Genetic and molecular markers of proteinuria and glomerulosclerosis

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Summary and general discussion

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The central aim of the work described in this thesis was to investigate the genetic and molecular mechanisms underlying the development of proteinuria and progressive glomerulosclerosis in animal models and human renal disease.

The clinical course of any renal disease depends on the type of renal disorder, genetic factors, environmental influences, and the severity of renal fibrosis. Excessive extracellular matrix (ECM) protein accumulation in the kidney may result from the increased synthesis of mRNA for ECM molecules, decreased ECM protein degradation due to reduced matrix metalloprotease (MMP) activity or a reduced ability of MMPs to degrade ECM proteins that have undergone posttranslational modifications, or from a combination of these factors. For adequate treatment, the early detection of renal disease, the type of renal disease, and prognostic factors is advisable. Clinical presentation and histopathological changes in the kidney do not always predict prognosis. An assessment of the expression of ECM molecules, ECM-regulating cytokines, and MMPs in renal biopsies may help discriminate between patients at risk for developing progressive renal failure and those who are likely to exhibit remodeling after insults to the kidney. Gene expression may function as an additive parameter supplementing clinical parameters and morphological information from the biopsy for predicting prognosis. **Chapter 2** provides a few examples of areas in which measuring mRNA levels has improved diagnosis and prognosis. In particular, predicting prognosis when no fibrosis is yet present improves the effective treatment and outcome. Of interest are biomarkers in the urine, a non-invasive technique with which prognostic markers have been identified. For example, the collagen I degradation activity in urine has been shown to predict the severity of renal fibrosis.[1]

The initiation and progression of fibrosis, and the eventual development of end-stage renal disease, is influenced by multiple factors. Proteinuria is an important independent risk factor for renal disease progression,[2] and age, hypertension, and genetic predisposition also contribute to progression. In **Chapter 3**, genetic predisposition to proteinuria and progressive glomerulosclerosis in anti-Thy-1 glomerulonephritis, a rat model of acute glomerular damage, is described.[3] A backcross population of a proteinuria and progression-susceptible Lewis substrain and proteinuria and progression-resistant substrain (Lewis/Maastricht and Lewis/Møllegard, respectively) demonstrates that genetic factors determine both proteinuria and glomerulosclerosis. Proteinuria levels 7 days after the induction of anti-Thy-1 glomerulonephritis were suggestively linked to a novel region on chromosome 6 that is not found by other linkage analyses. Heterozygosity of this region resulted in less proteinuria. Because we used a backcross population, we cannot conclude whether this genetic region contributes to proteinuria susceptibility in Lewis/Maastricht

(Lew/Maa) or resistance to proteinuria in Lewis/Møllegard (Lew/Moll). From the phenotype range in the backcross generation, we anticipated finding more loci associated with proteinuria. One possible explanation for not finding more loci is that proteinuria is influenced by multiple genes with small additive effects below the level of detection of our QTL-mapping strategy using 145 animals from the backcross generation. In addition, alleles protecting against or promoting proteinuria may complicate linkage analysis. Moreover, QTL may be missed due to the fact that genome areas were missing in our genome scan. In this study, we found a QTL on chromosome 1 that is associated with the development of glomerulosclerosis in Lew/Maa. This locus accounts for approximately 10% of the variance in the backcross population. Comparing these genetic results with the glomerular gene expression profile in the Lewis substrains indicated adrenomedullin, rab38, and synaptic vesicle protein 2b as possible candidates on chromosome 1. The rat QTL on chromosome 1 is concordant with regions of human chromosomes 10, 11, 15, and 16. Interestingly, human chromosome 10 is linked to a susceptibility to the development of end-stage renal disease.[4;5] In the future, testing candidate genes within the rat QTL may identify the gene or genes influencing glomerulosclerosis in this experimental model of glomerulonephritis and may aid in identifying genes involved in human susceptibility as well. In summary, **Chapter 3** shows that different chromosomal regions are linked to proteinuria and glomerulosclerosis in anti-Thy-1 glomerulonephritis, confirming proteinuria as an independent risk factor.

Candidate genes involved in the development of proteinuria and glomerulosclerosis can be expressed by renal cells, as well as cells of extra-renal origin. Both bone marrow-derived cells and intrinsic renal cells have been described as influencing the course of anti-Thy-1 glomerulonephritis. A previous study from our group found that progressive glomerulosclerosis in Lew/Maa rats is determined by genes expressed in renal cells.[6] To investigate which compartment determines proteinuria development in anti-Thy-1 glomerulonephritis in Lewis rats, we performed kidney and bone marrow transplantations between the Lew/Maa and Lew/Moll substrains **(Chapter 4)**. We found that both renal and bone marrow-derived cells contribute to proteinuria development or convey resistance to its development. Interestingly, transferring a kidney or bone marrow from the proteinuria-susceptible Lew/Maa rats to Lew/Moll rats did not lead to proteinuria in the Lew/Molls. This finding suggests that a combination of factors contribute to the development of proteinuria in Lew/Maa rats. Furthermore, we found that Lew/Moll in Lew/Maa bone marrow chimeras had reduced levels of proteinuria. This reduction was accompanied by a reduced number of glomerular microaneurysms, suggesting that proteinuria in anti-Thy-1 glomerulonephritis is linked to the development of

microaneurysms and that bone marrow-derived cells can influence this process. In the initial phase of anti-Thy-1 glomerulonephritis, polymorphic nuclear cells and macrophages infiltrate the glomerulus.[7] Phenotypic differences in the macrophage response to glomerular damage could influence the degree of initial damage. Several observations support this hypothesis. Intraperitoneal macrophages from Lew/Maa rats have been found to produce more inducible nitric oxide synthase (iNOS) than those of Lew/Moll.[8] A reduction in iNOS in Lew/Maa rats reduced proteinuria and the number of microaneurysms after inducing anti-Thy-1 glomerulonephritis.[9] In this study, we showed that transplanting a Lew/Moll kidney into Lew/Maa rats does not lead to proteinuria in the recipient, suggesting that Lew/Moll kidneys convey protection against proteinuria. More insights into the mechanisms that protect against proteinuria may lead to better treatment modalities in humans.

Regardless of the underlying cause of the glomerular damage, proteinuria can be the consequence of damage to all genes involved in the maintenance of the glomerular filtration barrier. Several genetic mutations have been found, for example in molecules expressed exclusively in podocytes, leading to congenital nephrotic syndrome in humans.[10-14] Podocytic gene mutations cause dysfunctional proteins or protein deficits, which lead to podocyte damage and a subsequently dysfunctional glomerular filtration barrier. In addition, other genetic defects that lead to glomerular damage other than the glomerular filtration barrier can also present as congenital proteinuria. The sequence of events in congenital proteinuria mediated by, for example glomerular hyperfiltration, is not well understood. Therefore, in **Chapter 5,** we investigated the sequence of events in the development of glomerular hypertrophy, proteinuria, and glomerulosclerosis in a model of congenital proteinuria accompanied by glomerular hyperfiltration**.** Munich Wistar Frömter rats (MWF) spontaneously develop abnormal albuminuria shortly after birth, and glomerulosclerosis has been described at a later phase.[15;16] Histological analysis of MWF rats at 4, 6, and 8 weeks of age showed that glomeruli first enlarge without signs of podocyte stress, changes in podocyte-associated molecules, or detectable albuminuria. Thereafter, at 6 weeks of age, segmental podocyte stress, as presented by podocyte desmin expression, was observed. This *de novo* desmin expression was accompanied by a loss of podoplanin in podocytes of the same glomerular segment. Interestingly, other podocyte-associated molecules remained unaffected, as determined by immunohistochemistry. Even more interesting is that, in the same affected glomerular segment, albumin entrapment was seen in the podocytes. These findings suggest that, in MWF rats, renal disease begins with glomerular hypertrophy, which is probably caused by nephron deficit-induced hyperfiltration. Subsequently, podocytes become stressed in glomerular segments, a process accompanied by desmin up-regulation in the podocytes and the

specific disappearance of the podoplanin protein in the same glomerular segment. Whether podocyte stress or the down-regulation of podoplanin protein expression occurs first cannot be determined from this study. In addition, because proteinuria appeared at the same age as when podocyte changes were present, we cannot conclude, based on a time sequence, whether podocyte damage is the cause or result of proteinuria. However, we speculate that the loss of podoplanin in podocytes is involved in a dysfunctional glomerular filtration barrier leading to proteinuria and, subsequently, the development of focal and segmental glomerulosclerosis. A recent histological analysis of Dahl rats has shown a similar pattern of podoplanin protein loss,[17] which supports our hypothesis. The data in **Chapter 5** suggest that segmental glomerulosclerosis starts with segmental podocyte damage. Segmental glomerular changes are also present in patients with focal segmental glomerulosclerosis (FSGS).[18] Although the exact mechanism underlying the development of segmental glomerulosclerosis is unknown, segmental podocyte damage is thought to precede its development in humans with FSGS.

Another interesting feature of MWF rats is the difference in the course of proteinuria between males and females. **Chapter 5** also describes the role of gender in renal disease. In humans, males are more susceptible to developing progressive renal disease compared to females. In MWF rats, compared to females, males exhibit a more severe phenotype with proteinuria and glomerulosclerosis that was associated with an increased percentage of desmin-positive podocytes in glomeruli. This finding suggests an increased podocyte workload in males compared to females, which could contribute to the development of glomerulosclerosis in males. It is not yet clear which gender-related factor contributes to the increased workload later in the life of male MWF rats. One possibility is the relatively fewer nephrons in males than females. Although the absolute number of nephrons is similar in male and female MWF rats, the number of nephrons corrected for body weight is less in males.[19] This difference may lead to more severe hyperfiltration in males and subsequent proteinuria and progressive glomerulosclerosis.

Progression to glomerulosclerosis is thought to be a final common pathway, independent of the underlying renal disease. However, several mediators can influence progression. The severity of the initial glomerular damage, level of proteinuria, and genetic factors influences the development of glomerulosclerosis and the rate of progression. A group of patients that is not yet well-defined are those with nephrotic syndrome and focal and segmental glomerulosclerosis upon renal biopsy without other causes of nephrotic syndrome. Renal histology varies immensely in these patients with “primary” FSGS. This morphological diversity is accompanied by a large range of clinical presentations, progression rates, and chances of developing

recurrent FGSG after transplantation. A new classification was proposed in 2004[18] in hopes of separating new disease subentities and to facilitate the diagnosis, treatment, and prediction of progression. These variants are: 1) the tip lesion variant, a lesion located near the origin of the proximal convoluted tubule; 2) the cellular variant, which is characterized by endocapillary hypercellularity; 3) the collapsing variant, which exhibits glomerular tuft collapse concomitant with epithelial cell hypertrophy and hyperplasia; 4) the perihilar variant, a lesion predominantly located at the vascular pole; and 5) FSGS not otherwise specified (FSGS NOS). If these five variants are separate pathogenetic entities, then the recurrence of FSGS in renal allografts should result in the development of the same histological variant. To test this hypothesis, morphological variants of FSGS were analyzed in biopsies from the native kidney and allografts with recurrent primary FSGS (**Chapter 6)**. In 81% of all cases, FSGS recurred as the same variant, supporting the "Columbia" classification. However, not all cases were consistent. Three categories of recurrence were observed: Type I, recurrence of the same variant of FSGS; type II, recurrence of the same FSGS variant preceded by minimal change disease-like features in the renal biopsy; and type III, recurrence of a different variant of FSGS in the allograft. Transitions to different variants occur in both native and allograft kidneys, especially between FSGS NOS and the collapsing variant. Thus, a potential evolution of the pathological phenotype should be considered in pathologic interpretation and clinical trials. Furthermore, the role of immunosuppressive agents and the chance of developing *de novo* collapsing FSGS in the renal allograft should be taken into account. Careful attention is required for the exclusion of other primary diseases associated with FSGS and FSGS secondary to chronic allograft nephropathy or calcineurin inhibitors, especially for FSGS NOS.[20] Further research with more patients and more subsequent allografts may lead to refining the Columbia classification. In addition, the hypothesis that minimal change disease is an early phase of FSGS has been a source of debate.[21;22] Our study provides evidence in favor of this hypothesis. Further research on larger groups is required to confirm these patterns of recurrence in FSGS.

Pathogenesis of proteinuria and glomerulosclerosis in anti-Thy-1 glomerulonephritis in Lew/Maa and Lew/Moll rats

In **Chapter 3** of this thesis, we showed that the development of proteinuria and glomerulosclerosis in Lewis substrains after the induction of anti-Thy-1 glomerulonephritis is governed by different chromosomal regions. **Chapter 4** showed that the development of proteinuria is determined by factors expressed in both renal

and extra-renal compartments. This finding is in contrast to the previous observation that genes expressed by the kidney determine progression to glomerulosclerosis in Lew/Maa rats. Therefore, the pathways involved in proteinuria and progressive glomerulosclerosis in anti-Thy-1 glomerulonephritis will be discussed separately.

Development of proteinuria

Proteinuria in anti-Thy-1 glomerulonephritis is the result of glomerular damage after the binding of anti-Thy-1 antibody to mesangial cells. Several factors contribute to the development of proteinuria or resistance to its development in Lew/Maa and Lew/Moll rats (Figure 1).

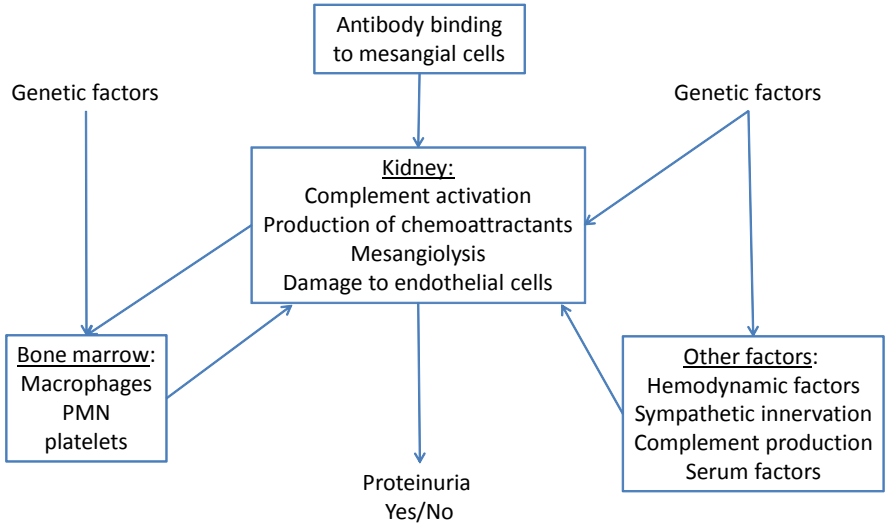

Figure 1. Schematic and speculative view of some factors involved in glomerular damage and proteinuria development in anti-Thy-1 glomerulonephritis. PMN, polymorphic neutrophils.

The amount of antibody binding to mesangial cells may influence the initial damage in the two Lewis substrains. Because the antibody binding leading to complement activation and complement deposition in both Lewis substrains is similar,[8] this explanation seems unlikely. This conclusion is further supported by the observation that the injection of higher concentrations of anti-Thy-1 antibody

into Lew/Moll rats could not induce proteinuria (unpublished results).

The response of renal cells to anti-Thy-1 antibody may be different in Lew/Maa and Lew/Moll rats. First, the amount of mesangiolysis could be different, which may be caused by genetic differences within the renal cells, such as, differences in the production of chemoattractants. Furthermore, the infiltrating inflammatory cell response may explain the difference in proteinuria levels between Lew/Maa and Lew/Moll rats. Although the number of glomerular macrophages is similar in both substrains, the inflammatory response may differ. This hypothesis is supported by the observation that intraperitoneal macrophages from Lew/Maa rats produce more iNOS compared to those from Lew/Moll rats.[8] Furthermore, decreasing iNOS in Lew/Maa reduces glomerular damage and proteinuria.[9]

In addition to differences in renal and bone marrow-derived cells, other extra-renal factors may contribute to differences in proteinuria. Increased glomerular capillary pressure induces additional sheer stress in glomerular cells, which may lead to increased capillary damage. This process may influence the development and severity of glomerular microaneurysms. Hemodynamic factors are influenced by the renin-angiotensin-system (RAS). Plasma renin activity and neuronal NOS expression in the glomeruli is increased in Lew/Maa rats compared to Lew/Moll rats,[8] suggesting that hemodynamics may play a role in the proteinuria susceptibility of Lew/Maa rats. The renal sympathetic innervation is also known to influence the RAS and renal inflammation.[23]

All factors that contribute to renal damage, such as those related to the kidney, bone marrow, and renal innervation, are genetically determined. Therefore, genetics can influence susceptibility or resistance to the development of glomerular damage. We hypothesize that a certain level of glomerular damage, including the induction of microaneurysms, is required for the development of proteinuria in anti-Thy-1 glomerulonephritis induced in the Lewis substrains.

Proteinuria is a common feature in many human renal diseases. However, proteinuria severity and treatment response vary between individuals. The development of glomerular injury and subsequent proteinuria depends on several renal and extra-renal factors, as has been described in the anti-Thy-1 glomerulonephritis model. Finding the factors that convey susceptibility or protect against the development of proteinuria may result in better treatment options with intervention at an early phase before renal disease progression is present.

Development of glomerulosclerosis

A unique feature of Lew/Maa rats is the development of progressive glomerulosclerosis after a single injection of anti-Thy-1 antibody. Previous studies have shown that this

susceptibility can be transferred with the kidney, suggesting that renal factors are important for the development of glomerulosclerosis.[6] In **Chapter 3**, we described a locus on rat chromosome 1 that is linked to glomerulosclerosis or resistance to its development. We hypothesize that glomerulosclerosis only develops when a certain degree of glomerular damage is present, with proteinuria as a result. However, additional strain-specific factors are required for progression to glomerular fibrosis (Figure 2). Mediators in the development of glomerular fibrosis may be hemodynamic factors, increased renal sympathetic nervous system activity, different responses to macrophage stimulation, increased ECM production or decreased levels of degradation, and a reduced ability to repair glomerular damage. These factors are also known to influence progressive renal disease in humans. Reducing hemodynamic stress by blood pressure control and reducing proteinuria is known to inhibit progressive renal disease.[24;25] Furthermore, patients with chronic kidney disease have increased sympathetic nervous system activity correlated with poor renal and cardiovascular outcomes.[26] Specific inhibition of the sympathetic nervous system, independent of blood pressure control, may further inhibit progression.[27;28]

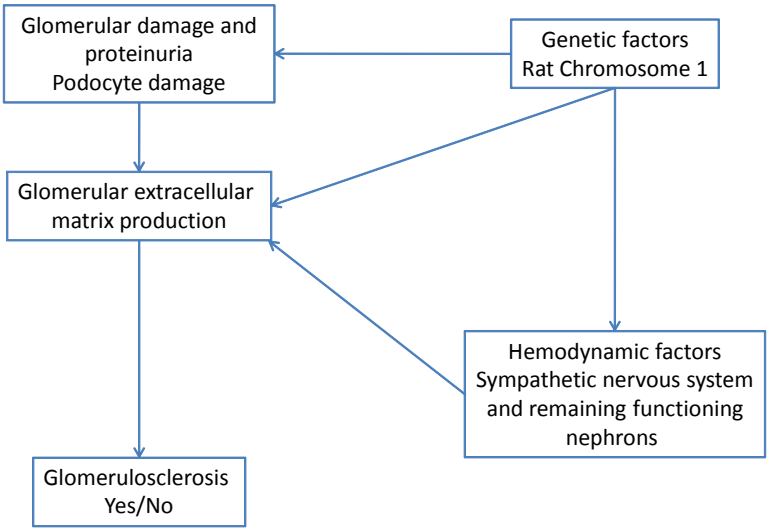

Figure 2. Schematic representation of factors involved in glomerulosclerosis development in anti-Thy-1 glomerulonephritis in Lewis substrains.

The factor on rat chromosome 1 that contributes to the development of progressive glomerulosclerosis needs to be identified. Previously, in Lew/Maa rats, intrinsic renal factors were found to determine glomerulosclerosis, whereas bone marrow-derived factors did not influence this process.[6] Therefore, the gene on chromosome 1 is likely expressed by renal cells. Another interesting gene, which is not located on chromosome 1, is Neuronal activity-regulated pentraxin (Narp), a protein present in mesangial and parietal epithelial cells. Gene expression analysis of Lew/Maa and Lew/Moll rats after the induction of anti-Thy-1 glomerulonephritis revealed Narp as a suitable candidate gene for involvement in the remodeling or progression of damaged glomeruli.[29] The down-regulation of Narp mRNA expression in Lew/Maa rats may shed light on the role of Narp in the development of glomerulosclerosis in anti-Thy-1 glomerulonephritis.

In humans, the level of proteinuria and the development of glomerulosclerosis are linked. Our studies suggest that proteinuria is required for development of glomerulosclerosis, but the presence of proteinuria does not always lead to glomerulosclerosis. Treatment is now focused on diminishing proteinuria and hypertension by inhibiting the RAS. Subsequently, this approach inhibits renal disease progression. Unfortunately, many patients still progress to end-stage renal disease. Identifying factors involved in protecting against progressive glomerulosclerosis may improve treatment modalities and the prevention of glomerulosclerosis.

Future perspectives

As proteinuria is an independent risk factor for end stage renal disease,[2] identifying the factors involved in proteinuria resistance may be a breakthrough in the treatment of chronic renal diseases. Until now, we have not found a locus or gene involved in protecting against proteinuria. In the genome scan of Lew/Maa and Lew/Moll rats (**Chapter 3**), phenotype analysis does not discriminate between loci that protect against or promote proteinuria. Instead of searching for proteinuria-inducing genes, we may focus on finding resistance-promoting genes. Further research, such as a gene expression analysis on designer cDNA chips,[30] of the genes present on the chromosome 1 QTL could provide more information about this issue. Also, silencing these genes could give more insight into the role of these genes in anti-Thy-1 glomerulonephritis. Gene expression can be down-regulated by short synthetic interfering RNA using electroporation[31] or a mesangial cell-specific siRNA-nanovector.[32] The injection of mesangial cell-specific NARP siRNA may provide more information on the role of Narp in the development of glomerulosclerosis in Lew/Maa rats.

Multiple genetic factors expressed by cells in renal and extra-renal

compartments contribute to the development of or resistance to proteinuria. From previous experiments, we concluded that complement activation after antibody binding is similar in Lew/Maa and Lew/Moll rats, and thus does not contribute to the differential development of proteinuria in these rats. Similarly, the number of glomerular macrophages was not different between the Lewis substrains. However, the differential induction of iNOS in these macrophages may explain differences in the level of proteinuria after the induction of anti-Thy-1 glomerulonephritis. This explanation suggests that bone marrow-derived genes contribute, in part, to the development of proteinuria. Because more specific antibodies for iNOS have recently become available, investigating iNOS expression in glomeruli is a logical next step.

In addition to bone marrow-derived factors, renal intrinsic factors are determinants of proteinuria. Hemodynamic factors and renal innervation may also contribute to endothelial damage and inflammation. All of these factors together determine whether or not proteinuria develops. The role of the sympathetic nervous system in proteinuria development needs to be investigated further.[23] Bilateral denervation in Lew/Maa rats, followed by the induction of anti-Thy-1 glomerulonephritis, is the appropriate experiment to investigate this role. Furthermore, the role of circulating permeability factors has not been thoroughly investigated. Recently, the circulating factor responsible for the development of primary FSGS has been found. Mutations in the soluble urokinase receptor has been identified as a cause of proteinuria.[33;34]

In **Chapter 4,** we showed that congenital proteinuria in MWF rats is linked to glomerular hypertrophy, *de novo* desmin expression in podocytes, and specific focal and segmental down-regulation of podoplanin. The role of hypertrophy in development of proteinuria in MWF was recently investigated. In these rats, both chromosomes 6 and 8 were linked to the development of proteinuria. When chromosome 6 from another hypertensive rat (SHR) was introgressed into the MWF background, the resulting rats exhibited suppressed albuminuria levels. This consomic strain had a greater number of nephrons compared to wildtype MWF,[35] suggesting that proteinuria development is linked to an inborn nephron deficit. However, MWF rats with chromosome 8 from SHR exhibited suppressed proteinuria with no change in nephron number.[36] From this data, we hypothesize that both chromosomes determine the level of proteinuria and that development of proteinuria is not exclusively linked to nephron deficit in MWF. The double consomic rats will provide more insight to the influence of both loci in the development of proteinuria. Histological analyses of all three consomic strains and immunohistochemistry for desmin and podoplanin are currently being investigated.

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