan, kallad perlecan, hade ett förändrat genuttryck som också korrelerade med patienternas läckage av protein i urinen och progress av sjukdomen. Detta fynd gör att vi tror att perlecan skulle kunna användas som en diagnostisk markör vid IgA nefrit och hjälpa oss att förstå hur patientens sjukdom kommer att utvecklas.

ACKNOWLEDGEMENTS

Finally I would like to express my sincere gratitude to everyone who has made this work possible, especially:

Min handledare, **Jenny Nyström** och min bihandledare **Börje Haraldsson**, ni har båda gett mig fantastisk uppmuntran och ovärderligt stöd under min doktorandtid. Tack för alla diskussioner, både vetenskapliga och alldagliga och för ert engagemang i projekten. **Anna Granqvist**, min andra bihandledare, som har lärt mig allt jag kan när det gäller celler och PCR. Jag hoppas att vi får tillfälle att åka på fler trevliga konferenser tillsammans!

Ulf Nilsson för all hjälp med granskning av min ram och ständigt stöd med hjälp av ett aldrig sinande förråd av choklad när jag bäst behöver det!!

The collaborators at Karolinska institutet, **Ying Sun**, **Christer Betsholtz** and **Karl Tryggvason**. Special thanks to Ying for all the help with the bioinformatics!

Johan Mölne för all hjälp med klassificering, bilder och att få fram snitt från alla patienter.

Elisabeth Rosqvist för all hjälp med att få med patienterna i biopsistudien, det hade inte gått utan dig och din entusiasm! **Lena Heidelberg**, för hjälp med att jaga patienter för blodgivning.

Nina (Kristina) **Levan**, för att du alltid finns där! Tack för all hjälp på sluttampen när vi dessutom äntligen fick jobba ihop, en riktig bonus i slutet av min doktorandperiod.

Alla som är och varit på njurcentrum för att det alltid är inspirerande och roligt att gå till jobbet! **Madeleine Ingelsten** för all hjälp med dataproblem och trevliga och intressanta samtal. **Heidi Hedman** för alla roliga tokigheter vi gör och för att du håller fika-fanan högt. **Annika Lindskog** för bra samarbete, du har varit saknad sista året. **Vincent Fridén** för all hjälp det sista året, det var skönt att du var först så jag kunde dra nytta av din planering! **Johannes Elvin** för bra samarbete**. Emelie Roos** för all hjälp på EBM. **Lisa Buvall**, **Jennie L-W**, **Marie Jeansson**, **Clara Hjalmarsson**, **Damerna** (Elisabeth, Lisbet och Inger) och **Christel Jansson** för trevlig samvaro!

Alla kollegor på **Wallenberglab** och det faktum att det alltid är någon som har ett svar när det är något man undrar över.

Mina vänner som alltid gör livet lite roligare; **Anna**, **Annika**, **Linda X 4**, **Sofia**, **Nina A**, **Louise** och inte minst min syster **Erika**.

Min familj för att ni finns och ställer upp! Det hade varit svårt att få ihop ”livspusslet” utan er.

Dominic ♥

Rasmus och **Maja**, nu är mammas ”bok” äntligen klar!

REFERENCES

1. njurregister, S. Aktiv uremivård i Sverige 1991-2009. (2010).
2. Johnsson C, T.G. *Transplantation*, (Studentlitteratur, 2002).
3. Medin, C., Elinder, C.G., Hylander, B., Blom, B. & Wilczek, H. Survival of patients who have been on a waiting list for renal transplantation. *Nephrol Dial Transplant* **15**, 701-704 (2000).
4. Winkelmayer, W.C., Weinstein, M.C., Mittleman, M.A., Glynn, R.J. & Pliskin, J.S. Health economic evaluations: the special case of end-stage renal disease treatment. *Med Decis Making* **22**, 417-430 (2002).
5. Donations rådet, S. statistik. (2011).
6. Haraldsson, B., Nystrom, J. & Deen, W.M. Properties of the glomerular barrier and mechanisms of proteinuria. *Physiol Rev* **88**, 451-487 (2008).
7. Kalluri, R. Proteinuria with and without renal glomerular podocyte effacement. *J Am Soc Nephrol* **17**, 2383-2389 (2006).
8. Eremina, V., Baelde, H.J. & Quaggin, S.E. Role of the VEGF--a signaling pathway in the glomerulus: evidence for crosstalk between components of the glomerular filtration barrier. *Nephron Physiol* **106**, p32-37 (2007).
9. Rostgaard, J. & Qvortrup, K. Electron microscopic demonstrations of filamentous molecular sieve plugs in capillary fenestrae. *Microvasc Res* **53**, 1-13 (1997).
10. Jeansson, M. & Haraldsson, B. Morphological and functional evidence for an important role of the endothelial cell glycocalyx in the glomerular barrier. *Am J Physiol Renal Physiol* **290**, F111-116 (2006).
11. Bjornson, A., Moses, J., Ingemansson, A., Haraldsson, B. & Sorensson, J. Primary human glomerular endothelial cells produce proteoglycans, and puromycin affects their posttranslational modification. *Am J Physiol Renal Physiol* **288**, F748-756 (2005).
12. Jeansson, M., Bjorck, K., Tenstad, O. & Haraldsson, B. Adriamycin alters glomerular endothelium to induce proteinuria. *J Am Soc Nephrol* **20**, 114-122 (2009).
13. Fogo, A.B. & Kon, V. The glomerulus--a view from the inside--the endothelial cell. *Int J Biochem Cell Biol* **42**, 1388-1397 (2010).
14. Abrahamson, D.R. Origin of the glomerular basement membrane visualized after in vivo labeling of laminin in newborn rat kidneys. *J Cell Biol* **100**, 1988-2000 (1985).
15. Weber, M. Basement membrane proteins. *Kidney Int* **41**, 620-628 (1992).
16. Miner, J.H. Glomerular basement membrane composition and the filtration barrier. *Pediatr Nephrol* (2011).
17. Barker, D.F.*, et al.* Identification of mutations in the COL4A5 collagen gene in Alport syndrome. *Science* **248**, 1224-1227 (1990).
18. Lemmink, H.H.*, et al.* Mutations in the type IV collagen alpha 3 (COL4A3) gene in autosomal recessive Alport syndrome. *Hum Mol Genet* **3**, 1269-1273 (1994).
19. Mochizuki, T.*, et al.* Identification of mutations in the alpha 3(IV) and alpha 4(IV) collagen genes in autosomal recessive Alport syndrome. *Nat Genet* **8**, 77-81 (1994).
20. Zenker, M.*, et al.* Human laminin beta2 deficiency causes congenital nephrosis with mesangial sclerosis and distinct eye abnormalities. *Hum Mol Genet* **13**, 2625-2632 (2004).
21. Noakes, P.G.*, et al.* The renal glomerulus of mice lacking s-laminin/laminin beta 2: nephrosis despite molecular compensation by laminin beta 1. *Nat Genet* **10**, 400-406 (1995).
22. Groffen, A.J.*, et al.* Agrin is a major heparan sulfate proteoglycan in the human glomerular basement membrane. *J Histochem Cytochem* **46**, 19-27 (1998).
23. Murdoch, A.D., Liu, B., Schwarting, R., Tuan, R.S. & Iozzo, R.V. Widespread expression of perlecan proteoglycan in basement membranes and extracellular matrices of human tissues as detected by a novel monoclonal antibody against domain III and by in situ hybridization. *J Histochem Cytochem* **42**, 239-249 (1994).

24. Goldberg, S., Harvey, S.J., Cunningham, J., Tryggvason, K. & Miner, J.H. Glomerular filtration is normal in the absence of both agrin and perlecan-heparan sulfate from the glomerular basement membrane. *Nephrol Dial Transplant* **24**, 2044-2051 (2009).
25. Adler, S. Characterization of glomerular epithelial cell matrix receptors. *Am J Pathol* **141**, 571-578 (1992).
26. Chen, S.*, et al.* Podocytes require the engagement of cell surface heparan sulfate proteoglycans for adhesion to extracellular matrices. *Kidney Int* **78**, 1088-1099 (2010).
27. Kestila, M.*, et al.* Positionally cloned gene for a novel glomerular protein--nephrin--is mutated in congenital nephrotic syndrome. *Mol Cell* **1**, 575-582 (1998).
28. Ruotsalainen, V.*, et al.* Nephrin is specifically located at the slit diaphragm of glomerular podocytes. *Proc Natl Acad Sci U S A* **96**, 7962-7967 (1999).
29. Boute, N.*, et al.* NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat Genet* **24**, 349-354 (2000).
30. Donoviel, D.B.*, et al.* Proteinuria and perinatal lethality in mice lacking NEPH1, a novel protein with homology to NEPHRIN. *Mol Cell Biol* **21**, 4829-4836 (2001).
31. Shih, N.Y.*, et al.* Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science* **286**, 312-315 (1999).
32. Cheng, H. & Harris, R.C. The glomerulus--a view from the outside--the podocyte. *Int J Biochem Cell Biol* **42**, 1380-1387 (2010).
33. van den Berg, J.G., van den Bergh Weerman, M.A., Assmann, K.J., Weening, J.J. & Florquin, S. Podocyte foot process effacement is not correlated with the level of proteinuria in human glomerulopathies. *Kidney Int* **66**, 1901-1906 (2004).
34. D'Agati, V. Pathologic classification of focal segmental glomerulosclerosis. *Semin Nephrol* **23**, 117-134 (2003).
35. Choi, S.Y., Suh, K.S., Choi, D.E. & Lim, B.J. Morphometric analysis of podocyte foot process effacement in IgA nephropathy and its association with proteinuria. *Ultrastruct Pathol* **34**, 195-198 (2010).
36. Xu, L.*, et al.* Podocyte number predicts progression of proteinuria in IgA nephropathy. *Mod Pathol* **23**, 1241-1250 (2010).
37. Schiffer, M.*, et al.* Apoptosis in podocytes induced by TGF-beta and Smad7. *J Clin Invest* **108**, 807-816 (2001).
38. Lai, K.N.*, et al.* Podocyte injury induced by mesangial-derived cytokines in IgA nephropathy. *Nephrol Dial Transplant* **24**, 62-72 (2009).
39. Wang, C.*, et al.* Mesangial cells stimulated by immunoglobin A1 from IgA nephropathy upregulates transforming growth factor-beta1 synthesis in podocytes via renin-angiotensin system activation. *Arch Med Res* **41**, 255-260 (2010).
40. Coppo, R.*, et al.* Aberrantly glycosylated IgA1 induces mesangial cells to produce platelet-activating factor that mediates nephrin loss in cultured podocytes. *Kidney Int* **77**, 417-427 (2010).
41. Wang, C.*, et al.* Mesangial medium with IgA1 from IgA nephropathy inhibits nephrin expression in mouse podocytes. *Eur J Clin Invest* **39**, 561-567 (2009).
42. Ye, Z.C.*, et al.* Serum IgA1 from patients with IgA nephropathy up-regulates integrin-linked kinase synthesis and inhibits adhesive capacity in podocytes through indirect pathways. *Clin Invest Med* **32**, E20-27 (2009).
43. Lai, K.N.*, et al.* Activation of podocytes by mesangial-derived TNF-alpha: glomerulo-podocytic communication in IgA nephropathy. *Am J Physiol Renal Physiol* **294**, F945-955 (2008).
44. Schlondorff, D. & Banas, B. The mesangial cell revisited: no cell is an island. *J Am Soc Nephrol* **20**, 1179-1187 (2009).
45. Barratt, J., Feehally, J. & Smith, A.C. Pathogenesis of IgA nephropathy. *Semin Nephrol* **24**, 197-217 (2004).
46. Mason, R.M. & Wahab, N.A. Extracellular matrix metabolism in diabetic nephropathy. *J Am Soc Nephrol* **14**, 1358-1373 (2003).
47. Pereira, P.C., Miranda, D.M., Oliveira, E.A. & Silva, A.C. Molecular pathophysiology of renal tubular acidosis. *Curr Genomics* **10**, 51-59 (2009).
48. Rosen, S. & Stillman, I.E. Acute tubular necrosis is a syndrome of physiologic and pathologic dissociation. *J Am Soc Nephrol* **19**, 871-875 (2008).

49. Risdon, R.A., Sloper, J.C. & De Wardener, H.E. Relationship between renal function and histological changes found in renal-biopsy specimens from patients with persistent glomerular nephritis. *Lancet* **2**, 363-366 (1968).
50. Rodriguez-Iturbe, B., Johnson, R.J. & Herrera-Acosta, J. Tubulointerstitial damage and progression of renal failure. *Kidney Int Suppl*, S82-86 (2005).
51. Lai, K.N., Chan, L.Y. & Leung, J.C. Mechanisms of tubulointerstitial injury in IgA nephropathy. *Kidney Int Suppl*, S110-115 (2005).
52. Reich, H.N.*, et al.* A molecular signature of proteinuria in glomerulonephritis. *PLoS One* **5**, e13451 (2010).
53. Wight, T.N. & Potter-Perigo, S. The Extracellular Matrix: An Active or Passive Player in Fibrosis. *Am J Physiol Gastrointest Liver Physiol* (2011).
54. Miner, J.H. Renal basement membrane components. *Kidney Int* **56**, 2016-2024 (1999).
55. Vahedi, K. & Alamowitch, S. Clinical spectrum of type IV collagen (COL4A1) mutations: a novel genetic multisystem disease. *Curr Opin Neurol* **24**, 63-68 (2011).
56. Durbeej, M. Laminins. *Cell Tissue Res* **339**, 259-268 (2010).
57. Iozzo, R.V. Matrix proteoglycans: from molecular design to cellular function. *Annu Rev Biochem* **67**, 609-652 (1998).
58. Prydz, K. & Dalen, K.T. Synthesis and sorting of proteoglycans. *J Cell Sci* **113 Pt 2**, 193-205 (2000).
59. Laurent, T.C. & Fraser, J.R. Hyaluronan. *Faseb J* **6**, 2397-2404 (1992).
60. Schaefer, L. & Schaefer, R.M. Proteoglycans: from structural compounds to signaling molecules. *Cell Tissue Res* **339**, 237-246 (2010).
61. Babelova, A.*, et al.* Biglycan, a danger signal that activates the NLRP3 inflammasome via toll-like and P2X receptors. *J Biol Chem* **284**, 24035-24048 (2009).
62. Ebefors, K.*, et al.* Role of glomerular proteoglycans in IgA nephropathy. *PLoS One* **6**, e18575 (2011).
63. Jeansson, M., Granqvist, A.B., Nystrom, J.S. & Haraldsson, B. Functional and molecular alterations of the glomerular barrier in long-term diabetes in mice. *Diabetologia* **49**, 2200-2209 (2006).
64. Jeansson, M. & Haraldsson, B. Glomerular size and charge selectivity in the mouse after exposure to glucosaminoglycan-degrading enzymes. *J Am Soc Nephrol* **14**, 1756-1765 (2003).
65. Bjornson Granqvist, A.*, et al.* Podocyte proteoglycan synthesis is involved in the development of nephrotic syndrome. *Am J Physiol Renal Physiol* **291**, F722-730 (2006).
66. Xian, X., Gopal, S. & Couchman, J.R. Syndecans as receptors and organizers of the extracellular matrix. *Cell Tissue Res* **339**, 31-46 (2010).
67. Greene, D.K., Tumova, S., Couchman, J.R. & Woods, A. Syndecan-4 associates with alpha-actinin. *J Biol Chem* **278**, 7617-7623 (2003).
68. Rops, A.L.*, et al.* Syndecan-1 deficiency aggravates anti-glomerular basement membrane nephritis. *Kidney Int* **72**, 1204-1215 (2007).
69. Filmus, J., Capurro, M. & Rast, J. Glypicans. *Genome Biol* **9**, 224 (2008).
70. Whitelock, J.M., Melrose, J. & Iozzo, R.V. Diverse cell signaling events modulated by perlecan. *Biochemistry* **47**, 11174-11183 (2008).
71. Conde-Knape, K. Heparan sulfate proteoglycans in experimental models of diabetes: a role for perlecan in diabetes complications. *Diabetes Metab Res Rev* **17**, 412-421 (2001).
72. Wight, T.N. Versican: a versatile extracellular matrix proteoglycan in cell biology. *Curr Opin Cell Biol* **14**, 617-623 (2002).
73. Thomas, G.J., Bayliss, M.T., Harper, K., Mason, R.M. & Davies, M. Glomerular mesangial cells in vitro synthesize an aggregating proteoglycan immunologically related to versican. *Biochem J* **302 (Pt 1)**, 49-56 (1994).
74. Yamaguchi, Y., Mann, D.M. & Ruoslahti, E. Negative regulation of transforming growth factor-beta by the proteoglycan decorin. *Nature* **346**, 281-284 (1990).
75. De Cosmo, S.*, et al.* The Decorin gene 179 allelic variant is associated with a slower progression of renal disease in patients with type 1 diabetes. *Nephron* **92**, 72-76 (2002).
76. Schaefer, L.*, et al.* Small proteoglycans in human diabetic nephropathy: discrepancy between glomerular expression and protein accumulation of decorin, biglycan, lumican, and fibromodulin. *Faseb J* **15**, 559-561 (2001).

77. Williams, K.J., *et al.* Decorin deficiency enhances progressive nephropathy in diabetic mice. *Am J Pathol* **171**, 1441-1450 (2007).
78. Hildebrand, A., *et al.* Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta. *Biochem J* **302 (Pt 2)**, 527-534 (1994).
79. Iozzo, R.V. The biology of the small leucine-rich proteoglycans. Functional network of interactive proteins. *J Biol Chem* **274**, 18843-18846 (1999).
80. Schaefer, L., *et al.* The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. *J Clin Invest* **115**, 2223-2233 (2005).
81. Nikitovic, D., Katonis, P., Tsatsakis, A., Karamanos, N.K. & Tzanakakis, G.N. Lumican, a small leucine-rich proteoglycan. *IUBMB Life* **60**, 818-823 (2008).
82. Orth, S.R. & Ritz, E. The nephrotic syndrome. *N Engl J Med* **338**, 1202-1211 (1998).
83. Neelakantappa, K., Gallo, G.R. & Baldwin, D.S. Proteinuria in IgA nephropathy. *Kidney Int* **33**, 716-721 (1988).
84. Beck, L.H., Jr., *et al.* M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy. *N Engl J Med* **361**, 11-21 (2009).
85. Barratt, J. & Feehally, J. IgA nephropathy. *J Am Soc Nephrol* **16**, 2088-2097 (2005).
86. Davin, J.C., Ten Berge, I.J. & Weening, J.J. What is the difference between IgA nephropathy and Henoch-Schonlein purpura nephritis? *Kidney Int* **59**, 823-834 (2001).
87. Hiki, Y., *et al.* Mass spectrometry proves under-O-glycosylation of glomerular IgA1 in IgA nephropathy. *Kidney Int* **59**, 1077-1085 (2001).
88. Allen, A.C., *et al.* Mesangial IgA1 in IgA nephropathy exhibits aberrant O-glycosylation: observations in three patients. *Kidney Int* **60**, 969-973 (2001).
89. Smith, A.C., Molyneux, K., Feehally, J. & Barratt, J. O-glycosylation of serum IgA1 antibodies against mucosal and systemic antigens in IgA nephropathy. *J Am Soc Nephrol* **17**, 3520-3528 (2006).
90. Giannakakis, K., Feriozzi, S., Perez, M., Faraggiana, T. & Muda, A.O. Aberrantly glycosylated IgA1 in glomerular immune deposits of IgA nephropathy. *J Am Soc Nephrol* **18**, 3139-3146 (2007).
91. Suzuki, H., *et al.* Aberrantly glycosylated IgA1 in IgA nephropathy patients is recognized by IgG antibodies with restricted heterogeneity. *J Clin Invest* **119**, 1668-1677 (2009).
92. Tomana, M., *et al.* Circulating immune complexes in IgA nephropathy consist of IgA1 with galactose-deficient hinge region and antiglycan antibodies. *J Clin Invest* **104**, 73-81 (1999).
93. Roccatello, D., *et al.* The fate of aggregated immunoglobulin A injected in IgA nephropathy patients and healthy controls. *Am J Kidney Dis* **18**, 20-25 (1991).
94. Schena, F.P., *et al.* Abnormalities of the IgA immune system in members of unrelated pedigrees from patients with IgA nephropathy. *Clin Exp Immunol* **92**, 139-144 (1993).
95. Gharavi, A.G., *et al.* Aberrant IgA1 glycosylation is inherited in familial and sporadic IgA nephropathy. *J Am Soc Nephrol* **19**, 1008-1014 (2008).
96. Silva, F.G., Chander, P., Pirani, C.L. & Hardy, M.A. Disappearance of glomerular mesangial IgA deposits after renal allograft transplantation. *Transplantation* **33**, 241-246 (1982).
97. Varis, J., *et al.* Immunoglobulin and complement deposition in glomeruli of 756 subjects who had committed suicide or met with a violent death. *J Clin Pathol* **46**, 607-610 (1993).
98. Suzuki, K., *et al.* Incidence of latent mesangial IgA deposition in renal allograft donors in Japan. *Kidney Int* **63**, 2286-2294 (2003).
99. Odum, J., *et al.* Recurrent mesangial IgA nephritis following renal transplantation. *Nephrol Dial Transplant* **9**, 309-312 (1994).
100. Berger, J. Recurrence of IgA nephropathy in renal allografts. *Am J Kidney Dis* **12**, 371-372 (1988).
101. Briganti, E.M., Russ, G.R., McNeil, J.J., Atkins, R.C. & Chadban, S.J. Risk of renal allograft loss from recurrent glomerulonephritis. *N Engl J Med* **347**, 103-109 (2002).
102. Kretzler, M., *et al.* Repuncturing the renal biopsy: strategies for molecular diagnosis in nephrology. *J Am Soc Nephrol* **13**, 1961-1972 (2002).
103. Cohen, C.D., *et al.* Laser microdissection and gene expression analysis on formaldehyde-fixed archival tissue. *Kidney Int* **61**, 125-132 (2002).

104. Hodgin, J.B. & Cohen, C.D. Experimental approaches to the human renal transcriptome. *Semin Nephrol* **30**, 455-467 (2010).
105. Pippin, J.W.*, et al.* Inducible rodent models of acquired podocyte diseases. *Am J Physiol Renal Physiol* **296**, F213-229 (2009).
106. Takemoto, M.*, et al.* A new method for large scale isolation of kidney glomeruli from mice. *Am J Pathol* **161**, 799-805 (2002).
107. Chabardes-Garonne, D.*, et al.* A panoramic view of gene expression in the human kidney. *Proc Natl Acad Sci U S A* **100**, 13710-13715 (2003).
108. Takemoto, M.*, et al.* Large-scale identification of genes implicated in kidney glomerulus development and function. *Embo J* **25**, 1160-1174 (2006).
109. Cuellar, L.M.*, et al.* Identification and localization of novel genes preferentially expressed in human kidney glomerulus. *Nephrology (Carlton)* **14**, 94-104 (2009).
110. Nystrom, J., Fierlbeck, W., Granqvist, A., Kulak, S.C. & Ballermann, B.J. A human glomerular SAGE transcriptome database. *BMC Nephrol* **10**, 13 (2009).
111. He, L.*, et al.* Glomerulus-specific mRNA transcripts and proteins identified through kidney expressed sequence tag database analysis. *Kidney Int* **71**, 889-900 (2007).
112. Higgins, J.P.*, et al.* Gene expression in the normal adult human kidney assessed by complementary DNA microarray. *Mol Biol Cell* **15**, 649-656 (2004).
113. Patrakka, J.*, et al.* Expression and subcellular distribution of novel glomerulus-associated proteins dendrin, ehd3, sh2d4a, plekhh2, and 2310066E14Rik. *J Am Soc Nephrol* **18**, 689-697 (2007).
114. Lindenmeyer, M.T.*, et al.* Systematic analysis of a novel human renal glomerulus-enriched gene expression dataset. *PLoS One* **5**, e11545 (2010).
115. Sun, Y.*, et al.* Glomerular transcriptome changes associated with lipopolysaccharide-induced proteinuria. *Am J Nephrol* **29**, 558-570 (2009).
116. Qian, Z., Okuhara, D., Abe, M.K. & Rosner, M.R. Molecular cloning and characterization of a mitogen-activated protein kinase-associated intracellular chloride channel. *J Biol Chem* **274**, 1621-1627 (1999).
117. He, L.*, et al.* The glomerular transcriptome and a predicted protein-protein interaction network. *J Am Soc Nephrol* **19**, 260-268 (2008).
118. Hinkes, B.G. NPHS3: new clues for understanding idiopathic nephrotic syndrome. *Pediatr Nephrol* **23**, 847-850 (2008).
119. Gbadegesin, R.*, et al.* Mutations in PLCE1 are a major cause of isolated diffuse mesangial sclerosis (IDMS). *Nephrol Dial Transplant* **23**, 1291-1297 (2008).
120. Sironen, A.*, et al.* Knobbed acrosome defect is associated with a region containing the genes STK17b and HECW2 on porcine chromosome 15. *BMC Genomics* **11**, 699 (2010).
121. Salmivirta, M., Lidholt, K. & Lindahl, U. Heparan sulfate: a piece of information. *Faseb J* **10**, 1270-1279 (1996).
122. Nakayama, K.*, et al.* Altered expression of NDST-1 messenger RNA in puromycin aminonucleoside nephrosis. *J Lab Clin Med* **143**, 106-114 (2004).
123. Couchman, J.R., Chen, L. & Woods, A. Syndecans and cell adhesion. *Int Rev Cytol* **207**, 113-150 (2001).
124. Woods, A. Syndecans: transmembrane modulators of adhesion and matrix assembly. *J Clin Invest* **107**, 935-941 (2001).
125. Fishman, J.A. & Karnovsky, M.J. Effects of the aminonucleoside of puromycin on glomerular epithelial cells in vitro. *Am J Pathol* **118**, 398-407 (1985).
126. Kasinath, B.S., Singh, A.K., Kanwar, Y.S. & Lewis, E.J. Effect of puromycin aminonucleoside on HSPG core protein content of glomerular epithelial cells. *Am J Physiol* **255**, F590-596 (1988).
127. Mynderse, L.A., Hassell, J.R., Kleinman, H.K., Martin, G.R. & Martinez-Hernandez, A. Loss of heparan sulfate proteoglycan from glomerular basement membrane of nephrotic rats. *Lab Invest* **48**, 292-302 (1983).
128. Ina, K., Kitamura, H., Nakamura, M., Ono, J. & Takaki, R. Loss of sulfated carbohydrate from the glomerular podocyte as a cause of albuminuria in experimental diabetic rats: ultrastructural histochemical study. *J Diabet Complications* **5**, 173-175 (1991).

129. Guasch, A., Hashimoto, H., Sibley, R.K., Deen, W.M. & Myers, B.D. Glomerular dysfunction in nephrotic humans with minimal changes or focal glomerulosclerosis. *Am J Physiol* **260**, F728-737 (1991).
130. Sakagami, Y.*, et al.* Analysis of glomerular anionic charge status in children with IgA nephropathy using confocal laser scanning microscopy. *Nephron Clin Pract* **96**, c96-104 (2004).
131. Okada, K.*, et al.* Ultrastructural alterations of glomerular anionic sites in IgA nephropathy. *Clin Nephrol* **31**, 96-102 (1989).
132. Raats, C.J.*, et al.* Expression of agrin, dystroglycan, and utrophin in normal renal tissue and in experimental glomerulopathies. *Am J Pathol* **156**, 1749-1765 (2000).
133. Bassols, A. & Massague, J. Transforming growth factor beta regulates the expression and structure of extracellular matrix chondroitin/dermatan sulfate proteoglycans. *J Biol Chem* **263**, 3039-3045 (1988).
134. Ruoslahti, E. & Yamaguchi, Y. Proteoglycans as modulators of growth factor activities. *Cell* **64**, 867-869 (1991).
135. Border, W.A. & Noble, N.A. Transforming growth factor beta in tissue fibrosis. *N Engl J Med* **331**, 1286-1292 (1994).
136. Border, W.A.*, et al.* Natural inhibitor of transforming growth factor-beta protects against scarring in experimental kidney disease. *Nature* **360**, 361-364 (1992).
137. van Det, N.F.*, et al.* Proteoglycan production by human glomerular visceral epithelial cells and mesangial cells in vitro. *Biochem J* **307 (Pt 3)**, 759-768 (1995).
138. van den Born, J.*, et al.* Distribution of GBM heparan sulfate proteoglycan core protein and side chains in human glomerular diseases. *Kidney Int* **43**, 454-463 (1993).
139. Cattran, D.C.*, et al.* The Oxford classification of IgA nephropathy: rationale, clinicopathological correlations, and classification. *Kidney Int* **76**, 534-545 (2009).
140. Roberts, I.S.*, et al.* The Oxford classification of IgA nephropathy: pathology definitions, correlations, and reproducibility. *Kidney Int* **76**, 546-556 (2009).
141. Chihara, Y.*, et al.* Roles of TGF-beta1 and apoptosis in the progression of glomerulosclerosis in human IgA nephropathy. *Clin Nephrol* **65**, 385-392 (2006).
142. Yang, Y.L.*, et al.* Bone morphogenetic protein-2 antagonizes renal interstitial fibrosis by promoting catabolism of type I transforming growth factor-beta receptors. *Endocrinology* **150**, 727-740 (2009).
143. Pegorier, S., Campbell, G.A., Kay, A.B. & Lloyd, C.M. Bone morphogenetic protein (BMP)-4 and BMP-7 regulate differentially transforming growth factor (TGF)-beta1 in normal human lung fibroblasts (NHLF). *Respir Res* **11**, 85 (2010).
144. Simic, P. & Vukicevic, S. Bone morphogenetic proteins in development and homeostasis of kidney. *Cytokine Growth Factor Rev* **16**, 299-308 (2005).
145. Ignotz, R.A. & Massague, J. Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J Biol Chem* **261**, 4337-4345 (1986).