Functional and molecular mechanisms behind glomerular kidney disease

Peidi Liu

Department of Physiology
Institute of Neuroscience and Physiology
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

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peidi.liu@neuro.gu.se

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“Our greatest glory is not in never falling, but in rising every time we fall.”

- Confucius

ABSTRACT

Glomerulonephritis (GN) is one of the most common causes of chronic kidney disease (CKD). In our studies, we investigated the molecular mechanisms behind GN on both transcriptomic and proteomic levels using a combined *in vivo* and *in vitro* approach. By doing so, our aim was to find new possible candidates for therapeutic intervention in CKD. IgA nephropathy (IgAN) is the most common type of GN worldwide. It is a proliferative glomerular kidney disease in which galactose-deficient IgA (gd-IgA) is deposited in the mesangial area of the glomeruli. Previous studies have pointed out that gd-IgA is not the only factor inducing the disease. Our hypothesis is that the mesangial cells are of great importance in IgAN development and that patients with IgAN have more susceptible mesangial cells to gd-IgA compared to healthy individuals. The deposition of gd-IgA is likely caused by interaction with a receptor on the mesangial cell leading to proliferation and inflammation. To study these mechanisms, we cultured primary human mesangial cells from IgAN patient biopsy samples and healthy controls. Our results showed that patient mesangial cells had a significantly increased release of the growth factors PDGF, TGFβ1 as well as the cytokines IL-6 and CCL5, when treated with gd-IgA. These cells also had a significantly higher proliferation rate compared to control cells. We investigated the mesangial cell transcriptomic and proteomic function in patients with IgAN using microarray and mass spectrometry techniques. We demonstrated that many inflammatory pathways were significantly regulated both in the glomeruli and in the gd-IgA treated mesangial cells. By using cell-type specific positive standard genes we found a dominant role in IgAN of the mesangial cell compared to the podocytes. The transformed z-scores based on mesangial cell standard genes showed significant correlation with patient clinical data (eGFR and serum creatinine). In order to know how gd-IgA is deposited in the mesangium, we investigated receptors from the mesangial cells interacting with gd-IgA. Interestingly, a transmembrane receptor was

identified to be associated with gd-IgA and it also regulated mesangial cell proliferation. Additionally, we investigated micro-RNAs in glomerular disease using a screening technique. MiR-x7 was found to regulate a specific podocyte protein and the level was correlated to disease. Since it is a small molecule, miR-x7 can be detected in urine samples and may be used as a diagnostic marker for CKD.

In conclusion, we have verified the importance of mesangial cells in IgAN. The correlation of mesangial cell standard genes with clinical data can potentially explain the progression of the disease. A specific receptor was found to regulate the proliferation of the mesangial cells and it may potentially be involved in the deposition of gd-IgA. Micro-RNAs are found to be promising markers for CKD and thus disease-specific micro-RNAs should be further investigated.

Keywords: Glomerular kidney disease, IgA nephropathy, mesangial cell, micro-RNA

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SAMMANFATTNING PÅ SVENSKA

IgA-nefrit är en av västvärldens vanligaste njursjukdomar och den bryter ofta ut när patienten är ung. Tyvärr saknas idag specifik behandling för IgA-nefrit och detta beror till stor del på att vi inte har kunskap om de mekanismer på molekylär nivå som ligger bakom sjukdomen. Man vet att IgA-nefrit delvis orsakas av att patienterna har ett IgA (en del av kroppens immunförsvar) som är annorlunda sammansatt och att denna typ av IgA bildar komplex och fastnar i njurens kärlnystan, där de orsakar inflammation och njurskada. Det är i njurens kärlnystan som filtrationen av blodet sker och urinen bildas. Mellan blodkärlen sitter mesangieceller, en unik celltyp som anses ha egenskaper som vissa immunologiska celler och som glatt muskelceller samt kan fungera som stödjevävnad. Inlagringen av IgA leder till strukturella förändringar och cellernas signaler till varandra förändras vilket i sin tur leder till en försämrad njurfunktion. Dock finns det personer som har denna typ av IgA men som ändå inte blir sjuka. Vi tror därför att de patienter som utvecklar IgA-nefrit har celler i kärlnystanet i njuren som är känsligare och reagerar annorlunda på IgA komplexen än de som inte utvecklar sjukdomen.

Vi ville därför bestämma den roll som mesangiecellerna spelar vid IgA-nefrit. Eftersom vi har utvecklat ett välfungerande arbetsflöde för att ta tillvara på små vävnadsbitar (biopsier) från njursjuka patienter kan vi ta reda på hur olika sjukdomstillstånd påverkar gen- och proteinuttrycket i njurens celler och jämföra med friskt vävnad. Vi kan också odla celler från biopsier och studera signalmekanismer i de olika celltyperna i njuren och hur de samverkar med varandra. För att utvärdera alla dessa komponenter och hur de kan sammankopplas har vi använt oss av storskalig analys både av gen- och av proteinuttrycket samt kliniska data. Vi har dessutom kombinerat dessa storskaliga analyser med cell och molekylärbiologiska analyser för att bättre förstå detaljerna i sjukdomsförloppet.

Våra fynd tyder på att mesangieceller från patienter med IgA-nefrit har ett ökat inflammatoriskt svar och en ökad tillväxttakt jämfört med celler från friska personer. De reagerar dessutom kraftigare på IgA komplexen än celler från kontroller. När vi undersöker gen- och proteinuttrycket kan vi se att hela signalvägar som innefattar inflammation, tillväxt och immunförsvarsaktivering är påverkade vid IgA-nefrit. Vi har också genom sofistikerade analysmetoder kunnat klarlägga att regleringen av ett antal utvalda gener från mesangieceller kan kopplas samman med hur njursjukdomen utvecklas. Detta är inte möjligt om man använder gener från en annan celltyp i kärlnystanet, podocyterna. Våra studier visar sammantaget att mesangiecellerna spelar en mycket viktig roll vid IgA-nefrit och vi har kunnat klarlägga några av mekanismerna bakom mesangiecellernas roll för den forsatta utvecklingen av sjukdomen.

Genom att öka förståelsen för de processer som ligger bakom IgA-nefrit så hoppas vi kunna utveckla bättre behandling av sjukdomen för att förhindra njursvikt och därigenom höja patienternas livskvalitet.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Mesangial cells from patients with IgA nephropathy have increased susceptibility to galactose-deficient IgA1**
Ebefors K, Liu P, Lassén E, Elvin J, Candemark E, Levan K, Haraldsson B and Nyström J.
BMC Nephrology (2016) 17:40

II. **Transcriptomic and proteomic profiling reveal insights of mesangial cell function in patients with IgA nephropathy**
Liu P, Lassén E, Sihlbom C, Nair V, Berthier C, Katayama M, Kretzler M, Betsholtz C, Ju W, Ebefors K and Nyström J.
Manuscript

III. **A potential receptor of IgA is involved in mesangial proliferation and development of IgA nephropathy**
Liu P, Lassén E, Müller-Lühlhoff S, Candemark E, Wallentin H, Hultenby K, Sihlbom C, Buvall L, Ebefors K and Nyström J.
Manuscript

IV. **Podocytes regulate expression of a specific glomerular basement membrane protein via micro-RNA in glomerular disease**
Müller-Deile J, Dannenberg J, Schröder P, Miner J, Chen R, Bräsen J, Thum T, Liu P, Nyström J, Haller H, Lorenzen J and Schiffer M.
Manuscript

CONTENT

ABBREVIATIONS

CKD	Chronic Kidney Disease
ESRD	End Stage Renal Disease
EPO	Erythroprotein
GBM	Glomerular Basement Membrane
IgA	Immunoglobulin A
IgAN	Immunoglobulin A Nephropathy
DN	Diabetic Nephropathy
FSGS	Focal Segmental Glomerulosclerosis
MN/MGN	Membranous Nephropathy
GN	Glomerulonephritis
LN	Lupus Nephritis
MCD	Minimal Change Disease
ESL	Endothelial Surface Layer
NO	Nitric Oxide
eNOS	endothelial Nitric Oxide Synthase
TBMD	Thin Basement Membrane Disease
HS	Henoch-Schönlein purpura
gd-IgA	Galactose-deficient IgA
cIgA	control IgA

GO	Gene Ontology
IPA	Ingenuity Pathway Analysis
PCR	Polymerase Chain Reaction
qRT-PCR	quantitative Real-Time PCR
LDA	Low Density Array
MS	Mass Spectrometry
TMT	Tandem Mass Tag
BrdU	5-Bromo-2'-deoxyuridine
PDGF	Patelet-derived Growth Factor
ELISA	Enzyme-linked immunosorbent Assay
shRNA	short-hairpin RNA
GFP	Green Fluorescent Protein
PAN	Puromycin Aminonucleoside
TGFβ	Transforming Growth Factor beta
IL-6	Interleukin 6
IL-8	Interleukin 8
CCL5	Chemokine (C-C motif) ligand 5
MCP-1	Monocyte Chemoattractant Protein-1
DCN	Decorin
RMA	Robust Multi-array Averaging
NUSE	Normalized Unscaled Standard Error

RLE	Relative Log Expression
PCA	Principal Component Analysis
PCoA	Principal Coordinate Analysis
SAM	Significant Analysis of Microarray
eGFR	estimated Glomerular Filtration Rate
TFRC	Transferrin Receptor
PEEC	Preeclampsia/eclampsia
ANCA	ANCA positive vasculitis
HUS	Hemolytic Uremic Syndrome

1 INTRODUCTION

Chronic kidney disease (CKD) is increasing world wide, partly due to life style related diseases but also because the population in general is getting older with increased risk for CKD (1, 2). Unfortunately, there are no curative treatments for advanced CKD and patients who progress to End Stage Renal Disease (ESRD) need to undergo dialysis or have kidney transplantation for survival. One reason for the lack of effective treatments is that the underlying mechanisms of the diseases leading to ESRD are not fully understood. It is therefore necessary to increase our understanding of the molecular mechanisms in the kidney leading to disease onset and progression to find new treatment targets that in the end can lead to curative treatment options (3).

1.1 The kidney

The kidneys are the homeostatic centers of the body with multiple essential regulatory functions. These include filtering of the blood,

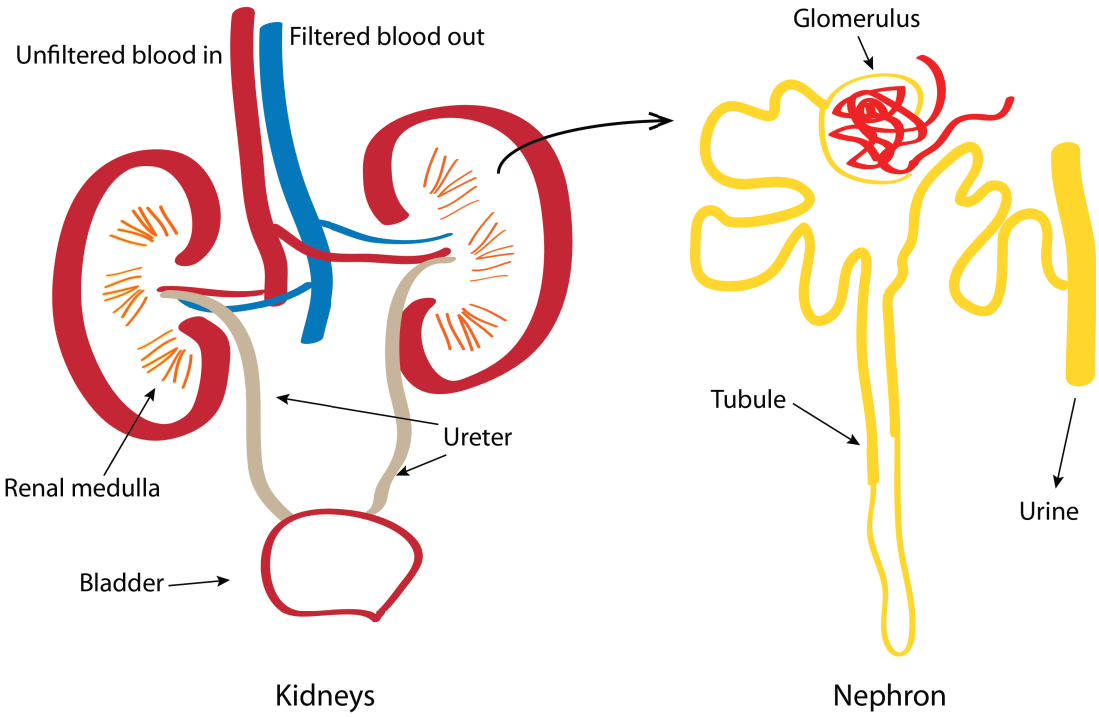


Figure 1. The structure of the kidneys and the nephron.

maintaining water and acid-base balance and regulating osmolality as well as producing several essential hormones (4, 5).

1.1.1 Structure

The kidneys are bean-shaped and placed retroperitoneally on both sides of the spine and partly covered by the last ribs of the ribcage (6, 7). They are enclosed in the renal capsules and are composed of a renal cortex and renal medulla. Each kidney contains over 1 million functional units, the nephrons. The nephrons filter the blood and produce primary urine. Most of the fluid and electrolytes are then reabsorbed back to the circulation through the peritubular capillaries. The nephron is built up by the glomerulus (filtering) and the tubular system (reabsorbtion), Figure 1.

1.1.2 Function

The kidneys filter nearly 180 liters of primary urine daily through the glomerular filtration barrier and around 1.5 liters of final urine is produced (8). The main task of the kidney is to regulate body homeostasis (4). By doing so, the kidneys reabsorb valuable nutrition back into the blood and excrete waste into the urine. The electrolyte distribution and acid-base balance is also regulated by the kidneys. Besides this, the kidneys have endocrine functions. Renin secreted by the kidneys regulates blood pressure and sodium homeostasis (9). Erythropoietin (EPO) produced in the renal cortex is necessary for erythropoiesis (10, 11). The kidneys also activate vitamin D and active vitamin D is needed for a stable calcium and phosphate balance (12, 13).

1.1.3 The glomerulus

The filtration of the blood takes place in the glomerulus, a capillary network that is enclosed by the Bowman's capsule (Figure 2). The capillary loops makes up the filtration barrier which is composed of 4 layers: The glycocalyx covering the highly fenestrated endothelium, the glomerular basement membrane and specialized epithelial cells

(podocytes) (14). The filtration barrier is highly selective and restricts almost all the large molecules and proteins from leaving the blood, while water and small particles are easily filtered (15-18). Between the capillaries of the glomerulus, there is a framework of mesangial cells supporting the capillary structure, contracting and reducing glomerular surface to regulate filtration rate (19-21).

Mesangial cells

The role of the mesangial cells include maintenance of the glomerular structure integrity and matrix homeostasis (22). In response to injury,

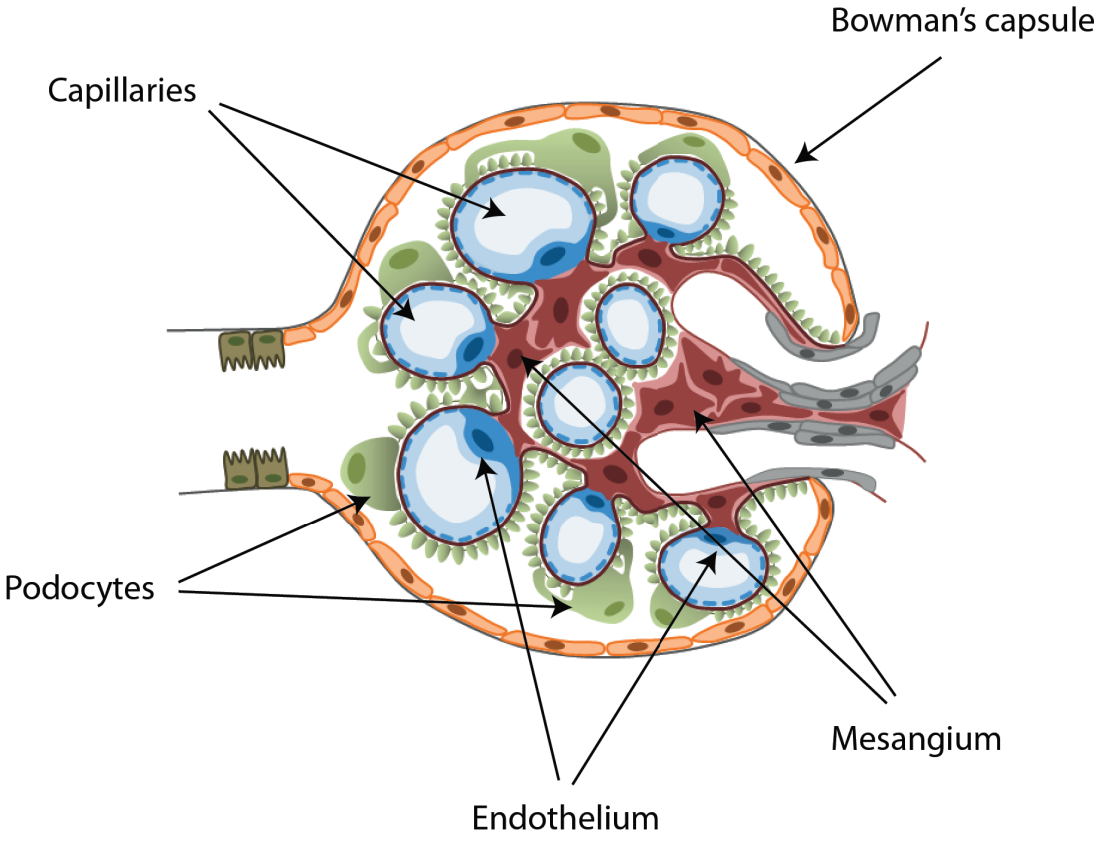


Figure 2. The structure of the glomerulus.

mesangial cells can undergo proliferation with release of a broad range of mediators including cytokines/chemokines, growth factors and matrix proteins affecting themselves and the surrounding cells (22-24). In diabetic nephropathy (DN) and IgA nephropathy (IgAN), one of the

key findings is mesangial cell proliferation and mesangial matrix expansion reducing the glomerular function (25-27). It has been suggested that the released mediators by the mesangial cells themselves, such as IL-6, PDGF, TGFβ, could cause the changes found in the mesangium in DN and IgAN (28-30). Mesangial cells are in direct contact with endothelial cells and may also be in contact with the podocytes in specific areas of the glomerulus (28). The mediators from mesangial cells may also influence endothelial cells, as well as podocytes through the basement membrane porous structure (22). It has been found that integrin αvβ8 produced by mesangial cells can regulate the local amount of TGFβ, which as a result affects endothelial cell growth (31). This leads to disturbance of the glomerular filtration barrier and cross-communication with the podocytes, inducing podocyte injury and albuminuria (32).

Podocytes

Podocytes are specialized epithelial cells found exclusively in the glomerulus (33). The podocyte has a highly differentiated structure, with major processes extending from the cell body and further dividing into foot processes that wraps around the glomerular capillaries (34, 35). Where the filtration takes place is between the foot processes with the slit diaphragm of proteins making up a highly selective part of the glomerular filtration barrier. Maintenance of the structure of the podocyte and the intactness of the slit diaphragm is crucial in maintaining a selective filtration barrier in the kidney. Thereby several proteins in the slit diaphragm, integrins that attaches the cell to the basement membrane, focal adhesion proteins and the actin cytoskeleton are dynamically interacting and changing to adapt to changes in the environment striving to keep the filtration barrier intact (16). Disruption of any of these structures results in podocyte dysfunction (podocyte foot process effacement), resulting in proteinuria. Podocyte dysfunction has been found to be a denominator for many kidney diseases, such as Focal Segmental Glomerulosclerosis

(FSGS), Membranous Nephropathy (MN), Lupus Nephritis (LN) and Minimal Change Disease (MCD) as examples (36).

Glomerular endothelial cells

Glomerular endothelial cells are highly fenestrated cells that are located on the inside of the glomeruli facing the blood stream. The glomerular endothelial cells are covered by a thick gel-like layer called the glycocalyx. The glomerular endothelial cells and the glycocalyx are the first components of the glomerular filtration barrier (37). It has been shown that damage to the glycocalyx induces proteinuria (38, 39) and dysfunction of endothelial cells plays an important role in many glomerular diseases, for instance pre-eclampsia, hemolytic uremic syndrome (HUS) and DN (40, 41). In IgAN, studies have demonstrated that nitric oxide (NO) plays an important role in the disease progression and endothelial NO synthase (eNOS) could be a potentially regulatory factor for inflamed glomeruli (42, 43). Podocytes produce factors, such as vascular endothelial growth factor (VEGF), that are essential for glomerular endothelial cell phenotype with e.g. fenestrations (44, 45). Even more interesting is that endothelial cells affect podocyte function for example in certain forms of FSGS (46) and most likely in DN as well.

1.2 Kidney diseases

CKD is categorized into 5 stages, where stage 1 is mild kidney damage and stage 5 is referred to as kidney failure. Stage 5 is often called end stage renal disease (ESRD) and when patients reach this level, dialysis or kidney transplantation is needed. The causes of CKD are various, among which diabetic nephropathy and glomerulonephritides are the most common ones (47).

1.2.1 Glomerular diseases

Glomerulonephritis (GN) is a kidney disease characterized by glomerular inflammation (48). It includes several subclasses, mainly subdivided into proliferative and non-proliferative categories. For

example, minimal change disease (MCD), thin basement membrane disease (TBMD) and focal segmental glomerulosclerosis (FSGS) are considered non-proliferative, while IgAN and Henoch-Schönlein purpura (HS) are considered proliferative glomerular diseases. Many of the glomerulonephritides are idiopathic with unknown underlying mechanisms. A kidney biopsy is in most cases needed for diagnosis. Unfortunately, there is still a lack of specific treatments for glomerulonephritides. Drugs against glomerular capillary hypertension such as ACE inhibitors (angiotensin converting enzyme) or ARBs (angiotensin receptor blockers) are considered standard of care and on top of this corticosteroids and other immune modulating therapies are sometimes used depending on the disease etiology and regional preferences (49-53).

1.2.2 Immunoglobulin A and IgA nephropathy

Imunoglobulin A (IgA) (Figure 3) has two subclasses, IgA1 and IgA2. In human serum, IgA1 is primarily presented (around 85%) (54). IgA

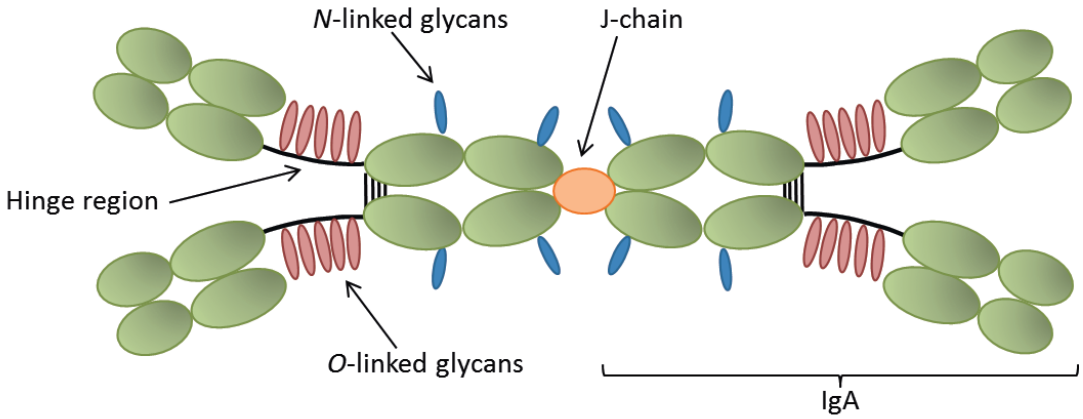


Figure 3. IgA can be found in the circulation as monomers, dimers and larger polymers. Dimeric IgA consists of two monomers of IgA that are linked together by a joining chain. IgA (gd-IgA) has 3-6 O-linked glycans in each hinge region and in patients with IgAN there is a large portion of the IgA that lacks galactose in this region (galactose-deficient IgA, gd-IgA).

exists normally in its monomic form while a small proportion, 1-2%, is polymeric (55). The IgA has an 18 amino-acid tail at the end of the C terminus that consists of a joining chain (J-chain), which makes IgA

possible to form polymers. Its heavy chain contains two *N*-glycans and a hinge region as potential attachment sites for *O*-glycans. Glycosylation studies have showed that aberrantly glycosylated IgA1 can be found in patients with IgAN and this special form of IgA1 exhibits galactose-deficient *O*-glycans on the hinge region (56-60).

IgAN is the most common form of GN around the world (61). Patients with IgAN have galactose-deficient IgA (gd-IgA) in the circulation and

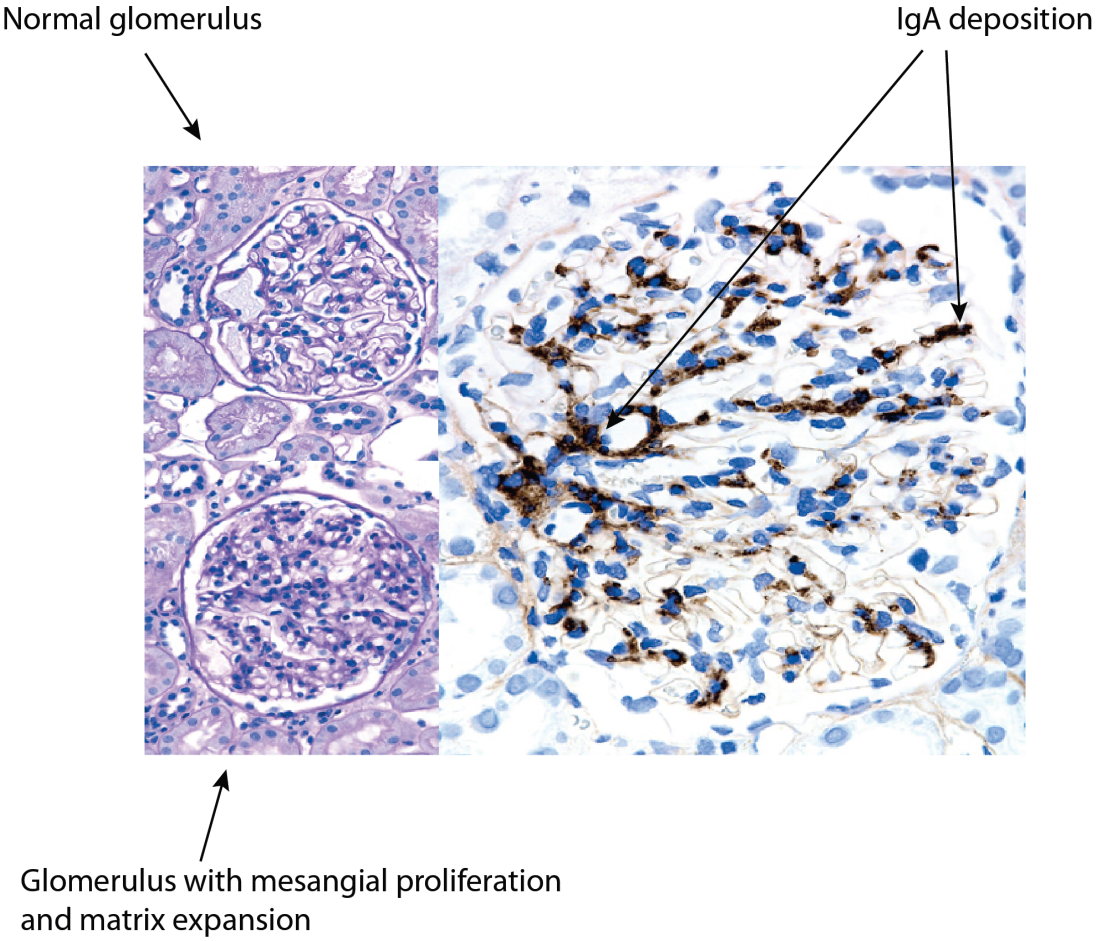


Figure 4. Kidney sections with glomeruli from a healthy person showing a normal glomerulus, and below from a patient with IgAN presenting with mesangial proliferation. Picture to the right shows staining for IgA deposition (brown) in a patient with IgAN.

the gd-IgA forms immune complexes that are deposited in the glomeruli, and more specifically in the mesangium (62). In IgAN, the key findings are proliferation of the mesangial cells together with

expansion of the mesangial matrix and this is coupled to the deposition of IgA-containing immune complexes (63) (Figure 4). The process is usually slow, but this may vary. However, with time a significant number of patients will end up with ESRD (64). It is interesting to note that of the patients with IgAN undergoing renal transplantation, around 40-60% will have recurrence of IgAN (65-71), the remaining part will not, although they still have the same gd-IgA in their circulation (66, 67, 72-75). In addition there are healthy individuals having gd-IgA in their circulation and even IgA deposits in their kidneys without developing IgAN (76). Thus, the deposition of gd-IgA is not the only factor triggering the disease.

Many new developments have made notable contributions to the field of IgAN, including discoveries of genetic loci, mechanisms of immune-complex induced kidney injury, improvements of the diagnosis methods, etc (77). The complement system has been studied extensively and it has been suggested to have a role in the pathogenesis of IgAN (78). Complement activation has been found to have direct interaction with gd-IgA immune complexes in the circulation and also in the depositions (78-80). C3 fragment is also found at increased levels in the plasma with circulating gd-IgA suggesting a possible association with IgAN (81-83). Hence, the complement C3 fragment can serve as a potential biomarker for IgAN. Regarding biomarkers, there are also many other studies conducted to search for suitable and accurate biomarkers for diagnosis and monitoring of IgAN. As described in Moresco et al (2015), many candidates such as IL-6, complement components, MCP-1, urinary EGF have been suggested (84). Micro-RNAs are also studied in IgAN, which may be used to diagnose and monitor the disease (85). In addition, different gd-IgA receptors are also widely studied. The transferrin receptor (TFRC or CD71) is generally accepted as a potential receptor for gd-IgA. In this case, FcalphaRI (CD89) is needed to initialize the formation of gd-IgA immune complexes, which bind to CD71 resulting in the deposition in the mesangium (86, 87). Furthermore, many genome-wide association

studies (GWAS) have identified several potential loci on chromosomes 6q22-23, 2q36, 4q26-31, 17q12-22 and 3p24-23 via a genetic linkage approach of familial forms (88). Even though no causal genes are found yet, the studies have shown that IgAN is a highly genetically related disease.

1.3 Global profiling studies on kidney diseases

The development of new technologies has enabled large-scale data collection in a way not possible before. The large data sets can include analysis of the genome, epigenome, transcriptome, proteome and metabolome (89). In order to interpret the connection of many targets at one time it is necessary to use different computational algorithms, commonly referred to as bioinformatics. By combining large data set analyses with traditional experimental approaches we can identify new potential drug targets or biomarkers and we believe in the end to use this for more precise diagnosis and prognosis, as well as more specific treatments of the patients.

1.3.1 Gene expression profiling

Gene expression profiling is the analysis of all the gene expression patterns in individual biological entities. It provides information about what genes that is active in a tissue/cell at a certain time point. Transcriptomic expression analysis is used in this thesis to analyze the mRNA expression level on a global scale for the whole genome. Microarray technique is widely used nowadays, but more and more replaced by a sequencing technology called RNA-seq (89). Many databases have been established to investigate pathways, function annotations (i.e. gene ontology), gene networks/interactions, etc. to help us understand the data derived from the profiling analysis.

1.3.2 Proteomic profiling

Large-scale proteomic analysis is a technique that recently has been substantially promoted both in academic research and in the pharmaceutical industry (90, 91). The advances of mass spectrometry

techniques enable analysis of all the proteins from biological samples at a global level (92). Proteomics can be applied to many different types of samples, such as blood, tissue, cells and even urine. Urine samples particularly, have been investigated extensively in order to find potential biomarkers for early diagnosis of kidney disease or responses to therapeutic treatments of kidney disease (93, 94). During the last decade, proteomic analysis has improved in accuracy, resolution, speed and also the ability of detecting isoforms and post-translational modification of the proteins, such as protein phosphorylation (95). Integrating with genomic approaches, it enhances the global understanding of the human diseases on a systems biology level (96).

1.4 Micro-RNA in kidney diseases

Micro-RNAs are small RNAs of 21-23 nucleotides without coding functions. But they work as regulators for gene expression at a post-translational level (97-99). Micro-RNAs have been utilized widely in searching for prognostic and therapeutic biomarkers due to their stability (100). They have been found to be important in kidney development, hemostatic conditions and disease progression (101). It has been suggested that micro-RNAs play a role in podocyte damage in diabetic nephropathy (102, 103). Additionally, many enriched micro-RNA candidates have been found to be potential non-invasive biomarkers for diagnosis of DN (104-107). In IgAN, the expression of miR-148 has been identified to have direct connection to the early stage of the disease (108). Studies also have demonstrated that certain micro-RNAs are important in many other kidney diseases such as lupus nephritis and hypertensive nephropathy (101, 109).

2 AIMS

The aim of the thesis was to investigate the molecular mechanisms behind glomerular kidney diseases with focus on IgA nephropathy. Specific aims are stated below:

- To define and understand the roles of mesangial cells and their relevant factors in the onset and progression of IgA nephropathy (paper I and paper II)

- To find and determine potential new gd-IgA receptors in the mesangium and investigate its role in gd-IgA deposition, cell proliferation and inflammation (paper III)

- To investigate micro-RNAs in kidney diseases and the regulative function of a specific micro-RNA in podocytes (paper IV)

3 METHODOLOGICAL CONSIDERATIONS

Detailed method and material description is given in the individual papers. This section contains methodological considerations regarding how the materials and methods were selected and a discussion of pros and cons of the methods used.

3.1 Ethics

All the experiments performed in this thesis were approved by an ethical board. For the first three papers we obtained ethical permits from the local regional ethics board in Gothenburg (#110-98; #653-05; #413-09; #432-09; #555-02). For the last paper the ethical permit for human urine sample was approved by the ethics committee of the Hannover Medical School (#1709-2013) and zebrafish experiments were approved by Institutional Animal Care and Use Committee of the Mount Desert Island Biology Laboratory, Maine (IACUC protocol#0804). All patients who participated in the studies signed written consents after oral and written information had been provided.

3.2 Patients and biopsies

Glomerular diseases are diagnosed by taking renal biopsies. Pathologists perform routine biopsy diagnosis of the patient material, but this leaves no material for research. In 2004, our group started a study at the Sahlgrenska University Hospital to pursue studies on patient biopsy material. Patients undergoing routine renal biopsies and who confirmed to participate in the study agreed to give material not needed for diagnostic purpose to research. One of the biopsies taken was therefore stored in RNAlater and put in refrigerator for 24 hours prior to transferring to -80°C storage if not needed by the pathologist. Those who were diagnosed with IgAN were selected for further analysis. Samples from healthy living kidney donors were also collected in a similar procedure and subjected to the same storage

conditions and used as controls in our studies. Routine clinical data were assembled at the time of biopsy and the patients were followed for up to 7 years after diagnosis. A special procedure was also introduced to harvest free glomeruli left on the biopsy needle (Paper I and section 3.3.1).

In paper IV, morning urine samples were collected from patients diagnosed with different glomerular diseases based on biopsy evaluations. Urine samples were pooled (including healthy controls) and used for micro-RNA screening.

3.3 Cell culture

In vitro cell culture is a powerful approach to study the contribution of individual cell types and the cells can be subjected to different

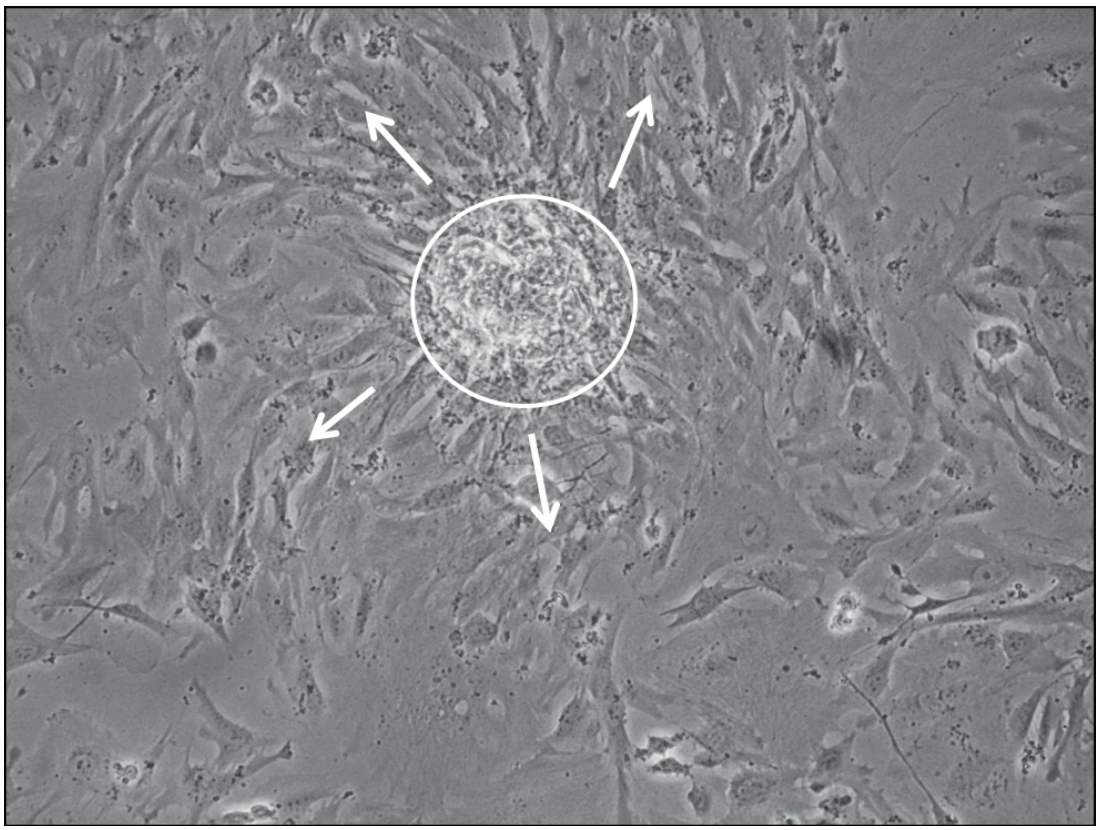


Figure 5. After 10-20 days of culturing mesangial cells can be seen growing out of the glomeruli. The glomerulus is marked with a white circle.

treatments, overexpression of genes or knockout of selected genes. The disadvantage is that the cells are isolated from their natural surrounding environments. However, it is the only efficient approach to study the individual cells and how they behave under different experimental conditions. In this thesis, we have cultured all three different cell types found in the glomerulus from human tissue.

3.3.1 Mesangial cell culture

Paper I – III focuses on IgAN, a glomerular kidney disease where the mesangial cells and their matrix environment are of great importance. In the thesis, we cultured primary mesangial cells both from patient biopsies and primary human mesangial cells obtained commercially (Lonza, Basel, Switzerland).

We developed a unique technique enabling culture of mesangial cells from kidney biopsies. The biopsies were dipped into saline solution and later transferred to RNAlater to preserve the RNA content. Loosely attached glomeruli that fell of the biopsy into the saline solution were moved to cell culture plates with attachment factor and medium with human serum and antibiotics. The mesangial cells grew from the edges of the glomeruli after 10 – 20 days (Figure 5) and were sub-cloned to new culture plates for further experiments. The mesangial cells were evaluated morphologically and by expression of smooth muscle actin protein while negative of synaptopodin expression (podocyte marker) and Ulex Europaeus (endothelial cell marker) (110-112).

3.3.2 Cell culture for other cells

In paper III and paper IV, we cultured human podocytes, endothelial cells, tubular cells and human embryonic kidney cells (HEK293) in addition to mesangial cells. Human podocytes were cultured in RPMI medium with insulin and antibiotics at 33°C and then transferred to 37°C for differentiation. Endothelial cells were cultured in endothelial growth medium containing cytokines, growth factors and supplements.

Human HEK293 cells were cultured for transfection experiments of protein knock-down and overexpression. The cells were cultured in DMEM medium with 4.5g/L glucose with L-glutamine.

3.4 Global gene expression analysis

Global gene expression analysis can give new insights of underlying mechanisms for disease, especially for those where the mechanisms behind the disease is lagely unknown. It is a significant dimension of this thesis. By using this approach, we think we can increase the understanding of the role of specific genes, as well as the signaling transduction, thus leading us to understand the molecular background of the disease.

3.4.1 Gene microarray

We used the Affymetrix microarray platform to investigate the transcriptomic profiling of patient biopsies for studies on IgAN. The study was designed to compare IgAN patient samples (age 42.91±15.49, female/male ratio 1:2) versus healthy living donors (age 47.76±12.55, female/male ratio 1:1.3). Biopsies and living donor tissues were micro-dissected under a light microscope to separate the glomerular from the tubular parts. RNA was extracted using RNA extraction kit and these samples were run on a microarray, the Affymetrix GeneChip Human Genome U133 Plus 2.0. The patient biopsy samples and healthy living donors were collected and run at different time points (as batches). The batches may give non-biological variation in multiple batch microarray experiments. So the biggest challenge in the analysis was in-batch normalization of the data and batch effect removal. We followed a routine bioinformatic normalization approach modified by the Kretzler group (Internal Nephrology, University of Michigan) (113) to generate solid normalized data. Also, we used Empirical Bayes method to remove the batch effects to gain accurate statistics. As a result, we analyzed 25 IgAN patient samples together with 26 healthy controls for comparison.

Ingenuity pathway analysis (IPA) software and Genomatix pathway tool were used to investigate the signaling pathways based on the microarray data. Part from that, we also checked Gene Ontology (GO) categories of all the significantly regulated genes using online GO term search engine Gene Ontology Consortium (www.geneontology.org).

In the gene microarray analysis, we also integrated an analysis using cell-type positive standard genes defined by Ju et al 2013 (113) to investigate the roles of cell specific genes in our dataset. We also correlated patient clinical data with the standard genes using a z-score transformation algorithm.

3.4.2 Quantitative real-time PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was used throughout all the four papers in the thesis. It is a very useful method to detect gene expression with high accuracy. Our experiments were carried out using Taqman® qRT-PCR technique on ViiA™ 7 or QuantStudio™ 7 flex systems (AppliedBiosystems, Waltham, MA, USA).

In paper II and III, qRT-PCR was used to evaluate the microarray results. Selected genes were pre-manufactured on Low-density array (LDA) cards (AppliedBiosystems, Waltham, MA, USA) and this ensured that small amount of RNA also could be detected, which might not be possible on microarray level.

In paper I, we used qRT-PCR to investigate the expression of extracellular matrix genes, growth factors and cytokines from the mesangial cells under different treatments. In paper IV, a special qRT-PCR was used for detection of micro RNAs from the urine samples and the cell samples. The platform used was Taqman® Array Human MicroRNA A+B card set 3.0.

3.5 Protein analysis

In this thesis, part from the gene expression analysis, another important aspect is analysis on protein level. Proteins are the actual functional elements in the cells. Many proteins are also post-translationally modified, and we need to analyze protein expression to gain functional data. There are many different methods to analyze protein expression and levels, such as Western Blot, immunohistochemistry, Mass Spectrometry (MS), ELISA and multiplex. Each technique is applicable for different purposes and the availability of protein and other factors may play in when choosing methods. We used all these techniques to gain a full view of the proteins of interest.

3.5.1 Western Blot

Western blot is one of the most widely used and valuable techniques to detect proteins. It uses gel electrophoresis to separate proteins according to individual molecular sizes. Later the protein is transferred to a membrane where specific antibodies can be applied to detect the target of interest. In paper III, western blot was firstly used to find a possible receptor of IgA in mesangial cells, using IgA itself as primary antibody. In paper IV, western blot was used to detect the amount of specific proteins in the podocytes after stimulation with TGFβ and transfection using micro-RNAs. The only down fall with western blot is that it is semi-quantitative and therefore additional techniques need to be used to confirm data.

3.5.2 Immunohistochemistry

Immunohistochemistry is a useful technique to visualize the expression and localization of proteins of interest using specific antibodies. The technique can both give a general impression of the protein localization but it can also be very sensitive when using a confocal microscopy for high magnifications. It is also a powerful approach to detect co-localization of proteins. First of all, we characterized the mesangial cells cultured form renal biopsies by positive staining of smooth muscle actin and negative staining for synaptopodin and Ulex

Europaeus Agglutinin I. In paper III, we used immunohistochemistry to detect the IgA receptor localization in kidney frozen sections as well as the expression pattern in different cell types. In paper IV, immunohistochemistry was used to detect co-localization of proteins in the podocytes and also expression of proteins in human kidney sections and zebrafish models.

3.5.3 Mass Spectrometry

Mass Spectrometry (MS) has become an advanced technique for protein analysis and generate data on a larger scale. With mass tandem tags (TMT), MS is capable of analyzing a mixture of multiple samples simultaneously.

In paper II, we analyzed the protein masses from cells lysates, which were IgA treated mesangial cells and untreated control mesangial cells. The samples were TMT labeled and run through an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific, Waltham, MA). The results were analyzed statistically to find out significantly regulated proteins in the treated cells compared to control cells. In paper III, MS was used for two different purposes. Firstly, to identify the proteins found using western blot for a possible IgA receptor, we cut out the bands and the samples were prepared for MS. By MS we detected the existing proteins from the sample and we matched them to protein databases. Secondly, the receptor candidate was selected based on a number of set criteria by literature search and previous gene expression analyses. MS was also used to determine the whole protein content in mesangial cells where the receptor gene was knocked down, as well as in control mesangial cells for comparison. The results were analyzed using IPA pathway software to investigate which pathways that were changed when knocking down the receptor.

3.5.4 Growth factors and cytokines expression analysis

Growth factors and certain cytokines have been reported to play important roles in glomerular kidney diseases, certainly also in IgAN. In order to measure the growth factors and cytokines released from mesangial cells with different treatments in a high sensitivity and high throughput manner, we used the Bio-plex 200 system (Bio-rad, Hercules, CA, USA). The system uses Luminex xMAP technology (Luminex, Austin, TX, USA) enabling multiplex analysis of several growth factors and cytokines simultaneously. This technique was used in paper I.

3.6 Cell proliferation

In IgAN mesangial cells, proliferation is one of the key features of the disease. Mesangial cell proliferation ability was investigated in paper I and III. To perform proliferation assays, cell proliferation ELISA, BrdU (colorimetric) technique was used. 5-bromo-2'-dexyuridine (BrdU) was used to label the DNA of the cells and chemiluminescence technology was applied to read out the proliferation rate. In paper I, mesangial cells derived from IgAN patients and controls were treated with platelet-derived growth factor (PDGF), a growth factor known to increase mesangial proliferation, and proliferation rates were compared to wild type cells from either patients or controls.

In paper III, mesangial cells were firstly treated with an antibody (blocking antibody for the potential gd-IgA receptor) or PDGF. BrdU was applied and proliferation rates were compared. Secondly, the mesangial cells were treated with IgA purified from patients with IgAN and PDGF to see how this affected proliferation of the cells. Lastly, we both knocked down and overexpressed the receptor in the mesangial cells. These cells were later treated with blocking antibody, IgA purified from patients and PDGF. The proliferation rates from each treatment were compared with proliferation rate from the individual controls.

3.7 Knock-down and overexpression techniques

In paper III, in order to investigate the function of the potential gd-IgA receptor in mesangial cells, we knocked down the protein and also overexpressed it. For knockdown, short hairpin RNA (shRNA) inserted in pLKO.1 cloning vector from Sigma-Aldrich (Saint Louis, USA) was used to silence the expression of the targeted gene. For overexpression, the targeted gene was amplified by PCR and cloned into the overexpression lentiviral vector VVPW EGFP to be able to overexpress the protein of interest fused to the fluorescent protein EGFP.

3.8 In vivo studies using animals

Animal models are sometimes necessary in order to study human diseases. For kidney diseases, there are many rodent animals used in experimental biology to examine certain dysfunctions. For examples, passive Heymann nephritis in rats is a model for human membranous nephritis (114) and podocytes injury induced by adriamycin or puromycin aminonucleoside (PAN) model for focal segmental glomerulosclerosis (115). However, there is still no suitable model for human IgAN, although there are models in development (116, 117). In paper IV, mice and zebrafish were used to examine micro-RNA functions in podocytes.

3.8.1 Mice

Mus musculus (mouse) is a widely used species for experimental purpose in many fields. In paper IV, 6 weeks old CD1 mice (Charles River) were injected with different micro-RNAs on different days. Urine samples and serum were collected at baseline, day 7, 14, 21, 28 and at day 28, kidneys were collected for further investigations of specific protein expressions, using western blot, immunohistochemistry and PCR.

3.8.2 Zebrafish

The zebrafish has become a very important and promising model for many aspects in medical research, such as target identification, pharmacology, toxicology, etc (118). Several phenotype-based drug developments using zebrafish have been successful during the recent decade (119-121). Zebrafish has also become broadly implemented in kidney research (122, 123) and many studies are related to cell migration (124) and transgenic studies (125). In paper IV, zebrafish was used to investigate the loss of plasma proteins and effacement of podocyte foot processes when knocking down a specific extracellular matrix gene or overexpressing micro-RNA.

4 RESULTS AND DISCUSSION

The thesis is based on four papers. Papers I – III deal with a specific glomerulonephritis, IgA nephropathy (IgAN). In these three papers, we investigated the molecular mechanisms of IgAN from different perspectives: susceptibility of mesangial cells, global transcriptomic/proteomic analysis of mesangial cell function and investigations of new IgA receptors. Paper IV focused on expression of micro-RNA in glomerular cells and their regulatory function in the podocytes.

4.1 Paper I: Mesangial cells have different susceptibility towards IgA purified from IgA nephropathy patients

The key feature of the IgA nephropathy (IgAN) is that self-aggregated galactose-deficient Immunoglobulin A (gd-IgA) forms immune complexes with Immunoglobulin G (IgG), which are deposited in the mesangial area. However, it is most likely that gd-IgA complex formation and deposition are not the only cause of the disease as stated previously.

In this paper, we hypothesized that the mesangial cells had a different susceptibility to gd-IgA compared to healthy controls. Patients with IgAN may have mesangial cells that react more to the gd-IgA complexes, which makes the complex deposition in the mesangium more pathogenic. This may thus be the reason for the onset of proliferative and inflammatory events in some individuals with gd-IgA in the circulation but not all. In order to investigate the susceptibility of mesangial cells in different individuals, we treated mesangial cells with gd-IgA purified from patients and healthy controls and analyzed the expression of matrix components, release of cytokines and growth factors and the proliferative response to PDGF stimulation.

Since there is a lack of good *in vivo* and *in vitro* models for IgAN, we cultured primary mesangial cells derived from IgAN patients and healthy control biopsies and treated the cells with gd-IgA to mimic disease conditions. In this study, we included 6 patients with gd-IgA deposits and one control without gd-IgA deposits. We also added two commercially available samples cultured from individuals without renal dysfunction as controls. Three patients with IgAN and three healthy volunteers donated blood from which we purified IgA. The patient IgA fraction contained more gd-IgA than the healthy and therefore is refered to gd-IgA in the study and the IgA from healthy as cIgA.

The major growth factor for mesangial cells is PDGF and it induces proliferation of the mesangial cells. It has been reported that increased expression of PDGF and its receptor can be found in patients with IgAN (126-128). To confirm this on a cellular level, we investigated the gene expression of PDGFB (PDGF subunit B) and PDGFRB (PDGF receptor beta) after treating the cells with cIgA or gd-IgA. We also evaluated the cell culture medium to check the amount of PDGF released under different treatments. The gene expression of PDGFB was not affected in control mesangial cells, but it was significantly increased in mesangial cells from IgAN patients when treated with gd-IgA. The release of PDGF-BB into the cell medium was significantly increased after gd-IgA treatment in mesangial cells both from controls and patients. The increase was significantly higher in patient mesangial cells compared to controls. To further investigate what effect the increased release of PDGF could have on the mesangial cells we treated the cells with PDGF-BB and AB and investigated their proliferative response. We found that that the mesangial cells from patients with IgAN increased their proliferation significantly more than the control cells. This tells us that patient mesangial cells were more responsive in terms of PDGF expression and release after gd-IgA treatment. Additionally, PDGF has also been reported to increase TGFβ1 expression in the mesangial cells, inducing matrix expansion

(28, 129). We first investigated the gene expression of TGFβ1 in mesangial cells with different IgA treatments. As for PDGF we found that only the mesangial cells from patients with IgAN increased their expression of TGFβ1 significantly when treated with gd-IgA. At the protein level the IgAN mesangial cells released increased amounts of TGFβ1 in the medium with treatment of either cIgA or gd-IgA.

The release of cytokines/chemokines has also been associated with IgAN in terms of proliferation and matrix expansion (130). In our analysis, we found that gene expression and release of interleukin-6 (IL-6) were significantly increased both in control and patient mesangial cells when treated with gd-IgA. The release of IL-6 into the medium was significantly higher in patient mesangial cells. The result is consistent with study done by Kim MJ et al (2012) where IL-6 is suggested to be involved in the pathogenesis of IgAN (131). However, the effect of cIgA was not investigated in that study. Another chemokine CCL5 (also known as RANTES) was reported to be related to proteinuria of the IgAN paitents (132). It was found to have an increased release in both control and patient mesangial cells after gd-IgA treatment. However, interleukin-8 (IL-8) and MCP-1 were found only significantly increased in control mesangial cells in our hands.

Mesangial cell matrix expansion is another key feature of IgAN. We analyzed expression of selected matrix genes under different treatment conditions. We discovered that decorin (DCN), a small proteoglycan, was significantly increased in untreated IgAN patient mesangial cells. Decorin functions as a natural inhibitor of TGFβ and in our previous studies of matrix genes, we have found increased gene expression of both DCN and TGFβ in IgAN (133). Several other matrix genes including BGN (biglycan), COL4A1 (collagen alpha-1(IV) chain), DCN (decorin), FN1 (fibronectin), HSPG2 (perlecan) and NDST1 (heparan sulfate N-deacetylase/N-sulfotransferase 1) also had elevated expression after IgA stimulation, but not to significant levels.

In conculsion we have shown that mesangial cells from patients with IgAN have increased reactivity to gd-IgA compared to normal cells. The cells from IgAN patients have an increased proliferative response to PDGF. Mesangial cells from IgAN patients also released more growth factors and cytokines when treated with gd-IgA. We speculate that this may be due to altered responsiveness to gd-IgA in patients with IgAN and this was further investigated in paper III.

4.2 Paper II: Global gene expression and proteomic analysis reveal important mesangial cell functions in IgA nephropathy

From previous studies, we and others have seen increased expression of matrix genes in IgAN compared to healthy controls. Also major growth factors such as PDGF and TGFβ1 are increased in IgAN

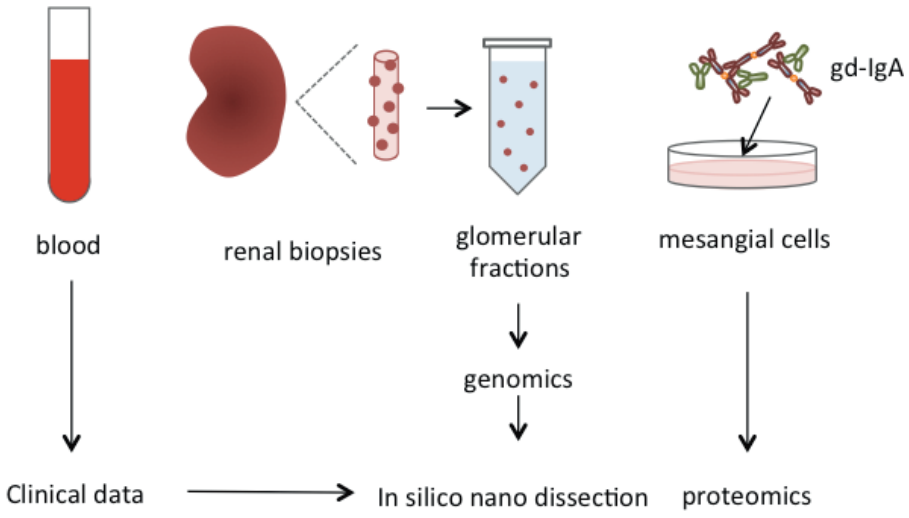
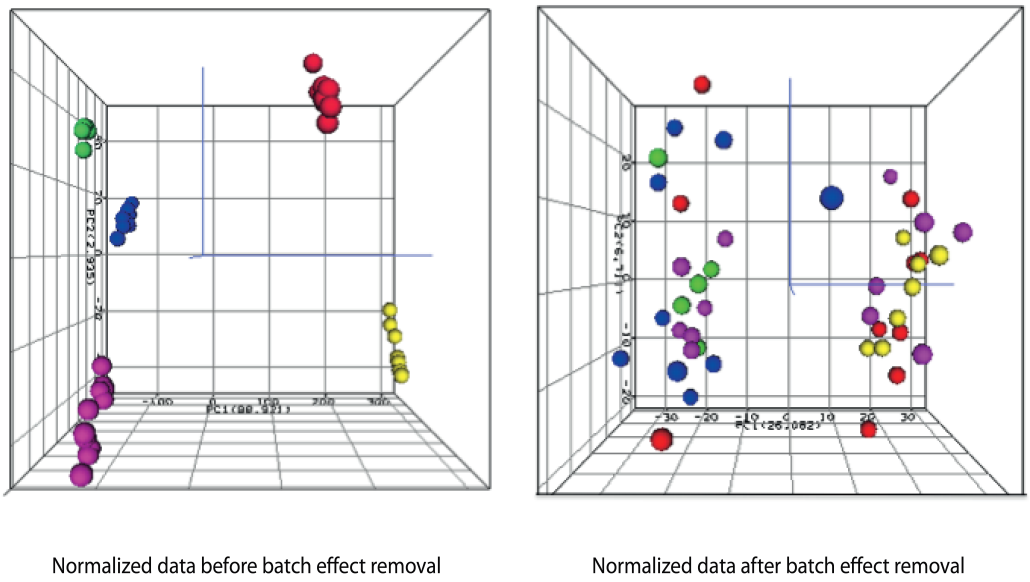


Figure 6. Workflow of the different steps involved in this study. Glomeruli were dissected from renal biopsies and mRNA was extracted for microarray analysis. Clinical data was at the same time collected and used to correlate with standard gene profiling that are identified by "In silico nano dissection". Proteomics of the untreated and gd-IgA treated mesangial cells were analyzed with mass spectrometry.

patients in terms of gene expression and cellular release. However, to be able to understand the complete structure of how the signals are transferred a broader approach is ultimately necessary. In this paper,

we conducted a global gene expression analysis and a proteomic analysis to investigate mesangial cell function in IgAN (Figure 6).

As stated previously, we started a study at the Sahlgrenska University Hospital to collect patient biopsies in 2004. As a result, there were in



Normalized data before batch effect removal

Normalized data after batch effect removal

Figure 7. Principal component analysis of normalized microarray data before and after removing the batch effect. Normalized data were clustered into individual batches before removing the batch effect. Using ComBat method could significantly reduce the effect and cluster the data into control and patient groups.

total 25 IgAN patients and 26 healthy living donors' samples used for Affymetrix GeneChip Human Genome U133 2.0 microarray. The samples were collected through the years and the microarray experiments were run at different time points. This sub-divided the raw data into different batches. According to the latest in-batch normalization method developed by Kretzler group, we normalized the raw data using Robust Multiarray Averaging (RMA) (134) within the samples in the same batch. Quality check was performed in individual batches as well using different visualization tools (NUSE, RLE, RNA degradation and PCA plots, details see paper II). The selection criterion for an outlier was 3 out of 4 plots showing bad quality. By these means, outliers were excluded from the analysis and in total we

included 20 patient samples and 22 controls for statistics. The data from different batches were merged using Empirical Bayes method based batch effect removing algorithm (Figure 7) (135). The patient samples with IgAN were well separated from the healthy living donors in the clustering analysis. One patient sample was run in two batches as endogenous control and they clustered together as well.

The normalized data was analyzed using the Significant Analysis of Microarray (SAM) method (136). It is a statistic approach designed for large-scale gene expression data. At a SAM q-value of <0.01, there were more than 700 genes significantly changed with more than half fold change for both up and down regulation. Many of these genes belong to extracellular matrix component reflected by Gene Ontology analysis, which proved the results in paper I where we found many matrix genes were regulated on cell level. Apart from that, the significantly regulated genes were included in many important pathways. We used Ingenuity Pathway Analysis (IPA) software to investigate the top regulated pathways. As a result, the top regulated pathways were related to proliferation and inflammation with many cytokines/chemokines involved, like IL-6 and IL-8 that we described in paper I. Complement system has been shown by others to play a role in IgAN (83, 137-139) and we also pointed out that in paper I. In the microarray data analysis, complement system was represented in the pathway analysis as well.

An excellent paper published by Ju et al in 2013 introduced an iterative machine-learning algorithm to define cell-type specific transcripts using whole tissue microarray data (113). By applying data obtained from analysis of kidney disease, cell-type specific positive standard genes were identified for mesangial cells and podocytes. A small set of genes were also defined for glomerular endothelial cells however they were not confirmed. There are in total 35 genes identified for mesangial cells and 50 for podocytes. The positive standard genes are important factors in individual cell types in terms of kidney disease. Recent studies have shown a tendency that podocyte injury is also

related to IgAN (140) but mesangial cells are problably the major cells affected. However, it may be argued that we do not know which compartment of the glomerulus that is mostly affected in IgAN. In our list of significantly regulated genes, more than ¾ of the mesangial cell positive standard genes were found significant, compared to less than ½ for the podocyte positive standard genes. In our clustering analysis, we used the gene expression values from these positive standard genes to cluster the patient samples and the controls. Using mesangial positive standard genes, the patient samples were well separated from the controls, while no such pattern was seen using podocyte positive standard genes.

At the time of taking biopsies, clinical data was recorded for further analysis. The patients were also followed from day of biopsy and new clinical parameters were registered every 6 months for the first years and then on a yearly basis. In order to correlate the clinical data with gene expression values, we used the expression data of the positive standard genes to calculate patient z-scores. The z-scores represented deviation of the standard gene expression for each patient based on standard gene expression of all the patients. It is an approach to measure the magnitude of gene expression change in individual entities. In our analysis, we found that the patient z-scores were significantly correlated with patient eGFR and serum creatinine values using mesangial cell positive standard genes. eGFR was calculated using CKD-EPI equation (141) and the lower the eGFR is, the worse renal function the patient has. Patients with kidney disease have a poor creatinine clearance ability that could likely increase serum creatinine level, which is a parameter used to determine the stage of disease. Together with eGFR, the correlation with z-scores both indicated that mesangial standard genes indeed affected patient disease conditions as output. The z-scores calculated based on podocyte positive standard genes gave no significant correlations at all. Therefore, we conclude that the mesangial cell is the dominant element with regards to IgA and not the podocytes.

There is no good *in vivo* or *in vitro* model for IgAN that perfectly mimicks the disease and all data must be interpreted with some

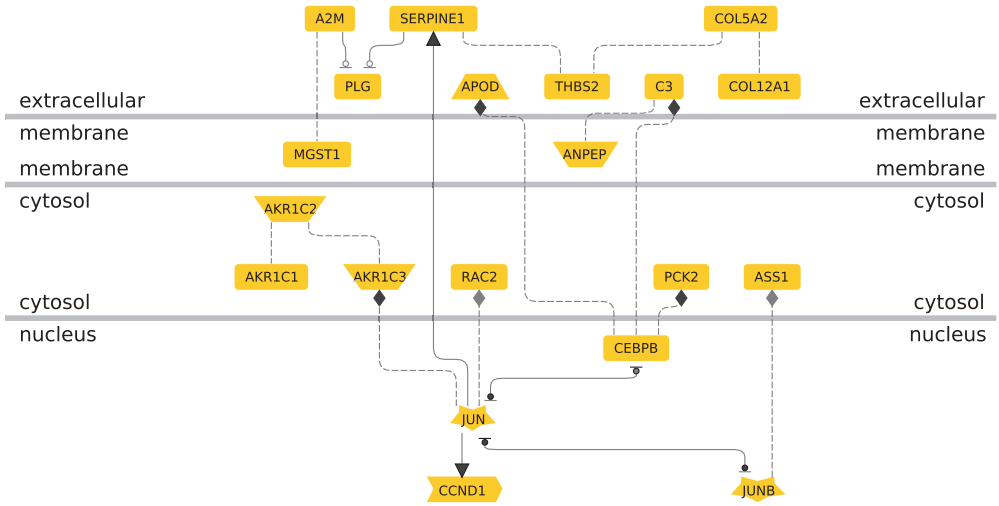


Figure 8. Pathway networks based on commonly found genes/proteins in both microarray and MS data analysis.

caution. In paper I, we could confirm previous studies (28, 142), that treating the mesangial cells with gd-IgA increases release of cytokines and growth factors, as well as increases the proliferation rate. In this paper, we designed an environment mimicking the conditions of IgAN. We cultured mesangial cells and treated them with purified IgA from patients with IgAN (higher level of gd-IgA was confirmed using ELISA) over 48 hours. Thus, the mesangial cells were incubated with *in vitro* gd-IgA in the medium and we could investigate how the treatments affect the cells. In this setup, we included 5 control mesangial cell samples without treatment and 5 treated with gd-IgA. Mass spectrometry (MS) was used in this experiment to analyze the protein contents in the samples. T-test with Benjamin-Hochberg adjustment (143) was applied to identify significantly regulated proteins from treated cells compared to controls. The significantly regulated proteins were analyzed using IPA software for pathways. Interestingly, 22 significantly regulated pathways found in the MS data were matched in the microarray gene expression data from whole glomeruli. These commonly regulated pathways both *in vivo* and *in*

vitro could give us a strong hint on the mechanisms of IgAN in proliferation and inflammation. They are also inter-connected via commonly found genes/proteins forming pathway networks (Figure 8). Further investigations are needed for individual pathways and the pathway network to define the specific functions.

In summary, this paper demonstrated the importance of mesangial cells in IgAN patients from both a transcriptomic and a proteomic perspective. The mesangial cell positive standard genes showed a prominent role in regulating the disease and correlated to the clinical conditions. These positive standard genes can be evaluated as potential drug targets or biomarkers for predicting the disease. The commonly found significant pathways both *in vivo* and *in vitro* may certainly be a key to understanding the mechanisms behind IgAN.

4.3 Paper III: A potential receptor of IgA is involved in the proliferation of the mesangial cells and the development of IgA nephropathy

From paper I and paper II, we hypothesize that certain types of mesangial cells are more reactive to gd-IgA and the mesangial cells themselves are key elements in IgAN. However, it still does not explain how the gd-IgA is deposited in the mesangial region. The transferrin receptor (TFRC or CD71) is the only known IgA receptor to be expressed by mesangial cells and it has consequensly been suggested as a potential candidate (86). Another protein, soluble FcalphaRI (CD89) was found to initialize the formation of gd-IgA immunue complexes in the circulation and subsequently, CD71 function as gd-IgA receptor mediating the depostition in the mesangium (144, 145). However, the results are still not fully confirmed (146).

Our hypothesis is that there is a receptor that is more common in the IgA susceptible mesangial cells compared to cells from healthy

controls and that this receptor has a direct or indirect interaction with gd-IgA causing the deposition. This interaction eventually leads to cell

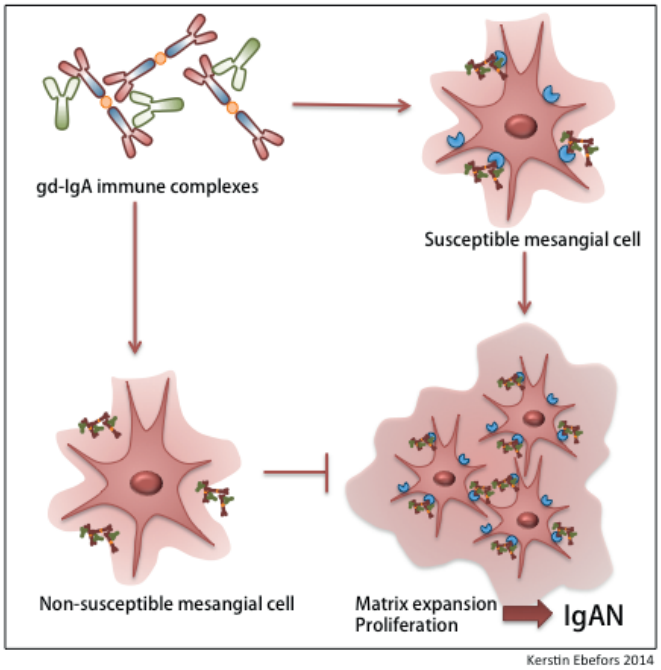


Figure 9. Our hypothesis. gd-IgA containing immune complexes deposit in the mesangium. Interaction with an IgA receptor on the cell membrane makes the deposits pathogenic leading to cell proliferation and inflammation, reducing kidney function.

proliferation and inflammatory responses (Figure 9). In our search for such a gd-IgA receptor, we used cell lysates from a patient with pathogenic IgA deposits and ran western blots against a healthy control sample. To detect proteins binding to gd-IgA, IgA purified from IgAN patients was used as primary antibody for the blot (details of detection gd-IgA content in the patient using ELISA is in paper I and II). In the result, there was a much stronger band in the patient samples compared to the control. The proteins in the band were identified using mass spectrometry. Based on extensive literature studies and our previous research, a specific transmembrane protein was found to be a potential candidate. This protein (named protein-R from now) was previously found to function as a receptor in epithelial cells, type II pneumocytes and vascular smooth muscle cells for different substances. Since mesangial cells have been characterized as a modified type of smooth

muscle cells (20), we believe that protein-R could also be a receptor in mesangial cells. In addition, it was found to be involved in with cell proliferation in other cell types. However, no solid studies have investigated this protein and its function in the mesangial cells. First of all, we used immunohistochemistry to determine the localization of protein-R on human frozen kidney sections. As a result, we found it located to all three cell types in the glomerulus. In our quantitative PCR results, the Ct values also showed an abundant amount of protein-R expression in all three cell types. In cultured mesangial cells from IgAN patients, we found a significant up-regulation of protein-R gene compared to controls and it was at a much higher expression level compared to the TFRC gene. This was also consistent with our microarray data described in paper II.

Proliferation is one of the key features of IgAN and generally speaking, it is believed to be a cause of kidney damage (77, 147, 148). We hypothesize that protein-R has a direct or indirect interaction with gd-IgA, which regulates the deposition of gd-IgA in the mesangium and consequently proliferation of the mesangial cells. Therefore, we wanted to investigate the cell proliferation rate under different conditions. We used a specific antibody that has a blocking function described in other studies to block protein-R on the cell membrane of mesangial cells. The proliferation was found significantly increased comparing to unblocked controls. This gave us a hint that the existence of protein-R on the cell surface could prevent cell proliferation. The same pattern was found when treating the cells with IgA purified from IgAN patients. This indicated that protein-R could function as a "break" for cell proliferation and gd-IgA might block/react with protein-R in a way that the "break" was malfunctioning.

To further investigate the proliferative response in regards to protein-R on mesangial cells, we tested if the response to the most important growth factor in mesangial cells, PDGF, was altered in mesangial cells depleted of protein-R. Cells were exposed to gd-IgA, PDGF and blocking antibody. Interestingly the increase in proliferation found by

gd-IgA was diminished in mesangial cells lacking protein-R while no alterations in PDGF response was shown. This indicated that the proliferative response downstream of protein-R is not directly coupled to PDGF signaling. Contradictory, overexpressing protein-R in mesangial cells did interfere with PDGF signaling with cells not being able to respond to PDGF-BB nor gd-IgA. Further investigations concluded that total protein level of the PDGF receptor was increased while no change was shown in regards to activated phosphorylated PDGF receptor in overexpressing cells. Akt signaling downstream of the PDGF receptor in overexpressing cells was disrupted, indicating that the overexpressed protein-R interferes with PDGF receptor signaling presumably by influencing PDGF receptor activation or by affecting PDGF receptor dimerization.

Further investigations of the relevant downstream pathways are needed. A detailed illustration is found in paper III. Comparing to the controls, knockdown of protein-R mainly influenced pathways related to proliferation, inflammation and cell migration, which can be correlated to matrix expansion pathways found in IgAN.

In conclusion, protein-R has been found to have multiple roles in different cells in the literature. In our study, we successfully demonstrate how protein-R was involved in cell proliferation and how it was related to PDGF. This is the first time that protein-R is investigated in the glomerulus. Based on our findings, an association of protein-R and PDGF (or its receptor) to gd-IgA is suggested to be a mechanism triggering development of IgAN.

4.4 Paper IV: Regulation of a certain micro-RNA (miR-x7) in podocytes is involved in glomerular diseases

Many glomerular diseases lack known molecular mechanisms and this makes diagnosis and treatment difficult. Micro-RNAs are small molecules regulating translation. Recently, micro-RNAs have been

payed a particular attention in many disease settings including the field of kidney research (149). This paper was conducted to investigate if a certain micro-RNA was involved in the onset and progression of glomerular diesase and if it could be a non-invasive diagnostic marker for specific kidney diseases.

First of all, urine samples from patients with different glomerular diseases were collected and a micro-RNA profiling using a large scale screening technique was applied. The diseases included FSGS, Minimal Change Disease (MCD), Membranous Nephropathy (MGN), preeclampsia/eclampsia (PEEC), ANCA positive vasculitis (ANCA), Diabetic Nephropathy (DN), Haemolytic Uremic Syndrome (HUS) and IgAN. Human podocytes, mesangial cells, endothelial cells and proximal tubular cells were cultured untreated or TGFβ treated and the same micro-RNA screening was run on at cellular level. TGFβ was used to induce stress on the cells. As a result, a certain micro-RNA, coded miR-x7, was identified to be significantly increased in FSGS, MCD and MGN urine samples compared to controls and other glomerular diseases, as well as in stressed (TGFβ treated) podocytes.. A potential target of miR-x7, an extracellular matrix protein, was identified using an online prediction tool called mirtarbase. This extracellular matrix protein (called em-protein here) is known to be a ligand to integrin during kidney development (details see paper IV). From the staining on human kidney sections, we found the em-protein mainly located in the podocytes and the glomerular basement membrane (GBM) area. The gene expression analysis also showed a much higher level of expression in the podocyte comparing to other cell types in the glomerulus. miR-x7 overexpressing podocytes expressed less amount of the em-protein while control podocytes showed no difference. It indicated that miR-x7 regulated em-protein by inhibition in the podocytes. From further investigation of the expression profile of different patient samples, we found that em-protein was down regulated, while miR-x7 was up regulated in MGN and FSGS. This confirmed the micro-RNA screening results and also

demonstrated miR-x7 regulating em-protein in the podocytes in specific diseases.

Next, two *in vivo* animal models were used to further investigate the expression of miR-x7 and em-protein in zebrafish and mice. Knocking down of em-protein gene (homolog of human em-protein in zebrafish) and overexpression of miR-x7 in zebrafish induced a severe edematous phenomenon. In addition, transgenic zebrafish experiments showed that knocking down of the em-protein gene or overexpression of miR-x7 decreased the amount of plasma proteins in the zebrafish, indicating protein leakage through the glomerular filtration area. Furthermore, podocyte effacement was observed in zebrafish with knocked down of the em-protein or overexpressed miR-x7. The structure of the GBM was also significantly changed. In the mice, knocking down of the em-protein or overexpression of miR-x7 increased the albuminuria significantly, induced podocyte effacement, as well as an altered protein expression (details see paper IV).

In conclusion, miR-x7 regulating em-protein in the podocytes could be a mechanism involved in glomerular disease including MGN and FSGS. The miR-x7 in the urine is potentially a non-invasive marker for detecting glomerular disease.

5 CONCLUDING REMARKS

This thesis is composed of studies aiming to understand the mechanisms of glomerular kidney disease, with a strong focus of IgA nephropathy (IgAN) (paper I, II and III) and a specific investigation of the importance of micro-RNAs in glomerular disease (paper IV). Our discoveries have given us new insights to the mechanisms behind the development of IgAN and helped to understand the role of mesangial cells in the disease. The micro-RNA study demonstrated a new important target and/or biomarker possibly involved in glomerular kidney disease, which could be a very good approach for early diagnosis and disease progression. The concept and technique can also be used in the investigation of IgAN to find out specific micro-RNA biomarkers.

Our studies of IgAN were conducted using a general-to-specific methodology, from an overview of global transcriptomic and proteomic profiling, mesangial genes/proteins expression and cytokines/chemokines functions to a specific potential IgA receptor and its proliferative properties affecting the disease. From paper I, we hypothesized that a proportion of mesangial cells were more susceptible towards gd-IgA by the release of more relevant growth factors and cytokines, which stimulated the cells to a proliferative onset. Mesangial cell extracellular matrix genes were also found to be significantly upregulated. In paper II, we continued to investigate the transcriptomic and proteomic information on a global level. The combined *in vivo* and *in vitro* profiling analysis showed many important inflammatory pathways that could have a potential role in disease development. Based on the positive standard genes from different cell types, we confirmed that mesangial cells are likely to be the most important cell type in IgAN, certainly when compared to podocytes. Additionally, the z-scores of mesangial standard genes correlated with clinical eGFR and serum creatinine values, which further proved that mesangial cells play an important role in IgAN.

Many of these genes could be potential factors for the disease, but need further confirmation of their specific function. In paper III, we went on identifying an IgA receptor, discovered protein-R, a protein that was detected in a western blot using gd-IgA as primary antibody. The protein was found to highly correlate with the proliferative rate of the mesangial cells when applying different stimuli. Overexpressing protein-R in mesangial cells altered PGDF receptor response with disruptions in Akt downstream signaling, indicating involvement of protein-R in PDGF signaling.

In paper IV, instead of focusing on a specific glomerular disease, we investigated a number of glomerular kidney diseases, but from a micro-RNA perspective. It is a modern but rather fundamental area in the research of kidney diseases. In our study, a specific micro-RNA miR-x7 was found to regulate em-protein in the podocytes leading to podocyte damage and loss of kidney function. It was found to be related to progression of diseases such as MGN, MCD and FSGS. Since miR-x7 may be detected in the urine it may be used as a diagnostic marker for the patients.

In conclusion, it is not an easy task to understand the molecular mechanisms of glomerular kidney disease due to the complexity and diversity. Using a general-to-specific methodology combined with *in vivo* and *in vitro* data analysis, we hope to have increased the understanding of certain underlying mechanisms in IgAN.

6 FUTURE PERSPECTIVES

From the global transcriptomic and proteomic analysis, we identified genes and proteins that were significantly regulated in IgAN patients compared to healthy controls. In relation to this, certain pathways were involved in the signaling transduction. In order to find specific target(s), experimental work needs to be conducted to study individual genes and proteins for their function in the disease. Studies of signal transduction related to different stimuli are required to find out upstream and downstream factors. Certain genes or proteins may be identified as markers of mesangial cell susceptibility.

Another important aspect is the clinical data. In order to gain a solid result of clinical correlations, clinical data need to be carefully collected. The accuracy of the clinical data will help to establish more precise correlation to the experimental data and give a better understanding of disease development. In paper II, mesangial cell positive standard genes showed significant correlation with the clinical data. These standard genes could possibly be used for diagnosis and prognosis in the clinic. Furthermore, endothelial cell positive standard genes need to be identified since there is evidence that the endothelial cells may also be involved in IgAN (42, 150, 151).

In addition to the points above, the human material is needed to be handled with utmost care for the global analysis. Many individual groups including us have developed their own cohorts. However, more solid statistical results would be gained from larger sample size and closer collaboration. Moreover, cross-validation among different cohorts is also extremely important. Due to the fact that different cohorts vary in age, sex, race, diagnostic methods etc, non-biological variances may impact the analysis. By conducting collaboration among scinetists with different cohorts and cross-validating the data, this problem can be markedly reduced or even avoided.

As for the receptor of gd-IgA, further investigation of the protein-R binding ability to gd-IgA needs to be done. We also found a potential relationship between PDGF and protein-R, which is interesting since both are involved in cell proliferation. Pathway signaling of these two factors needs to be investigated to understand how the proliferative events are being transducted in the cells. The study of micro-RNAs suggests that these are important in kidney disease. Further investigation of disease-specific micro-RNAs is needed. Urine data is important in this context to detect possible micro-RNAs excreted for early diagnostic purposes or progression of the disease. The study in paper IV was focused on podocytes only. It would also be interesting to investigate micro-RNAs in mesangial and endothelial cells for a more complete picture of how they influence renal disease.

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