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GLOMERULAR EXPRESSION PROFILING AND NOVEL PROTEINS IN NORMAL MOUSE KIDNEY AND ADRIAMYCIN-INDUCED NEPHROSIS

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To Fernando,

My Friend

My Science Teacher and My Lifetime Mentor

El Cant del Barça

Tot el camp
és un clam
som la gent blaugrana
Tant se val d'on venim
si del sud o del nord
ara estem d'acord,estem d'acord,
una bandera ens agermana.
Blaugrana al vent
un crit valent
tenim un nom el sap tothom:
Barça, Barça, Barça!

Jugadors, seguidors,
tots units fem força.
Són molt anys plens d'afanys,
són molts gols que hem cridat
i s'ha demostrat, s'ha demostrat,
que mai ningú no ens podrà torcer.
Blaugrana al vent
un crit valent
tenim un nom el sap tothom:
Barça, Barça, Barça!

ABSTRACT

Kidney glomeruli function as high-capacity molecular sieves through which plasma is filtered into the Bowman's space as primary urine. The glomerular filtration barrier is composed of glomerular endothelial cells, the glomerulus basement membrane and the podocyte cell layer. Dysfunction of the glomerulus is a central component of renal complications leading to end-stage renal disease. However, the molecular composition of the glomerulus and how it changes during disease are still mostly unknown. To elucidate the picture of molecules involved in the biology and pathology of the glomerulus, large-scale approaches including two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry analysis and microarray profiling were applied to normal and diseased glomeruli.

A proteome analysis of healthy glomeruli in mouse was performed using 2-DE with two different staining methods and subsequent mass spectrometric identifications. Altogether, 414 protein spots were identified, revealing 232 different proteins representing a wide spectrum of activities. Only 53 of the proteins identified here were detected in another proteome study, showing the value of analysis utilizing different methodologies. 80 of the proteins were not identified in a separate transcriptome analysis, while ten of the present proteins were identified as genes implicated in glomerulus development and function, allowing direct correlation with expression data.

Characterization of five glomerulus-upregulated transcripts/proteins, ehd3, dendrin, sh2d4a, plekhh2, and 2310066E14Rik was performed. The expression pattern of these novel glomerular transcripts in various mouse tissues was studied, and the distribution of corresponding proteins was examined. All five transcripts/proteins were expressed in the kidney exclusively by glomerular cells. Ehd3 was expressed only by glomerular endothelial cells. Importantly, *ehd3* is the first gene ever shown to be expressed exclusively by glomerular endothelial cells and not by other endothelial cells in the kidney. Dendrin, sh2d4a, plekhh2, and 2310066E14Rik were transcribed specifically in podocytes within the glomerulus. With the use of polyclonal antibodies, dendrin, sh2d4a, and plekhh2 proteins were localized to the slit diaphragm and the foot process, whereas 2310066E14Rik protein was localized to the podocyte major processes and cell body.

Comparison of the normal glomerular transcriptome with its changes during progression of glomerular disease can yield information about molecular pathomechanisms. The adriamycin (ADR)-induced proteinuric mouse model allows the precise timing of the onset of proteinuria and of morphological changes in glomerulus. Overt proteinuria was observed from four days after ADR injection, and reached maximum at seven days. Blood urea nitrogen (BUN) gradually elevated indicating the failure of renal function. TUNEL staining of kidney section revealed an increase in apoptotic positive cells in glomeruli. Transcriptional profiling of kidney glomeruli revealed that nine p53 target genes were up-regulated probably due to DNA damage caused by ADR at four days and several glomerular enriched genes were differentially expressed indicating glomerular injury at seven days.

These studies provide fresh insights into glomerular biology and reveal new possibilities to explore the role of glomerular specific proteins in renal physiology and pathophysiology. Furthermore, these studies shed light on the pathomechanisms of proteinuria, which eventually results in end-stage kidney disease as a result of progressive glomerular damage.

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Cell Mol Life Sci. 2007 Dec;64(24):3317-35.

II. **Expression and subcellular distribution of novel glomerulus-associated proteins dendrin, ehd3, sh2d4a, plekhh2, and 2310066E14Rik.**
Patrakka J, Xiao Z, **Nukui M**, Takemoto M, He L, Oddsson A, Perisic L, Kaukinen A, Szigyarto CA, Uhlén M, Jalanko H, Betsholtz C, Tryggvason K.
J Am Soc Nephrol. 2007 Mar;18(3):689-97.

III. **Stage specific glomerular transcriptome profiling in ADR-induced nephropathy mice.**
Nukui M, He L, Patrakka J, Takemoto M, Betsholtz C, Tegnér J, Tryggvason K
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LIST OF ABBREVIATIONS

2-DE	Two-dimensional gel electrophoresis
ADR	Adriamycin
BUN	Blood urea nitrogen
CBB	Coomassie brilliant blue
CID	Collision-induced dissociation
CNF	Congenital nephrotic syndrome of the Finnish type
DIGE	Differential gel electrophoresis
DN	Diabetic nephropathy
ESI	Electrospray ionization
FSGS	Focal segmental glomerulosclerosis
FTICR	Fourier transform ion cyclotron resonance
GECs	Glomerular endothelial cells
GBM	Glomerular basement membrane
HPLC	High pressure liquid chromatography
HSPGs	Heparan sulphate proteoglycans
ICAT	Isotope-coded affinity tags
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
IT	Ion trap
iTRAQ	Isobaric tags for relative and absolute quantification
KO	Knock out
LIT	Linear ion trap
LPS	Lipopolysaccharide
MALDI	Matrix assisted laser desorption/ionization
MDLC	Multidimensional liquid chromatography
MN	Membranous nephropathy
MS	Mass spectrometry
m/z	Mass-to-charge ratio
NS	Nephrotic syndrome
PAN	Puromycin
PECs	Parietal epithelial cells
PMF	Peptide mass fingerprinting
PS	Protamine sulphate
PTMs	Post-translational modifications

Q	Quadrupole
RNA-seq	RNA sequencing
RPLC	Reverse phase liquid chromatography
SAGE	Serial analysis of gene expression
SD	Slit diaphragm
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SILAC	Stable-isotope labeling by amino acids in cell culture
TOF	Time of flight
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

1 Introduction: Review of literature

1.1 The kidney

The kidneys are paired, bean-shaped organs, whose primary function is to filter metabolic waste products from the blood into urine. In adult humans about 180 liters of blood is filtered through the kidneys every day as primary urine, which is devoid of macromolecules that are essential for maintaining homeostasis. The primary urine is comprehensively modified in the renal tubular system, both in composition and volume, and the daily excretion is normally about 1–1.5 liters. In addition, the kidneys have multiple other functions such as maintenance of acid-base homeostasis, osmolality and blood pressure regulation, vitamin D metabolism and production of hormones like erythropoietin and renin.

1.1.1 Glomerulus

The glomerulus is the filtration unit of the kidney. It is a capillary tuft surrounded by the Bowman's capsule at the proximal end of the nephron. Each adult human kidney contains about a million glomeruli, which are located in the cortex. The glomerulus contains four resident cell types: parietal epithelial cells (PECs) of the Bowman's capsule, whilst mesangial cells, endothelial cells, and podocytes are within the glomerular tuft [1-2]. Figure 1 illustrates the anatomical details of kidney glomerulus.

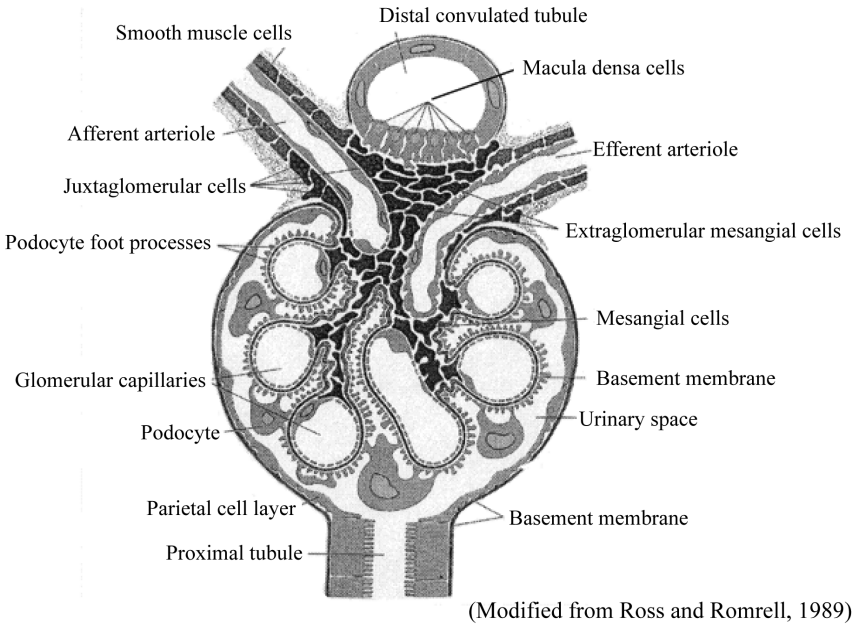


Figure 1. Schematic picture of the kidney glomerulus

The glomerular filtration barrier is composed of three layers: the fenestrated glomerular endothelial cells (GECs), the glomerular basement membrane (GBM) and the slit diaphragm (SD) located between the interdigitating podocyte foot processes (Figure 2). Plentiful evidence from biochemical and physiological analysis of the glomerular filter barrier has emerged, suggesting that the glomerular filter barrier functions in a size- and charge-selective manner [3-4], but its literal character and intricate properties have been and still are a matter of debate [5].

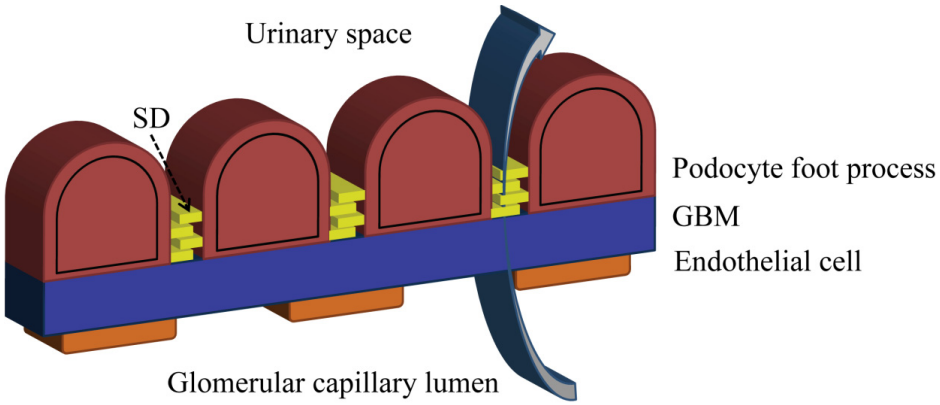


Figure 2. The glomerular filtration barrier

The filtration barrier of the capillary wall contains the innermost fenestrated endothelium, the glomerular basement membrane (GBM) and the podocyte. The slit diaphragm (SD) is located between the foot processes. The arrow indicates the direction of flow of primary urine.

1.1.2 Podocytes and slit diaphragm

Podocytes, also called glomerular visceral epithelial cells, are located on the outer surface of the GBM and contribute the outermost layer of the glomerular filtration barrier. They are highly polarized cells with an extremely unique trait; the mature podocytes are post-mitotic cells, which cannot proliferate since they arrest in the G2/M phase of the cell cycle [6]. With regards to their cytoarchitecture, the podocyte can be morphologically segregated into three different portions; cell body, major processes and foot processes. A capacious cell body, which lumps into the urinary space, possesses a well-developed endoplasmic reticulum, a large Golgi apparatus, and abundant mitochondria and lysosomes. This indicates that they have a high capacity for protein synthesis and post-translational modifications to maintain the dynamic and complex structure and function of the slit diaphragm [7]. The cell body induces long primary processes, which divide into secondary processes and eventually form the foot processes [8]. Interdigitated foot processes of adjacent podocytes leave narrow slits

between them, and those slits are bridged by a well-organized extracellular structure with a constant width of 30-45 nm [7, 9]. This specialized intercellular junction is known as the slit diaphragm (SD).

Previously, the structure of the SD was proposed to be a zipper-like membrane with pores smaller than the size of albumin molecules on both sides of a central filament [9-10]. Recently, new information on the structure of the SD has been revealed by electron tomography combined with immunogold-labeling that the SD may be composed of a uniformly organized network of convoluted strands [11]. Furthermore, accumulating evidence on protein complexes specifically located in the SD has supported the notion of the SD as being a genuine macromolecular barrier. CD2AP [12], nephrin [13], Neph1 [14] and podocin [15] have been found to be specifically localized to the SD region, and abnormal function or absence of any of these proteins leads to proteinuria and progression to renal failure in humans or mice. Thus, the podocytes and the SD are considered to have a central role in glomerular filtration.

1.1.3 Glomerular endothelial cells and glomerular basement membrane

The glomerular endothelial cells (GECs) form the innermost layer of the glomerular capillary wall with a large fenestrated area representing 20–50% of the entire endothelial surface [16]. The diameter of the fenestrae is about 60 nm [17]. Thus, GECs do not seem to be a direct barrier for the passage of plasma proteins in a size-selective manner [18-19]. It is possible that the GECs contribute to the physical glomerular filtration barrier, but detailed knowledge about its function remains unclear.

The glomerular basement membrane (GBM) is a 300 nm thick acellular matrix membrane located between GECs and podocytes [20]. Its main constituents are type IV collagen, laminin, nidogen and proteoglycans. Collagens and laminins form a huge structural network by self assembly, and nidogen is believed to be the connector of these networks in the GBM [21]. The most abundant proteoglycans in the GBM are heparan sulphate proteoglycans (HSPGs), such as agrin and perlecan [22-23]. They contribute to the negative charge of the GBM [24]. This trait of the GBM has been considered to play an important role in preventing negatively-charged plasma proteins from passing through the glomerular filtration barrier [25]. However, the role of HSPGs in glomerular filtration has been interrogated as genetically modified HSPGs deficient mice do not develop proteinuria [26-28].

1.2 Proteinuria and nephrotic syndrome

Nephrotic syndrome (NS) is not a disease itself, but the symptom of many different proteinuric diseases of the kidney glomerulus, which can be acute and transient or chronic and progressive. NS is an exceedingly heterogeneous disorder, which is triggered either by acquired or by genetic defects in the glomerular filtration barrier. NS is characterized by the triad of overt proteinuria, hypoalbuminemia, and edema, and often the conditions related to NS eventually result in end-stage kidney failure as a result of progressive glomerular damage [29]. Proteinuria is characterized by the presence of excess plasma proteins in the urine, and it is one of the most prominent indications of glomerular filtration barrier dysfunction. When protein excretion exceeds 3.5 g or more per day, it is considered as nephrotic-range proteinuria in human adults [30]. The pathogenic mechanisms of proteinuria have been clarified in some rare inherited diseases, but the causes of most human proteinuria cases are still unclear. Elucidation of the pathomechanisms and specific treatments for these diseases are the most important premises in the nephrology field. Recent discovery and identification of genes causing NS and consequent research on the function of the proteins encoded by these genes have increased the current understanding of NS [31].

1.2.1 Acquired proteinuria syndromes in humans

The majority of proteinuria syndromes in humans are acquired, and these are divided into immune and non-immune mediated [32]. The most common immune-mediated forms are membranous nephropathy (MN) and minimal change disease. MN is the most common cause of NS in adults. The traits that characterize MN as a histopathological entity are caused by the immune deposits that form at the base of the foot processes of podocyte. The name membranous originated from the thickened GBM that is often observed by light microscopy in later stages of the disease. The immune deposits and the additional matrix material laid down by the injured podocytes are responsible for thickening of the GBM as the disease progresses [33]. The target antigen podocyte foot processes in patients with MN has been under intense research for decades. Very recently, Beck *et al.* identified protein termed M-type phospholipase A_2 receptor (PLA_2R) as the target antigen in idiopathic membranous nephropathy [34]. PLA_2R is expressed on normal glomerular podocytes and is present in the glomerular immune deposits in patients with the disease. Their discovery is a major breakthrough

and may have implications for the diagnosis and treatment of the idiopathic membranous nephropathy.

Minimal change disease is considered immune-mediated because it is likely in consequence of an abnormality in T cells, although the precise mechanisms are not well defined [32]. It is by far the most common cause of NS in children under 10 years of age [35]. The name minimal change disease derived from no morphrogical changes in kidney observed under light microscopy. However, the hallmarks for this disease, podocyte foot processes effacement, vacuolation, and growth of microvilli on podocyte were observed with the advent of electron microscopy.

The most common non-immune mediated proteinuria syndrome is diabetic nephropathy (DN). DN, characterized by initial development of progressive albuminuria followed by a later decline in glomerular filtration in association with glomerulosclerosis, is the most common cause of end-stage renal disease [36], and it is also an independent risk factor for cardiovascular disease [37]. Renal extracellular-matrix accumulation and thickening of the GBM and the tubular basement membrane are distinctive traits of DN pathology. It is suggested that inheritance plays an important role in the pathogenesis of DN based on a high concordance rate for DN in families, and different rates of DN in disparate racial groups [38-39]. The search for identification of genetic variants underlying heritable risk of DN by either genome-wide scans or candidate-gene approaches has been conducted for a long time. Several studies have reported that the 3q locus is likely to harbor susceptibility gene(s) for DN [40-41]. Recently, He *et al*. reported the association of genetic variants at 3q22 with an increased risk of DN by genotyping highly dense single-nucleotide polymorphisms [42]. Despite intensive research efforts actual DN causative genes have not been identified yet.

1.2.2 Inherited proteinuria syndromes in humans

The inherited forms of proteinuria in humans have diverse courses. Some have moderate proteinuria and focal segmental glomerulosclerosis (FSGS), while others have rigorous proteinuria or congenital nephrotic syndrome. Regardless of the causes for those symptoms, the patients often end up with end-stage renal disease, which requires hemodialysis treatment or a kidney transplantation [31]. Furthermore, the trait of these hereditary proteinuria syndromes can be sorted in a clearly age-dependent manner.

Steroid-resistant nephrotic syndrome is an autosomal recessive inherited disorder, which is characterized by early childhood onset of proteinuria, rapid progression to end-stage renal disease and focal segmental glomerulosclerosis [15]. This disease is caused by mutations in *NPHS2* gene in about 25% of children and nearly 15% of adults [43]. *NPHS2* encodes a protein termed podocin [44-45]. Podocin, a 42-kDa hairpin-like protein, is localized at the slit diaphragm. It is a raft-associated component of the slit diaphragm and interacts directly with nephrin and CD2AP [46]. Mutations in podocin disrupt the slit diaphragm as it may serve a scaffolding function and serves in the structural organization of the slit diaphragm [47].

Congenital nephrotic syndrome of the Finnish type (CNF) is also an autosomal recessive inherited disorder, which is characterized by massive proteinuria already *in utero*, premature birth and edema just after the birth [48]. The incidence of this disease in Finland is 1 in 8,200 births [49], but has been diagnosed worldwide. CNF is caused by the mutations in *NPHS1*, which encodes a protein termed nephrin [13, 50]. Nephrin, a 180 kDa transmembrane protein, is localized at the podocyte SD, and functions as structural component that maintains and organizes podocyte cytoskeleton [51]. Mutations in the *NPHS1* gene hinder the formation of the slit diaphragm and lead to massive proteinuria and renal failure.

Hereditary forms of focal segmental glomerulosclerosis (FSGS) are often characterized by the onset of mild proteinuria during adolescence or early adulthood, with slow progression to segmental glomerulosclerosis [31]. Mutations in two genes have been identified as responsible for two types of FSGS, FSGS1 and FSGS2. FSGS1 is caused by mutations in *ACTN4,* which encodes α-actinin-4 [52]. α-actinin-4, a 105 kDa cytosolic protein, is located in the podocyte, and it cross-links F-actin filaments in the foot processes. Known mutations in the *ACTN4* gene increase the affinity of α-actinin-4 for F-actin, which may interfere with the normal assembly and disassembly of actin filaments in the glomerular podocytes [53]. FSGS2 is caused by mutations in *TRPC6,* which encodes transient receptor potential cation channel 6 (TRPC6) [54-55]. TRPC6, a 106 kDa transmembrane protein, is localized to the podocyte, and it is a member of a family of nonselective cation channel proteins that are involved in the increase of intracellular calcium concentration. Mutations in the *TRPC6* gene cause abnormally high current amplitudes, which may have a role in the pathogenesis of focal segmental glomerulosclerosis [56]. Mutations in a few other podocyte genes have been reported to cause progressive proteinuria. Table 1 summarizes some of these genes.

Table 1. Summary of genetic form of nephrotic syndrome

Disease	Gene	Protein	Protein location	Ref(s)
Steroid-resistant NS	*NPHS2*	Podocin	Podocyte	[44-45]
Congenital NS of Finnish type	*NPHS1*	Nephrin	Podocyte SD*	[48, 57]
Focal segmental glomerulosclerosis 1	*ACTN4*	α-Actinin 4	Podocyte	[52]
Focal segmental glomerulosclerosis 2	*TRPC6*	Transient receptor potential cation channel C6	Podocyte	[54-55]
Denys–Drash syndrome	*WT1*	WT1 protein	Podocyte	[58-60]
Pierson syndrome	*LAMB2*	Laminin β2	GBM**	[61]

* Slit diaphragm ** Glomerular basement membrane

1.2.3 Experimental proteinuric rodent models

Experimental proteinuric rodent models can be used to explore mechanisms of proteinuria induction and progression, and the development and better depiction of these models has advanced our understanding of human glomerular diseases. It is ideal to employ human sample/tissue to clarify the whole picture of proteinuric diseases, but there are some drawbacks with utilizing human tissue. These include limitations with sample collection, restriction to the time of disease onset and exceptional routines in follow-up [62]. On the other hand, using proteinuric rodent models overcomes these impediments, as they are easily accessible to sample collection and examination of development and progression of the disease over time. It is also desirable to control the onset of proteinuria or to disrupt/overexpress a particular gene that might be involved in the pathogenesis of proteinuria [62]. Furthermore, the use of rodent models has advantages such as low cost, easy handling and short reproductive periods. Experimental rodent proteinuric models induced by numerous nonimmunological procedures have been well characterized and include partial nephrectomy [63-64], protein overload [65] and administration of puromycin (PAN) [66], adriamycin (ADR) [67-68], protamine sulphate (PS) [69-70], and lipopolysaccaride (LPS) [71-74]. Table 2 summarizes details of these methods.

Table 2. Summary of rodent experimental proteinuric models

Models	Species	Human Disease	Type of PU**	Onset of PU	Ref (s)
protein overload	Rat/Mouse	NS*	Transient	6-24 hours	[65]
PAN nephrosis	Rat	FSGS	Transient	5-8 days	[66]
ADR nephrosis	Rat/Mouse	FSGS	Progressive	4-7 days	[67-68]
PS model	Rat	NS	Transient	40-70 minutes	[69-70]
LPS model	Mouse	NS	Transient	24 hours	[71-74]

* No specific human disease, ** Proteinuria

(Modified from Pippin et al., 2009)

1.2.3.1 Adriamycin induced proteinuric mouse model

Adriamycin (ADR), the trade name of Doxorubicin, is an anthracycline antibiotic that works by intercalating DNA. Injecting ADR intravenously to rodents induces proteinuria and histological features, which mimic human FSGS. Hence the ADR-induced proteinuric model is considered an experimental form of human FSGS. This model has been well characterized in most rat strains since 1970 [72], however, most mouse strains do not show complete susceptibility to ADR. In mice, the first ADR-induced proteinuric model was described in the BALB/c strain in 1995 [68], and Wang *et al*. established a stable and reproducible model in 2000 [67]. In 2005, Zheng *et al*. figured out that a single gene defect results in ADR susceptibility with recessive inheritance by mapping the trait locus to chromosome 16A1-B1 (*DOXNPH* locus) [75]. They identified the gene encoding protein arginine methyltransferase 7 (*Prmt7*), which is located in the region of the *DOXNPH* locus, following ADR exposure. Their theory is that direct methylation of ADR by Prmt7 mediates protection from kidney injury, as methylation of ADR has been reported to decrease its cytotoxicity aptitude [76]. Since then, it is known that BALB/cJ and 129/SvJ mice have severe susceptibility to ADR, while other mouse strains are completely resistant to ADR [77].

To induce ADR nephropathy, ADR is administered as a single intravenous injection of 10-11 mg/kg body weight. ADR nephropathy is characterized by persistent proteinuria, increased serum creatinine and progressive renal injury [67]. A distinctive benefit of using the ADR nephropathy model is that it enables specific timing of the onset of renal injury. In general, overt proteinuria emerges at 4-5 days after ADR injection. Segmental podocyte foot process fusion is observed by electron microscopy after a week. Apparent histological changes, including glomerular hypertrophy, reabsorption droplets and intratubular protein casts, are observed by light microscopy after two weeks. The sizes of glomeruli are reduced with glomerular vacuolization, tuft collapse and mesengial expansion after four weeks. FSGS and rigorous interstitial fibrosis and inflammation are observed after six weeks [62].

Even though the scientific evidence about ADR nephropathy is accumulating, the pathogenesis of renal damage by ADR is still not clear. Dai *et al*. showed that Wnt/β-catenin signaling might be a decisive player in the pathogenesis of podocyte dysfunction and proteinuria in ADR nephrosis [78]. They demonstrated that up-regulation of the Wnt1 gene in mice by ADR aggravated podocyte injury and albuminuria, while blockade of Wnt signaling with its antagonist DKK1 improved

podocyte lesions. Also, they illustrated that mice with podocyte-specific ablation of β-catenin, the principal downstream effecter of Wnt signaling, were protected against the development of albuminuria by ADR. Other groups have suggested that phosphorylation of certain proteins, in the JAK/STAT signaling pathway [79] or in the MAPK pathway [80], may exacerbate experimental acute renal injury. They demonstrated that phosphorylation of the protein in either pathway is enhanced in glomeruli of clinical nephrotic syndrome and of mice with ADR nephropathy and inhibition of phosphorylation of those proteins results in marked suppression of renal injury and proteinuria. Therefore, they concluded that activation of the JAK/STAT or the MAPK signal pathway is involved in the progression of glomerular diseases with proteinuric state.

Several groups have suggested that the alternative pathway of the complement cascade is vital in mediating the early podocyte injury and late onset glomerulosclerosis in the ADR nephropathy model. They used KO mice for various complement cascade components, including C3, C3 receptor, C1q, factor D, factor B or the complement regulatory protein CD59a [81-84], and concluded that renal injury by ADR was dependent on the activation of complement through C3. One group has suggested that activation of the complement system is implicated in the development of tubulointerstitial injury, which has the common pathway leading to renal failure. The other group has suggested that lack of component in complement cascade reduces early glomerular injury and proteinuria.

1.2.3.2 Lipopolysaccaride induced proteinuric mouse model

Lipopolysaccaride (LPS), a major component of the outer membrane of gram-negative bacteria, is an endotoxin that induces a variety of inflammatory responses in rodents. LPS binds to the Toll-like receptor 4 (TLR-4) complex, which contains CD-14 and MD-2, and these interactions trigger the secretion of pro-inflammatory cytokines [85-86]. The LPS-induced sepsis model has frequently been studied in rat [87], but Reiser *et al*. have described a transient proteinuric mouse model induced by LPS [74].

In this model, proteinuria is induced by administering LPS as a single intraperitoneal injection of 200 μg of LPS diluted in PBS at a concentration of 1 mg/ml. Three mouse strains, BALB/c, WT 129 and C57BL/6, are known to be susceptible to LPS [74]. Ordinarily, a milder form of proteinuria than in the ADR-induced model appears within 24 hours after LPS injection, but kidney function returns to baseline by

72 hours. Podocyte foot process effacement is observed by electron microscopy within 24 hours.

Sun *et al*. have reported global glomerular gene expression profile changes in the LPS-induced proteinuric mouse model [88]. A transcriptome analysis revealed significant changes in mRNAs encoding proteins involved in the regulation of adherens junctions, actin cytoskeleton and survival in podocytes. Also, they showed significant downregulation of podocyte-specific genes suggesting that podocytes and the podocyte actin cytoskeleton were culprits in the proteinuric response to LPS. Even though scientific evidence about this model has been accumulating, the mechanisms of how LPS induces proteinuria in mice are still unknown.

The relevance of the LPS-induced proteinuric model to human kidney disease has been questioned. Comper [89] has claimed that the small changes in protein excretion in LPS-mediated proteinuria, three-fold in proteinuria and five- to seven-fold in albuminuria [90], is not comparable with any human proteinuric diseases, in which 100-10,000 fold changes in albumin excretion are observed [91]. Furthermore, he has claimed that the distinct effect of LPS on glomerular permeability have not been supported experimentally [89]. In response to Comper's statement, Reiser and Mundel made an objection that this model was a significant tool to study podocyte biology and proteinuria, based on the facts that key effectors of this model, including B7-1 [74], cathepsin L [92] and urokinase-type plasminogen activator receptor [93] are induced in human proteinuric diseases and those proteins have been detected in podocytes *in vivo* [94].

1.3 Omics in the post genomic era

The importance of determining the entire genome sequence of humans has been well acknowledged since the draft human genome sequence was completed ahead of schedule in 2001 [95-96]. Genomics provides sequence information on the full complement of genes in an organism, and to date, there have been more than 180 genomes fully sequenced [97]. Therefore, genomics has facilitated the power of high-throughput and comprehensive analysis of biological systems.

In the post human genome era, several "omics" fields have emerged to facilitate our understanding of transcriptional regulation (transcriptomics) and the biochemical functions of all the gene products (proteomics). In contrast to traditional biochemical methods, omics techniques offer the potential to cross-examine thousands of independent variables in a single study, and thereby guarantee accelerated manners in identifying novel drug targets and in understanding the underlying mechanisms of health and disease [98].

1.3.1 Gene expression profiling and microarray analysis

The central genetic doctrine utters that genomic DNA is first transcribed into mRNA, after which it is translated into protein. While the genome, the genes and the non-coding sequences of the DNA are generally common to all the cells in an organism and are normally static over its lifetime, the transcriptome, the collection of mRNA, is highly dynamic and changes in response to external stimuli and in disease [99]. Therefore, measuring the expression levels of mRNA provides a more accurate view of gene expression.

Gene expression profiling is the term that describes the analysis of the transcriptome by simultaneous quantification of mRNAs levels expressed from large numbers of genes [99]. A wide range of technologies, both low and high throughput techniques, exist for the measurement of gene expression at the level of mRNA, including Northern blotting [100], quantitative real time RT-PCR (qRT-PCR)[101], real competitive PCR [102] and serial analysis of gene expression (SAGE) [103].

In 1995, the technique called DNA microarray was reported [104], and this technology has revolutionized research in gene expression analysis. Compared to conventional methods for gene expression measurements, which were performed at a gene-by-gene level, microarray technology allows simultaneous analysis of the expression of thousands of genes. Since the advent of this technology, microarray

techniques have been rapidly accepted as one of the most promising methods for gene expression profiling, and the application of this technique in molecular biology research has grown exponentially.

By definition, a microarray is a large collection of gene specific DNA fragments of known sequences aligned in an orderly fashion on a solid surface to which labeled samples are hybridized [105]. In general, DNA microarray platforms are generated by cDNAs or short synthetic oligonucleotides (25-nucleotide-long for Affymetrix gene chip) onto membranes or glass slides. Before hybridization onto the array chips, the cDNA of interest is labeled with a fluorescent or radioactive dye (with biotin for Affymetrix oligonucleotide GeneChips). After hybridization, mRNA expression levels are measured by means of quantitative detection of fluorescent dye (by the comparison of samples hybridized on different chips for Affymetrix oligonucleotide GeneChips). The signal intensity for each spot is assumed to be proportional to the amount of each specific mRNA in the original sample. For that reason, the intensity difference between different samples can be used as the measurement of differential gene expression [106]. Figure 3 illustrates detailed comparison of two different microarray methods [107].

Regardless of their ubiquity and tremendous usefulness, microarrays have certain limitations. First, no information can be obtained from microarray studies regarding the importance of translational regulation and the role of post-translational modification (PTMs) [108]. Second, it has been proven that the correlation between expression levels of mRNA and those of protein is not always perfect [109-110]. Therefore, it is not always accurate to conclude that changes in gene expression are direct evidence of consequent changes in protein expression.

Recently, a new method called RNA sequencing (RNA-seq) has been developed for both mapping and quantifying transcriptomes [111-112]. In this method, cDNAs generated from the RNA of interest are directly sequenced using next-generation sequencing technologies. Briefly, a library of cDNA fragments with adaptors attached to one or both ends is made from a population of RNA, and then each molecule is sequenced in a high-throughput manner. There are major advantages of this method over microarray-based methods. First, RNA-seq can be used to detect transcripts that correspond to non-existing genomic sequence. Second, RNA-seq has better sensitivity and larger dynamic range. An increasing number of studies have demonstrated the potential for this method [113].

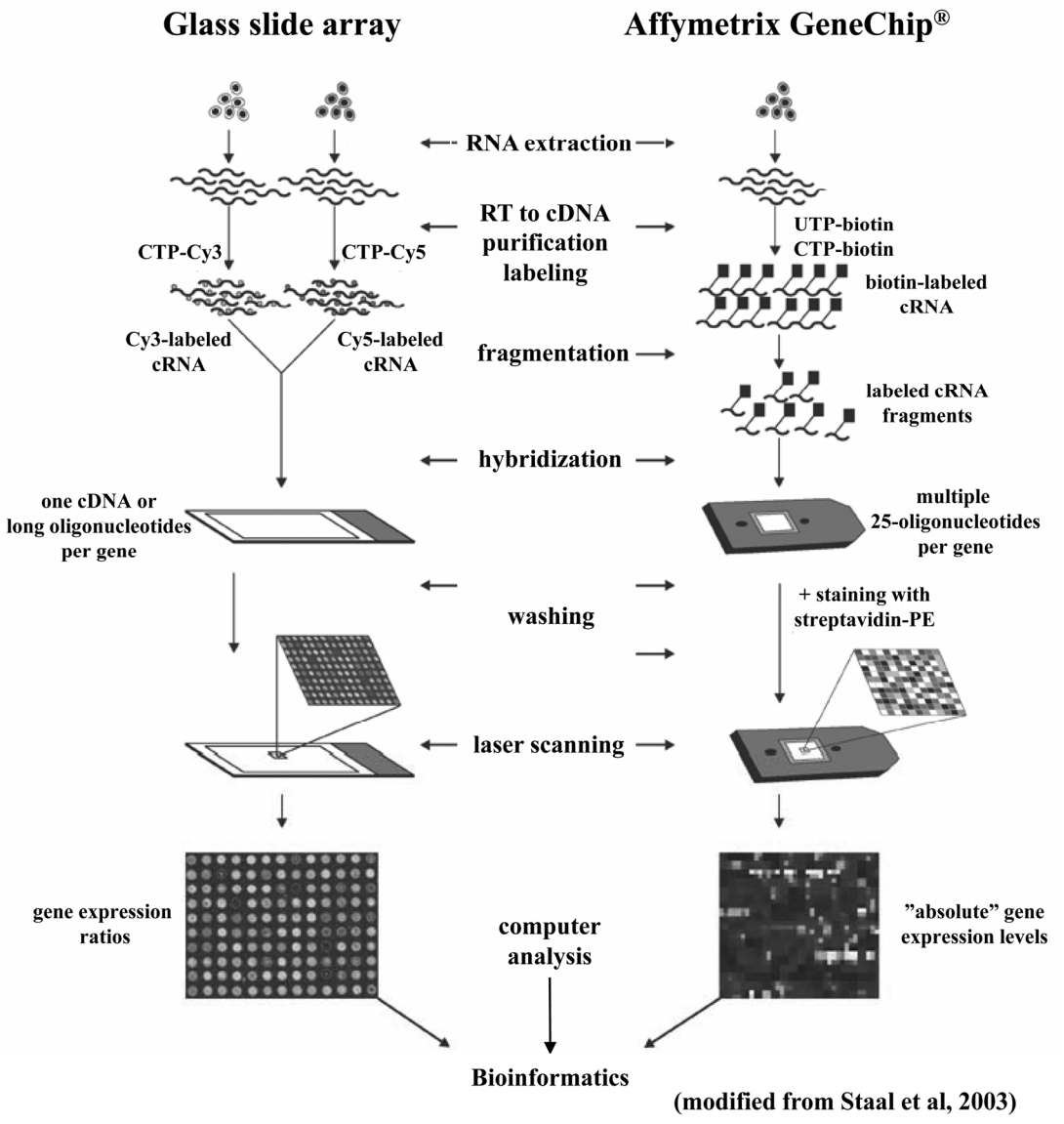


Figure 3. Comparison of two different cDNA microarray experiments

For glass slide experiments, cDNA is prepared from two samples. After *in vitro* transcription (IVT), the two labeled cRNA samples are mixed and hybridized on a glass slide array. This is then scanned with a laser, followed by computer analysis of the intensity image. In Affymetrix arrays, the cDNA is used in an IVT reaction to generate biotinylated cRNA. After fragmentation, this cRNA is hybridized to microarrays, washed and stained with PE-conjugated streptavidin, and subsequently scanned on a laser scanner. The different abundance of a specific transcript between two samples can be obtained by comparing the signal from one sample to the other.

1.3.2 Proteomics

Transcriptomics employs DNA microarray technologies to investigate gene expression by measuring transcriptional regulation of genes via their mRNA levels. However, it is the proteins that act as the cellular building blocks that directly assert the

potential function of genes, such as enzymatic catalysis, molecular signaling, and physical interactions [97].

The term proteome originally emerged to depict “the set of PROTeins encoded by the genOME” in 1996 [114]. The study of the proteome, proteomics, differs from protein analysis in that it is the characterization of the complete repertoire of individual protein species that comprise the proteome rather than focusing on a single protein. Proteomics aims to identify not only all the proteins in any given cells, but also the set of all protein isoforms and modifications, the interactions between them, the structural description of proteins and their higher-order complexes, and for that matter almost everything post-genomic [115].

However, the analysis of a full proteome poses inevitable challenges due to the limitation and variability of sample material, sample degradation, vast dynamic range and a plethora of post-translational modifications (PTMs). For instance, the number of proteins exceeds by far the number of genes in the corresponding genome due to the fact that a particular gene can generate multiple distinct proteins as a result of alternative splicing of primary transcripts, the presence of sequence polymorphisms, PTMs, and other protein-processing mechanisms. Moreover, the proteome covers a concentration range that surpasses the dynamic range of any single analytical method or instrument. For example, the concentration range of serum proteins has been estimated to exceed 10 orders of magnitude [116]. To overcome these significant challenges in proteomics, new analytical strategies have been developed in which mass spectrometry (MS) is the central element with increased performance and versatility of the instrumentation.

1.3.3 Mass spectrometry

Mass spectrometry (MS)-based proteomics is a discipline made possible by the availability of gene and genome sequence databases and technical and conceptual advances in many areas, most notably the discovery and development of protein ionization methods, as recognized by the 2002 Nobel prize in chemistry. By the mid 1990s MS had become a fundamental analytical technique for proteomics research. Since then, driven by the need to identify, characterize, and quantify proteins at ever increasing sensitivity and in ever more complex samples, a wide range of new MS–based analytical platforms and experimental strategies have emerged [117].

Fundamentally, a mass spectrometer measures the mass-to-charge ratio (*m/z*) of gas-phase ions. By definition, it consists of three essential parts; ion source, mass analyzer and detector (Figure 4). An ion source converts analytes into gas-phase, a mass analyzer separates ionized analytes on the basis of *m/z* ratio, and a detector records the number of ions at each *m/z* value [118].

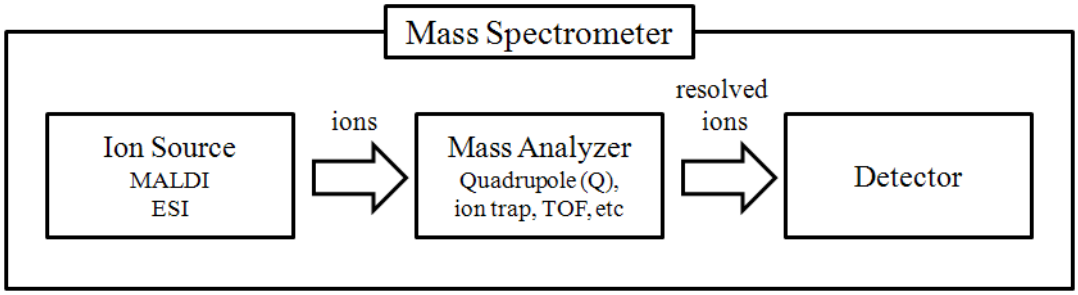


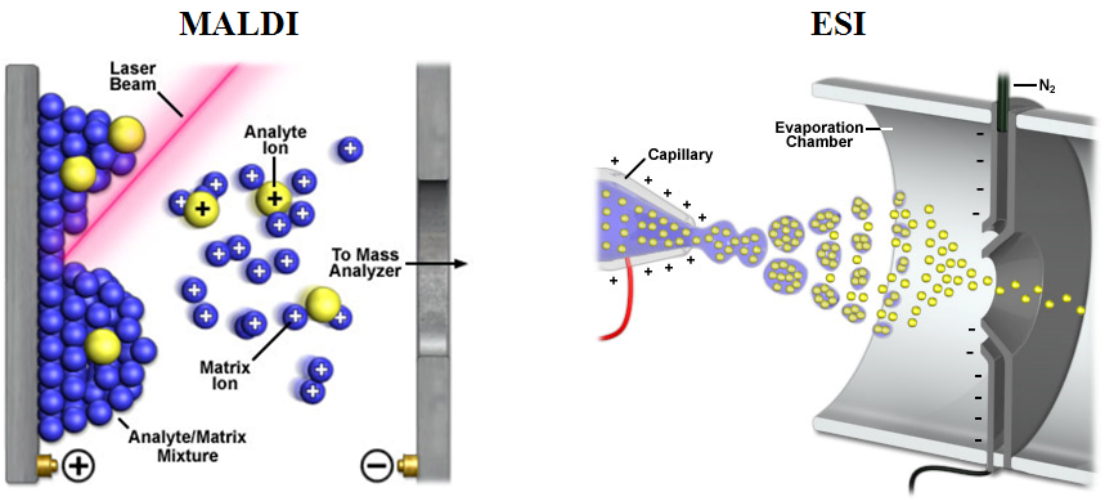
Figure 4. Schematic presentation of a mass spectrometer

A mass spectrometer has three essential parts, ion source, mass analyzer and detector. The ion source produces ions from the sample, the mass analyzer resolves ions based on their mass/charge (*m/z*) ratio, and the detector detects the ions resolved by the mass analyzer.

1.3.3.1 Ionization techniques

Proteins and peptides are polar, nonvolatile, and thermo-unstable molecules. To transfer those species into the gas phase without extensive degradation, a soft ionization technique is required. As an ion source, two ionization techniques, matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), are generally utilized to volatize and ionize the proteins or peptides [119-120]. MALDI sublimates and ionizes the analytes out of a dry, crystalline matrix via a laser pulse, conversely ESI ionizes the analytes out of a solution and is typically coupled to liquid chromatography (LC) [121] (Figure 5).

Preference of these two ion sources depends on the complexity of samples; MALDI is used for relatively simple peptide mixtures and ESI coupled to LC for complex samples.



(Modified from National High Magnetic Laboratory website , http://www.magnet.fsu.edu/)

Figure 5. Schematic picture of two kinds of ion sources, matrix assisted laser desorption/ionization (MALDI-left) and electrospray ionization (ESI-right)

MALDI: After the matrix and the analyte (e.g. peptides) are mixed and co-crystallized on a metal plate, the laser is fired at the crystals. The matrix absorbs photons from the laser beam, and then excess energy is transferred to the analyte, which is ejected from the plate into the gas phase. **ESI:** The analyte is mixed with a solvent and introduced into a capillary. A very high voltage is applied to this capillary, which charges the molecules in the solvent. The charged molecules pass from the capillary into the mass analyzer, whereas the solvent is pumped away by the vacuum system.

1.3.3.2 Mass analyzers

The mass analyzer is the essence of MS technology. Four types of mass analyzers, time-of-flight (TOF), quadrupole (Q), ion trap (IT) and Fourier transform ion cyclotron resonance (FTICR) are regularly employed for proteomics research. Today in research, it is fundamental to combine mass analyzers. Figure 6 illustrates common MS instruments.

Fundamentally, a mass analyzer separates ions based on the mass-to-charge (*m/z*) ratios: TOF uses flight time, Q uses *m/z* stability, IT and FTICR use *m/z* resonance frequency [97]. Each mass analyzer has unique properties, such as analysis speed, dynamic range, ion transmission, mass range, resolution and sensitivity. In recent years, hybrid instruments, in which two or more mass analyzers are put together in tandem, have been designed to combine the capabilities of different mass analyzers to do more complicated protein analysis. Table 3 summarizes comparative features of the instruments currently most commonly used in proteomics.

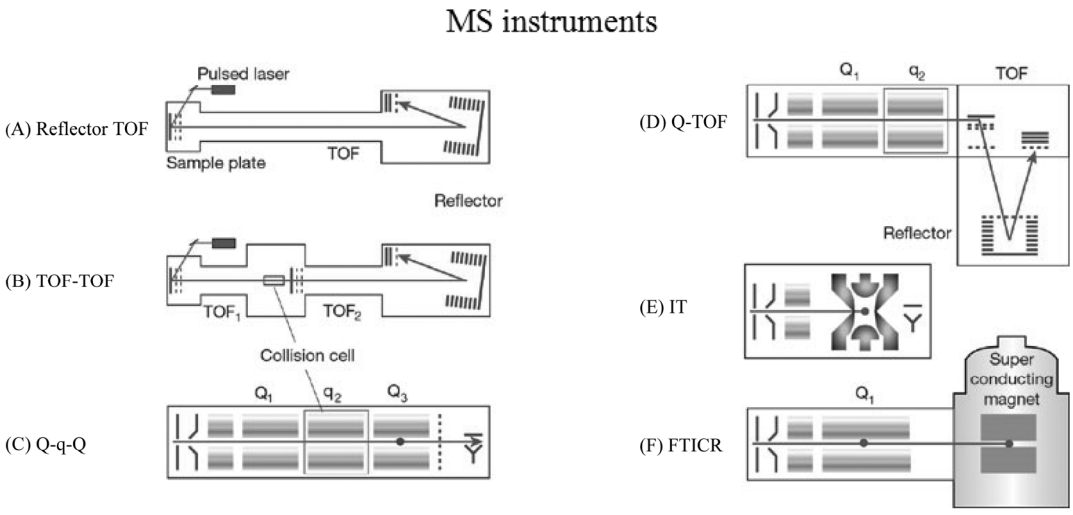


Figure 6. MS instrumental configurations with different mass analyzers

(A) Reflector time-of-flight (TOF): it accelerates ions by high kinetic energy, and it separates along a flight tube as a result of their different velocities. **(B)** TOF-TOF: it incorporates a collision cell between two TOF sections. In the first TOF section ions of one mass-to-charge (*m*/*z*) ratio are selected, fragmented in the collision cell, and the masses of the fragments are separated in the second TOF section. **(C)** Quadrupole: it selects ions by time-varying electric fields between four rods, which permit a stable trajectory only for ions of a particular desired *m*/*z*. Again, ions of a particular *m*/*z* are selected in a first section (Q_1), fragmented in a collision cell (q_2), and the fragments separated in Q_3. **(D)** Quadrupole TOF: it combines the front part of a triple quadruple instrument with a reflector TOF section for measuring the mass of the ions. **(E)** Ion trap: it captures the ions as in the case of the linear ion trap, fragments ions of a particular *m*/*z*, and then scans out the fragments to generate the tandem mass spectrum. **(F)** Fourier transform ion cyclotron resonance: it traps the ions with the help of strong magnetic fields.

Table 3. Comparison of commonly used mass spectrometers for proteomics

Instrument	Mass accuracy (ppm)	Sensitivity	*m/z* range	MS/MS capability	Ion source
TOF	10-20	Femtomole	NUL*	n/a	MALDI
TOF-TOF	10-20	Femtomole	NUL*	MS/MS	MALDI
Q-q-TOF	10-20	Femtomole	NUL*	MS/MS	MALDI/ESI
Q-q-Q	100-1000	Attomole to Femtomole	10-4000	MS/MS	ESI
QIT [a]	100-1000	Picomole	50-2000; 200-4000	MS^n **	ESI
Q-q-LIT [b]	100-500	Femtomole	5-2800	MS^n **	ESI
FTICR	<2	Femtomole	50-2000; 200-4000	MS^n **	MALDI/ESI

* No upper limit, ** n>2, up to 13, [a] quadrupole ion trap, [b] linear ion trap

(Modified from Han *et al*., 2008)

The Q-q-TOF instruments demonstrate high resolution and mass accuracy in MS and MS/MS mode. In the MS mode, the quadrupole acts as an ion guide to the TOF

analyzer where the mass analysis takes place. In the MS/MS mode, on the contrary, the precursor ions, which are typically a multiple charged ion in ESI, are picked up in the first quadrupole and go into fragmentation through collision-induced dissociation (CID) in the second quadrupole. The product ions are analyzed in the TOF analyzer [117].

The IT analyzers trap ions, and ions can therefore be accumulated and stored over time. The linear ion trap (LIT) analyzers have been replacing Q trapping devices because they cover a wider dynamic range and have better overall sensitivity. Characteristically, LIT instruments have an optional slow scanning function to increase resolution, and have multiple-stage sequential MS/MS capabilities, in which fragment ions are repeatedly isolated and further fragmented. This approach has proved to be very useful for the analysis of posttranslational modifications such as phosphorylation [122].

1.3.4 Sample separation techniques for MS

Protein MS highly depends on sample separation technologies that are needed to simplify complex biological samples prior to MS analysis. It should be emphasized that sample separation is the key to success for identification of proteins. For instance, low-abundance species cannot be detected without appropriate sample separation, otherwise they will be overshadowed by higher abundance signals [97].

There are two major approaches to separate proteins prior to MS analysis, gel-based and gel-free separation methods. Traditionally gel-based methods have been used in analysis with MALDI instruments. In this method the protein band can be excised, digested, and off-line sampled with MALDI source [123]. High-pressure liquid chromatography (HPLC) is the most common gel-free method, and is usually directly coupled to instruments with an ESI source. In this method protein samples can be digested, and on-line sampled with ESI source.

1.3.4.1 Two-dimensional gel electrophoresis (2-DE)

In 1975, the basic technology for two-dimensional gel electrophoresis (2-DE) was illustrated by O'Farrell [124]. Since then, 2-DE has become a powerful and commonly used methodology to study proteomic expression followed by mass spectrometry (MS) for protein identification [125]. 2-DE is based on the separation of proteins in two dimensions. In the first dimension, proteins are separated according to their charge (isoelectric point, pI: the pH at which a protein carries no net electrical charge) using a

technique called isoelectric focusing (IEF). In the second dimension, proteins are separated according to their size (molecular weight) using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [126]. Figure 7 illustrates the general principle of 2-DE [108].

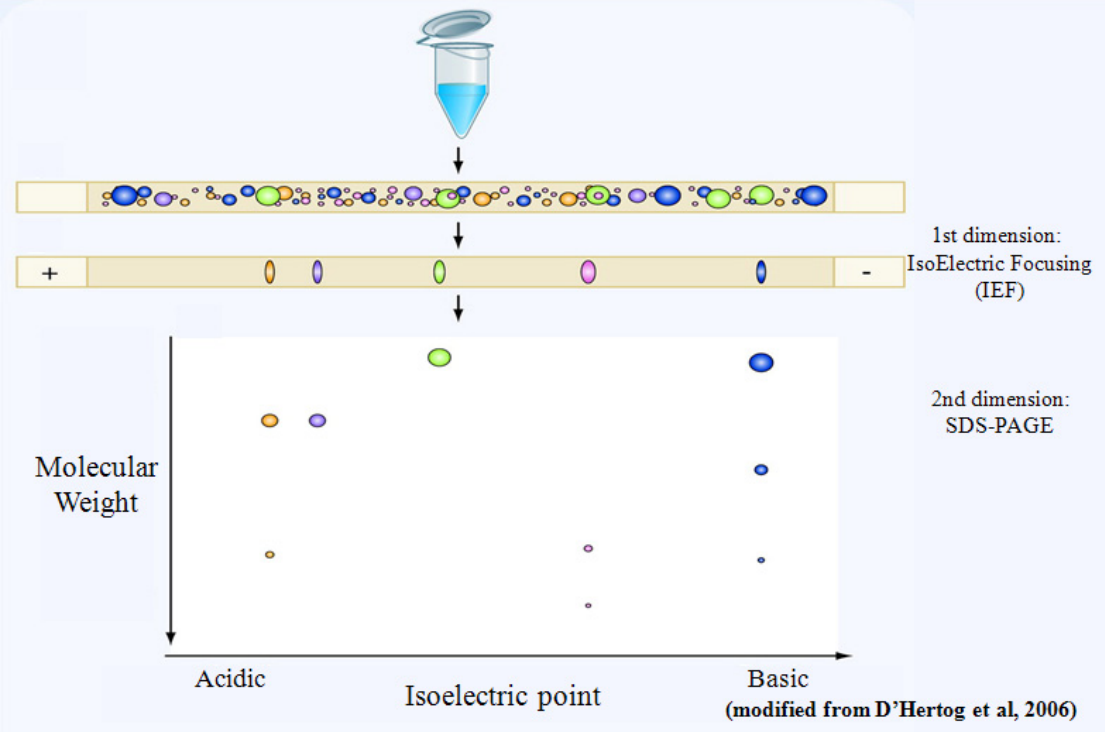


Figure 7. General principle of 2-DE

Proteins solubilized in buffer, typically containing high concentration of urea and detergent, are applied to an immobilized pH gradient strip for IEF (1st dimension). IEF separates proteins by their isoelectric points (pIs). SDS-PAGE (2nd dimension) separates proteins by their molecular weights.

A major advantage of separation of proteins by 2-DE is its ability to visualize proteins that have undergone post translational modifications (PTM) or mutation. Those proteins appear as spot trains in the horizontal and/or vertical axis of the gel (e.g., phosphorylation, glycosylation and limited proteolysis or point mutations). Another advantage is that 2-DE can separate a high amount of proteins due to the commercial availability of a whole range of different pH strips for first dimension separation [108]. Therefore, 2-DE can simultaneously separate about 2,000 proteins in a single gel depending on the chosen pH gradient and gel size [125]. In contrast, one of major drawbacks of 2-DE is the spot visualization of low abundant proteins since proteins have a wide dynamic range in abundance [127]. Another major drawback of 2-DE is the resolution of membrane and large hydrophobic proteins since most of them

are not soluble in aqueous solution and thus do not enter the pH strips during the first dimension [128-129].

1.3.4.2 High pressure liquid chromatography (HPLC)

High pressure liquid chromatography (HPLC) separates analytes based on their idiosyncratic polarities and interactions with the column's stationary phase. In MS-based proteomics, the following types of HPLC chromatographic materials are most commonly used: reverse phase (RP), ion exchange (IEX), hydrophilic-interaction chromatography (HILIC), affinity, and hybrid materials [97].

The reverse phase liquid chromatography (RPLC) is as indispensable to gel-free LC/MS as 2-DE is to gel-based proteomics [130]. RPLC separates peptides based on their hydrophobicity. The significant advantages of RPLC are that the buffers used are compatible with ESI and that RPLC desalts the sample [131-132]. The disadvantage is that it seldom provides enough resolution. However, by decreasing the diameter of the packing materials or by using narrow-bore, long columns under ultra-high pressure conditions, this issue has been overcome and separation performance has been improved dramatically [133].

Multidimensional separation, which combines several separation techniques coupled to improve the resolving power, is employed to tackle high sample complexity. In reality, analytical samples like tissue lysate or bio-fluid contain thousands of proteins that can range upward of five orders of magnitude in their abundance [127]. Moreover, the complexity of protein samples becomes even higher after being digested by trypsin, which, in turn, yields multiple peptide products [134]. It is essential for multidimensional separation to consider the orthogonality of the individual separation methods in which each dimension uses different properties of molecules as a basis for separation [135].

1.3.5 Analytical techniques for protein identification

Typically, two different analytical techniques are used to identify proteins in MS analysis. One is Peptide Mass Fingerprinting (PMF) in which the experimental spectrum obtained by MALDI (Figure 8) is compared with theoretical ones. These are computed from protein sequences stored in databases and *in silico* digested using the same cleavage specificity of the protease employed in the experiment [136]. The PMF method is suitable for identification of isolated proteins. Complicated mixtures of proteins typically require the use of tandem mass spectrometry (MS/MS) based protein identification to achieve sufficient specificity of identification [137].

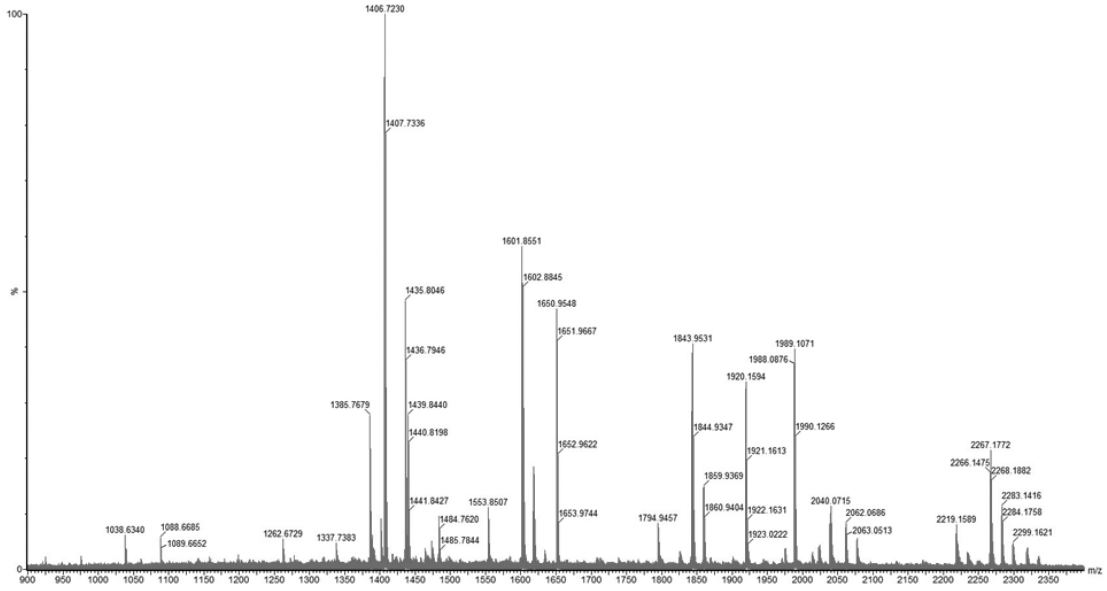


Figure 8. A typical MS spectrum obtained by MALDI

After a MALDI spectrum has been de-isotoped, a list of peaks, each of which represents a peptide, is created. A list of peaks is submitted to the database for analysis using a library of protein sequences.

MS/MS is another technique for protein identification by sequencing peptides (Figure 9). To fragment peptides, the technique called collision-induced dissociation (CID) [138] has been widely used. In general with this method, the precursor ions are picked up first, and go through peptide backbone fragmentation of C-N bonds resulting in a series of b-fragment and y-fragment ions [118]. However, the usage of CID is not suitable for sequencing peptides containing more than fifteen amino acids and intact proteins.

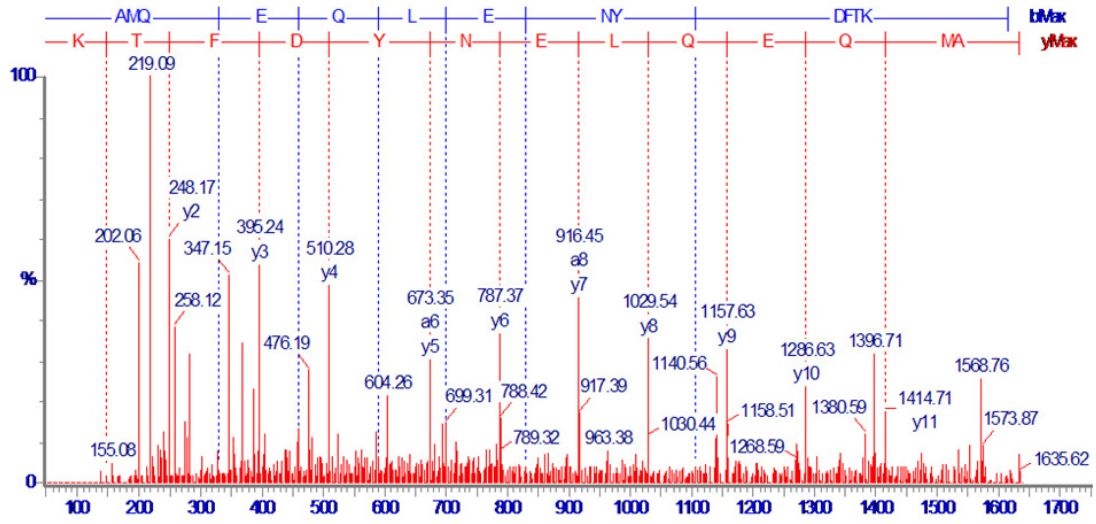


Figure 9. A typical MS/MS spectrum obtained by ESI

The MS/MS spectrum of mouse “EH-domain containing 4” peptide "AMQEQLENYDFTK". The sequenced peptide is blasted in the database to identify the protein.

1.3.6 Relative quantitative proteomics

In the past decade, the trend of proteomics research has shifted from simple identifications to complex quantitation of proteins for the understanding of cellular activities, disease developments and physiological responses to therapeutic interventions and environmental perturbations. Relative quantitative proteomics allows for simultaneous identification of hundreds of proteins and reveals differences in protein abundance between distinct samples [139] With the aim of investigating differential protein expression in complex biological samples, there are two approaches employed in MS-based proteomics; a gel-based method for intact proteins and a non gel-based method for enzymatically digested peptides.

1.3.6.1 Differential gel electrophoresis (DIGE)

Optical detection of proteins with a fluorescent label has an excellent dynamic range, and 2-DE is well-suited to separate and analyze proteins and their isoforms. Combining these two methods, Differential Gel Electrophoresis (DIGE) was invented for quantitative analysis of intact proteins [140]. DIGE is a technique allowing quantitative proteomics of two or more samples by optical fluorescence detection of differentially labeled proteins that are electrophoretically separated on the same gel [141]. This methodology involves pre-labeling several samples with spectrally distinct

fluorescent dyes (e.g. Cy2, Cy3, and Cy5) coupled to primary amines such as the N-terminus and lysine residues, followed by sample multiplexing and co-resolution on the same 2-DE gel to eliminate gel to gel variations [142]. Moreover, multi-variant analysis can be inter-compared with a relatively small number of coordinated DIGE gels using internal standards and experimental repetition.

DIGE is an extremely sensitive method for detection of proteins. It can detect less than 1 fmol of protein, and allows linear detection of over more than 10^4 fold protein abundance range [143-144]. Conversely, this methodology has some limitations including low-throughput and labor-intensive operation with high dependence on the expertise of the operator. In addition, 2-DE does not perform well for hydrophobic and very acidic or basic proteins [145].

1.3.6.2 Stable isotope-labeling methods

Two general approaches are utilized to incorporate stable isotopes into proteins/peptides for relative quantitation, metabolic labeling and chemical derivatization. For metabolic labeling, cells are cultured in isotopically defined and distinct medium containing unique isotope-labeled amino acids (heavy Arg, Lys, Leu and Ile). The relative-abundance ratio of peptides with identical primary sequence from mixed cell lysates is measured by comparing heavy/light peptide pairs by MS [97, 146]. One of the commonly used approaches is stable-isotope labeling by amino acids in cell culture (SILAC).

In an experiment employing SILAC, the labeled SILAC amino acids, for instance ^{13}C labeled L-lysine, are supplied to cells in culture instead of the natural amino acids, and the SILAC amino acids are incorporated into all newly synthesized proteins. After at least five cell divisions, replacement of amino acids will be completed [147]. In view of the fact that there is hardly any chemical difference between the labeled amino acid and the natural amino acid isotopes, the two cell populations should behave exactly the same in the presence of normal or labeled amino acid. In general, much less sample preparation for quantitative studies is required for metabolic labeling experiments, although tissue samples or body fluids cannot be used [145].

Chemical derivatization strategies, including isotope-coded affinity tags (ICAT) [148] and isobaric tags for relative and absolute quantification (iTRAQ) [149] allow measurement of changes in expression levels between two proteomes in a single MS-based experiment. Concisely, for the relative quantitation of peptides from proteolytic

digestion of complex protein mixtures, an isotopically unique functional group is added chemically to a peptide to distinguish separate samples by their unique mass [145].

In the ICAT method, proteins from two different biological samples are labeled with a biotin-containing heavy (typically ^{2}H or ^{13}C) or light (native) version of a reagent that selectively couples to the side chain of a reduced cysteine residues [148]. Subsequently, the labeled proteins are combined and proteolytically digested into peptides, and affinity purification using streptavidin is applied for enrichment of labeled peptides. Utilizing the ratio of the signal intensities from the 'heavy' and 'light' forms of each peptide, the relative abundance of peptides is determined by MS analysis. Subsequently individual peptide ratios from the same protein are combined to produce abundance ratios of identified proteins in the sample [150]. A major advantage with ICAT is that it enhances the detection of low-abundance proteins due to the enrichment of the modified peptides via affinity purification of the biotin moiety. Nonetheless, because of selectiveness of the ICAT reagent, which only labels proteins/peptides containing cysteine residues, quantitation of proteins not containing a cysteine residue will not occur [146].

In the iTRAQ method, amine-specific isobaric reagents are used to label the primary amines of peptides for the concurrent quantitative comparison of up to eight different biological samples [151-152]. This method was developed to solve some of the limitations of ICAT. Compared to the ICAT method which produces multiple MS peaks per peptide, the MS spectrum of each peptide in this method does not increase in complexity owing to the isobaric nature of the tagging reagents. Relative peptide abundance measurements between the samples are made based on the relative intensity of each reporter ion with distinct m/z, which is released during peptide fragmentation. In contrast to ICAT, iTRAQ theoretically labels all peptides in a mixture. However, labeling after protease digestion in the iTRAQ method limits the use of reporter ions as internal standards since experimental variations at prior sample preparation steps are not accounted for [146]. Table 4 summarizes three stable isotope-labeling methods [153].

Table 4. Comparison of three methods: SILAC, ICAT and iTRAQ

	how to label	suitable sample	when to digest	where to label
SILAC	metabolically	cell culture	after labeling	depends*
ICAT	chemically	anything	after labeling	cysteine residues
iTRAQ	chemically	anything	before labeling	primary amine

*One or several amino acids depends on the media used

	advantages	disadvantages
SILAC	• replacement of AA** occurring in the biological system • flexible sample preparation	• applicable only to cell cultures
ICAT	• any biological materials can be used • detection of low-abundance proteins	• relatively extreme chemical conditions • only labeling proteins/peptides containing cysteine residues
iTRAQ	• completion of the tagging reaction without major side reactions • up to eight different samples can be used	• expensive in large-scale experiments • variation of sample preparation

**Amino acids (modified from Wilm, 2009)

1.3.6.3 Label-free methods

Label-free quantification requires only minimal modification of the sample and is therefore the most compatible method for quantifying the expression changes of proteins in large-scale proteomics studies [153]. The foundation of label-free approaches is the comparison of distinct regions of *m*/*z* and retention time which represent the same analyte across multiple analyses. This method functions based on the observation that signal intensity from ESI correlates with ion concentration [154] and that an ion with a particular *m/z* is detected at a particular time.

There are some practical constraints to this method for the quantitation of protein abundances in several complex biological samples. One drawback is the difference in the peak intensities of the peptides from the same sample between runs, caused by experimental variation such as sample injection. The other hurdle is chromatographic shifts in retention time which occur as a result of multiple sample injections onto the same reverse-phase LC column [155]. Therefore, the thorough strategy, including meticulous experimental design, cutting-edge computational method, and highly reproducible technology, is vital in this comparative approach to normalize and correct experimental variances which affect MS intensity and retention time [146].

2 Aim of the study

The overall aim of this study was to investigate the kidney glomerulus at mRNA and protein level and to provide new insights into the biological and pathological processes of the glomerulus. To accomplish this goal, glomeruli were isolated from both healthy and diseased kidneys and studied by expression profiling and protein analyses. The specific aims were to:

I: Identify glomerular proteins from mouse kidneys using two dimensional gel electrophoresis and mass spectrometry.

II: Characterize novel proteins in the kidney, which are highly expressed in mouse glomeruli.

III: Perform glomerulus specific transcriptome profiling in the adriamycin-induced proteinuric mouse model.

3 Experimental procedures

In this section, various methodologies employed throughout this study are briefly described. Detailed descriptions can be found in the "Materials & Methods" sections of the corresponding papers (I, II and III).

Isolation of mouse kidney glomeruli (I and III)

Glomeruli were isolated by the Dynabeads® perfusion method [156] using female C57BL/6 mice for paper I and female BALB/c mice for paper III. Figure 10 illustrates the procedure of isolation of glomeruli.

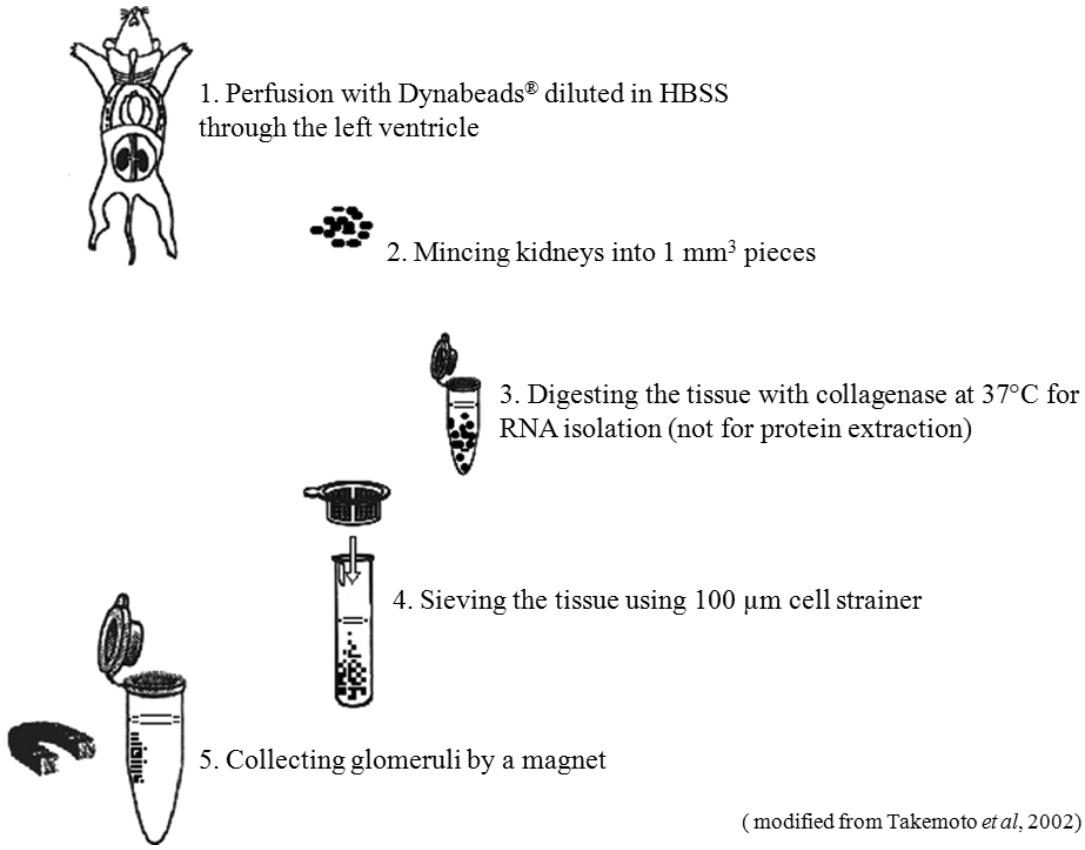


Figure 10. Flow chart of mice glomeruli isolation technique

Briefly, an anesthetized mouse was perfused through the left ventricle with HBSS containing $8x10^7$ Dynabeads® (Invitrogen, CA). The kidneys were removed, minced and digested with collagenase A and DNase I for 30 min at 37 °C for RNA isolation (III). For extraction of the whole proteome from the glomeruli, collagenase

treatment was eliminated to avoid digestion of extracellular proteins (I). Minced kidney tissue was then sieved through a 100 μm cell strainer, and the glomeruli-containing magnetic Dynabeads were collected with a magnetic particle concentrator. The glomeruli were counted and checked for purity under a light microscope.

Extraction of proteins from isolated mouse glomeruli (I and II)

Isolated glomeruli were suspended in water, and the suspension underwent four freeze-thaw cycles using liquid nitrogen to disrupt the cells. The samples were then freeze-dried under vacuum. Dried samples were solubilized in a buffer containing urea, DTT, and detergents in a shaker at 20 °C for 4 h. After centrifugation, supernatants were collected and protein concentrations measured with the Bradford assay.

Two-dimensional gel electrophoresis (I)

Protein extracts, 75 μg for silver-stained gels and 500 μg for the CBB-stained gels, were applied onto 17 cm IPG strips, and IEF was carried out with total voltage hours (Vh) of 51000 for the pI 4–7 strips and 35000 Vh for the pI 3–10 strips. The second dimension was performed on homemade 8–16% gradient SDS-PAGE gels. The gels were stained with silver or CBB. Molecular weights of proteins were determined by comparisons with positions of protein standards.

Sample preparation for mass spectrometry (I)

Protein spots were excised manually from both the CBB-stained and the silver-stained gels. Gel pieces were cut into small pieces and digested with trypsin using a protocol previously published [157]. Peptides were extracted from gel spots with acetonitrile solution containing formic acid. Acetonitrile was removed, and then samples were desalted manually with ZipTips® (Millipore, MA). For MALDI analysis, desalted samples were mixed 1:1 with a matrix solution containing α-cyano-4-hydroxycinnamic acid. For reduced matrix adduct formation in spectra, ammonium phosphate was added to the matrix [158].

Mass spectrometry analysis of trypsin digested proteins (I)

A MALDI instrument, Q-TOF Ultima (Waters, MA), was employed for peptide mass fingerprinting (PMF). The instrument was calibrated with a polyethylene glycol standard between 80–2500 *m/z*, and was operated in single reflector mode and with positive ion data. The MS software, MassLynx Version 3.4, was used to create a peak list, and MaxEnt3 was applied for each list before submitting into the Mascot search engine (http://www.matrixscience.com/).

A LC-API instrument, Q-TOF Ultima tandem mass spectrometer was utilized for amino acid sequences of relevant peptide samples for the identification of samples, which could not be confirmed by PMF experiment. Data-dependent acquisition (DDA) was used at 300–1600 *m/z* in MS mode and 50–1600 in MS/MS mode with a scan rate of 1 s and automatic switching from MS to MS/MS mode on multiple charged ions. PLGS 2.2.3 was used to a pkl-file for protein data searches.

Production of Polyclonal Antibodies (II)

Antibodies were made using tagged recombinant proteins as antigens. Briefly, after antigens were expressed as tagged recombinant proteins (His-tag for dendrin, GST-tagged for ehd3) in bacteria, they were purified by affinity chromatography, followed by ion-exchange chromatography. Purified antigens were utilized for production of antibodies by immunization of NZW rabbits using standard protocols.

Western Blotting (II)

Western blotting was carried out following standard procedures using polyvinyl difluoride (PVDF) membranes. Briefly, 10 μg of proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Then, the membrane was blocked, and incubated with a primary antibody. After being washed for 2 hrs, the membrane was incubated with horseradish peroxidase–conjugated secondary antibody. The protein of interest was detected by chemiluminescence.

Immunohistochemistry (II)

Adult mouse kidney was used for immunofluorescence staining. Kidneys were snap-frozen, and the cryosections (10 µm) were postfixed with cold acetone, followed by blocking in 5% normal goat serum. The primary antibodies were incubated overnight at 4 °C, followed by the incubation with fluorescent secondary antibody.

Induction of ADR nephropathy in mice (III)

Six-week-old female BALB/c mice, weighing 16-20 g, were used. Adriamycin (ADR, doxorubicin hydrochloride) was diluted with isotonic saline solution (0.9% NaCl) to 2 mg/ml, and was injected as a single dose via the tail vain at a dosage of 10.5 mg/kg body weight.

Urine and blood analysis of ADR-induced proteinuric mice (III)

Spot urine was collected from mice. To confirm proteinuria, 1 µl of urine was applied to an SDS-PAGE gel, and the gel was stained with PageBlue™. Total urinary protein concentration was quantified using Coomassie Plus. Urinary creatinine was quantified using the QuantiChrom™ Creatinine Assay Kit to calculate protein/creatinine ratio. Blood samples for blood urea nitrogen (BUN) measurements were collected under anesthesia by cardiac puncture. BUN was quantified using the QuantiChrom™ Urea Assay Kit.

Histological assessment (light- and electron-microscopy) (III)

For light microscopy, kidneys were fixed in 4% paraformaldehyde (PFA) at 4 °C for 24 h and then dehydrated in graded alcohols and embedded in paraffin. Sections (4 µm) were stained with hematoxylin and eosin (HE). For electron microscopy, small kidney pieces were fixed in 0.05 M sodium cacodylate buffer with 2% PFA and 2.5% glutaraldehyde.

TUNEL staining and positive cell counting inside glomerulus (III)

Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) staining was performed to assess the presence of apoptotic cells. Anti-podocalyxin antibody was used for labeling the secondary foot processes of the podocytes. In each sample, 100 glomeruli were counted randomly, and glomeruli containing TUNEL positive cells were analyzed using Volocity software. The counting was repeated three times for each sample.

Microarray analysis (III)

Total RNA from glomeruli was extracted using RNeasy Mini kit (Qiagen, Hilden, Germany). Extracted RNA was labeled and hybridized on Affymetrix Mouse Genome 430 2.0 chip. Affymetrix data were processed using the R software (version 2.9.0) and the Bioconductor packages. Affymetrix raw data were normalized with the gcrma method (R package version 2.16.0). After normalization, samples were subjected to hierarchical clustering (Euclidian distance, average linkage).

To identify differentially expressed genes between samples, all probe sets with expression changes more than two-fold at any time point were selected. The student t-test (two sided) was applied to examine the significance. Multiple test correction was done using the False Discovery Rate (FDR) method and a FDR <0.1 was used to select the differentially expressed genes.

4 Results and discussion

In Paper I, we described the use of 2-DE gel electrophoresis and mass spectrometry analysis to generate new information about the healthy glomerular proteome in mouse as a basis for later studies on the glomerular proteome from diseased kidneys.

After the isolation of glomeruli, the purity and number were checked under a light microscope. The glomeruli appeared to be highly pure and devoid of Bowman's capsule. The number of glomeruli obtained from one mouse was approximately 20,000. The amount of glomerular protein extracted from one mouse was approximately 100 μg.

Total glomerular, denatured protein extracts were subjected to two different pH ranges of immobilized pH gradient (IPG) strips for IEF, and two different methods were used to stain the gels (Table 5).

Table 5. Summary of three 2-DE experiments

pH range of IPG strip	Gel-staining method	number of spots detected	number of spots excised
3 to 10	CBB*	342	96
4 to 7	CBB*	625	192
4 to 7	silver	922	480

* Coomassie Brilliant Blue

Each excised spot was digested with trypsin and analyzed individually first by MALDI-TOF MS for peptide mass fingerprinting (PMF), using a minimum of four matching peptides, a coverage of 12% of the protein, minimal scores and maximal expected values as given for approval of the identification of protein. Furthermore, relevant protein spot identifications were checked by amino acid sequence analysis using LC-MS/MS. Eventually, 414 protein spots, representing 232 different proteins, were identified. 72 out of 232 proteins were identified by silver staining.

Most of the identified proteins are ubiquitous, housekeeping proteins whereas several are cytoskeletal structural proteins highly expressed in podocytes (actin, F-actin, vimentin, α-tubulin, β-tubulin), as reported previously [159]. Even though previous 2-DE studies of the kidney glomeruli have not been able to identify any proteins expressed specifically in the kidney podocyte slit diaphragm, we did identify

α-actinin-4 and nephrin, which are known to play important roles in the glomerular filtration barrier and its diseases.

Surprisingly, only 53 out of the 232 identified proteins had been detected in other glomerular proteome studies [159-160]. This result could be due to many factors, including the use of several identification methods and the quality of tissue preparations. Altogether, our result demonstrates the value of repeated proteome analyses using different methodologies.

Comparison of proteome analyses of endothelial and mesangial cells to that of ours revealed that these proteome studies detect many of the housekeeping proteins shared by similar tissues [160-161]. In addition, comparison of the results of these direct proteome studies with those of separate transcriptome analyses exposed that an independent glomerular-specific cDNA library analysis [56] could not identify all of these proteins. This shows that multiple approaches are indispensable to complement each other in final interpretations of functional relationships.

Miyamoto *et al*. analyzed the human glomerular proteome using a different approach from ours [162] . They separated proteins using 1-D (SDS-PAGE) and 2-D (solution-phase IEF in combination with SDS-PAGE) prefractionation prior to the shotgun analysis with LC−MS/MS. As a result, they identified around 3,000 unique proteins. This fact proves that prefrationation of the sample before applying to MS analyses is vital. Due to drawbacks of 2-DE, a limited dynamic range for detection, and a poor resolution of membrane and large hydrophobic proteins, more effective and sensitive methods have emerged and are now replacing 2-DE. Multidimensional liquid chromatography (MDLC) is one of these. An HPLC based MDLC platform can reduce the sample complexity prior to the MS analysis, and can therefore maximize the chances that the MS will detect peptides present in tryptic digest of a proteome [163].

In Paper II, we characterized five novel transcripts/proteins that were identified among more than 300 glomerulus-upregulated transcripts in a large-scale sequencing and microarray profiling analysis of the glomerular transcriptome [56]. The transcripts/proteins, ehd3, dendrin, sh2d4a, plekhh2, and 2310066E14Rik were characterized with respect to their expression profiles in detail. RT-PCR and Northern blotting experiments demonstrate the presence of four transcripts, except plekhh2, in kidney, and the size of each transcript was in agreement with the predicted cDNA size described in the NCBI database. In addition, Western blotting experiments revealed the presence of five proteins in glomerular lysate but not in the rest of kidney, and the size

of each protein was in agreement with its predicted protein size. The glomerulus specificity of three mRNAs, dendrin, ehd3 and2310066E14Rik, was verified using *in situ* hybridization, and the localization of five proteins was revealed to be specific to the glomerulus based on immunofluorescence staining. Dendrin, Sh2d4a, and Plekhh2 proteins were localized to the slit diaphragm and the foot process, 2310066E14Rik protein was localized to the podocyte major processes and cell body, and the Ehd3 protein was localized to glomerular endothelial cells (GECs).

It is known that several critical glomerular proteins, such as nephrin, podocin and NEPH1 have very restricted expression patterns, and these crucial proteins are expressed exclusively by podocytes in the kidney glomerulus [13, 15, 164]. Therefore, the limited expression pattern of the proteins discovered in this study advocates that they may have a highly specific role in the glomerulus.

We hypothesize that dendrin may act as a linker molecule between the slit diaphragm and the actin cytoskeleton based on our finding that dendrin is located at the cytoplasmic face of the slit diaphragm. This hypothesis is supported by recent data showing that dendrin interacts *in vivo* with two cytoskeletal components, α-actinin and membrane-associated guanylate kinase inverted (MAGI) [165]. We found that ehd3 was expressed specifically in GECs, and to our knowledge Ehd3 is, thus, the first protein shown to be expressed only in GECs and not by other endothelial cells. This discovery is intriguing because the use of the ehd3 promoter may enable the generation of a GEC-specific Cre mouse line, and such a mouse line could be a vital tool for future research. We discovered that both cytosolic proteins, Sh2d4a and Plekhh2, are expressed only in podocytes, and at the subcellular level, we localized these proteins specifically to the foot processes. Our hypothesis is that Sh2d4a protein may be involved in slit diaphragm signaling and that the Plekhh2 protein may be involved in connecting the slit diaphragm to the actin cytoskeleton.

In the work described in paper III, we compared the glomerular transcriptome in normal and disease states, as well as its changes during progression or recovery of glomerular disease utilizing the adriamycin (ADR)-induced proteinuric mouse model. This model is highly reproducible and allows the precise timing of the onset of proteinuria and the change of morphology in glomerulus, therefore, is excellent to study expression profiles during disease progression.

All animals injected with a single dose of ADR developed nephrotic syndrome characterized by body weight loss, massive proteinuria, and elevated BUN. Overt

proteinuria was observed four days after ADR injection, and the leakage of serum albumin reached its maximum at seven days. Electrophoresis of urinary proteins using SDS-PAGE revealed that proteinuria in ADR-injected mice consisted mainly of albumin with a molecular weight of about 70 kDa.

Apparent morphological changes in ADR-injected mice kidney were observed at day seven in electron microscopy as segmental podocyte foot process effacement was detected. Light microcopy revealed first pathological changes at day 14 as resorption droplets and intraluminal casts were detected in the tubular compartment. TUNEL staining revealed an increase in apoptotic positive cells in glomeruli of ADR-injected mice, and the number of apoptotic positive cells gradually increased after ADR injection, reaching a maximum at 14 days after ADR injection.

Affymetrix microarray experiments were performed using mRNA from isolated kidney glomeruli at four time points (0, 4, 7 and 14 days). Four days after ADR injection a total of 698 genes were differentially expressed in the acute injury phase. Nine p53 target genes were found to be up-regulated probably due to DNA damage caused by ADR. Five inflammatory response genes were found to be up-regulated and one gene, Socs3, was found to be down-regulated due to ADR toxicity. Two apoptosis related genes, Serpina3g and Tnfrsf12a, were found to be up-regulated. Three AP-1 (active protein-1) genes, belonging to the family of dimeric transcription factors binding to the AP-1 binding site of DNA, Atf3, Fos and Fosb, were all found to be down-regulated.

Since ADR is known to cause oxidative DNA damage [166], it was not surprising to observe a major increase in the levels of transcripts related with the p53 protein pathway in the acute injury stage at 4 days after ADR injection [167-168]. Although the expression of p53 itself did not significantly change, the expression of nine p53 target genes was changed at 4 days after ADR injection. The change of these genes expressions and the number of apoptotic cells inside glomeruli suggest that DNA damage due to injection of ADR has occurred and led to apoptosis of glomerular cells. However, since the number of apoptotic cells inside the glomeruli is still relatively small four days after ADR injection, and the proteinuria has not reached maximum, the podocytes and the glomerular filter have probably not yet become extensively damaged.

275 genes were differentially expressed at the seven days stage. Several genes indicating glomerular injury were differentially expressed seven days after ADR injection. Three genes related to human kidney diseases and proteinuria, Ankrd1[169],

Cyr61[170] and Serpine1 [171], were found to be up-regulated. Two DNA damage response genes were found to be up-regulated. Four podocyte enriched/specific genes were found to be down-regulated, suggesting podocyte injury caused by ADR [172].

At 7 days after ADR injection, the ratio of urine protein/creatinine had reached its maximum and there was extensive effacement of podocyte foot processes. At that time one could observe a marked change in the expression of several podocyte specific genes. As could have been expected, several genes expressed specifically in podocytes were down-regulated at this stage. Ddn (dendrin) is one of them, and is a specialized cell junction component present at the slit diagram (SD), where interdigitating foot processes of podocytes are connected [173]. As dendrin is known to bind directly to nephrin and CD2AP in the slit diaphragm [174], we examined the expression levels of those genes. Dendrin is relocated from the slit diaphragm to the nucleus of injured podocytes in experimental glomerulonephritis [174], but we could not detect the protein in the nuclei of podocytes in ADR-induced nephropathy. Considering the present data that show changes in the expression of podocyte specific genes at seven days after ADR injection, it is clear that ADR causes extensive podocyte injury, which leads to end stage renal failure.

A total of 600 genes were differentially expressed 14 days after ADR injection, at a stage when slight recovery of glomerular injury and the proteinuria had initiated. Two genes, Fgfbp1 and Acvrl1, were found to be up-regulated only at this time point. The majority of genes up-regulated at this point were found to be up-regulated from day 4 or day 7 after ADR injection.

At 14 days after ADR injection, overt proteinuria was still present and the number of apoptotic cells in glomeruli had further increased. Also, morphological changes were still observed in the kidney cortex by light microscopy. The proteinuria caused by ADR-induction is not transient and reversible like it is in LPS-induced proteinuria mice model [88].

Several genes were significantly expressed at all three time points, days 4, 7 and 14. Cdkn1a was one of those genes, which interestingly is known to play dual roles. Cdkn1a protein has prosurvival or proapoptotic function depending on its relative protein levels, subcellular localization and protein–protein interactions [175-178]. To verify which role Cdkn1a plays in the ADR-induced nephropathy model, we examined the expression of genes that are directly or indirectly linked with Cdkn1a. 57 genes were categorized into two groups; 20 genes to be positively correlated to and 37 genes to be negatively correlated to Cdkn1a expression profiles. Among them, five p53-

related genes were found to be positively correlated to Cdkn1a expression profile indicating that p53 was induced by ADR injection followed by DNA damage. Two anti-apoptotic genes were also positively correlated with Cdkn1a expression profile indicating these genes restrained the number of apoptotic cells in glomeruli. On the other hand, eight podocyte specific genes were found to correlate negatively with the Cdkn1a expression profile indicating that the podocyte was the direct target of ADR-induced DNA damage.

We conclude that ADR causes DNA damage inside glomeruli and it may lead to podocyte injury. It is not exactly clear which part of glomeruli is primarily affected by ADR, or which pathways are involved to induce overt proteinuria. Further investigations are needed to reveal which genes and pathways play important roles in the development of proteinuria in the ADR-induced proteinuric model.

5 Conclusions and future perspectives

Identification of proteins in mouse glomeruli using 2-DE gel electrophoresis and mass spectrometry with two different staining methods provided new knowledge about proteomes in glomeruli, but the amount of identified proteins was highly limited. Characterization of five novel genes, specifically expressed in glomeruli, rendered new information on novel proteins. They will be interesting to study further for function in mouse models and expression in glomerular diseases. Comparison of the glomerular transcriptome in normal and disease states, as well as its changes during progression or recovery of glomerular disease utilizing the ADR-induced proteinuric model in mouse gave us additional intuition toward understanding the pathogenesis of proteinuria. It is apparent that extensive transcriptome analysis of isolated glomeruli will yield immense new information on various glomerular diseases. Such analyses may have diagnostic value in the future.

Our knowledge about the gene and protein expression complexity in the glomerulus has been accumulating steadily over the years. At the same time, new technologies are arising to explore new information concerning the structure and development of the renal filter and its cellular and extracellular components. Furthermore, transcriptome and proteome analysis of human diseases affecting glomerular function, and of animal models of these diseases, will lead to the identification of critical developmental and maintenance processes, and unique and common pathogenic pathways involved in glomerular disease.

In conclusion, it is crucial to combine large-scale expression profiling platforms (RNA-seq), quantitative proteomics using mass spectrometry, transgenic mouse lines, and other *in vivo* gene delivery methods. That will further expand our understanding of the physiology and pathphysiology of the glomerular filtration barrier, and reveal novel target molecules for the therapy of kidney diseases.

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2010. 8.11
Massa

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