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Proteins Lost and Found: Not Only a Tubular Matter

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Abstract

Proteinuria, manifested predominantly as albuminuria, has been recognized as an independent risk factor for both renal and cardiovascular disease. The loss of proteins is the hallmark of tubular and glomerular diseases and can be due to structural and/or functional alterations involving different cell types. Several studies have underlined the involvement of gene mutations in the pathogenesis of glomerulopathies and tubulopathies.

In the kidney, proximal convoluted tubular epithelial cells are the main responsible for the uptake of filtered macromolecules. Protein endocytosis needs an active receptor-mediated pathway which involves Megalin (LRP2), Cubilin, Amnionless (AMN), Disabled-2 (Dab2), the low density lipoprotein receptor adaptor protein 1 (ARH) and the Cl^-/H^+ antiporter ClC-5. Although the role of the tubular cells in the protein uptake is a well-known process, less is known regarding the role of glomerular cells (podocytes, mesangial, endothelial and parietal epithelial cells) in protein rescue. In the last few years, major attention has been focused on albumin handling by glomerular cells in particular by podocytes and parietal epithelial cells.

In this review we described the principal mechanisms leading to protein loss and we examined in depth the mechanisms involved in their rescue both at tubular and glomerular levels. The actual knowledge scenario supports the idea of a partnership between tubular and glomerular cells in albumin uptake, *via* the same way of internalization.

Keywords: Proteins; Proteinuria ; Podocytes; Dab2; Glomerulopathies; Megalin

Introduction

Proteinuria, manifested predominantly as albuminuria, is not only a marker of kidney disease but also a known risk factor associated with kidney disease progression, cardiovascular disease and death in people with renal disease, hypertension, diabetes, vascular disease and in general population [1-3]. Increases in albuminuria also significantly associated with cardiovascular death, composite cardiovascular outcomes (atherosclerotic lesions, cardiovascular death, myocardial infarction, stroke, and hospitalization for heart failure) independently of its baseline values [3,4]. Chronic kidney disease (CKD) was defined by the Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines as persistent kidney damage usually marked by albuminuria or reduced glomerular filtration rate [5]. CKD is recognized as a major public health problem affecting 10-16% of the adult population worldwide, increasing the risk of all- cause mortality, cardiovascular disease and progression to kidney failure [6]. This Review discusses the main mechanisms leading to protein loss and subsequently analyzes in depth the mechanisms involved in their rescue both at tubular and at glomerular compartments.

How Kidney Lose Proteins

The Glomerular Filtration Barrier

Glomerular protein handling mechanisms have received much attention in the study of proteinuria [7]. The renal corpuscle (glomerulus) is composed by different cell types: parietal epithelial cells, podocytes, endothelial cells and mesangial cells. Mesangial cells are irregularly shaped and surrounded by an extracellular matrix (mesangial matrix) that lies between the mesangial and endothelial cells or the glomerular basement membrane (GBM) [8]. The glomerular barrier is by far the most complex biological membrane, with properties that allow for high filtration rates of water and not-restricted passage of small and middle-sized molecules [9]. Endothelial cells and podocytes have a negatively charged surface glycocalyx which, together with GBM sialoproteins and heparan sulfate, gives the glomerular filtration barrier an overall negative charge at physiological pH. Moreover, recent detection of mutations in the main component of the podocyte glycocalyx, podocalyxin, in familial nephrotic syndrome supports charge selection through electrostatic

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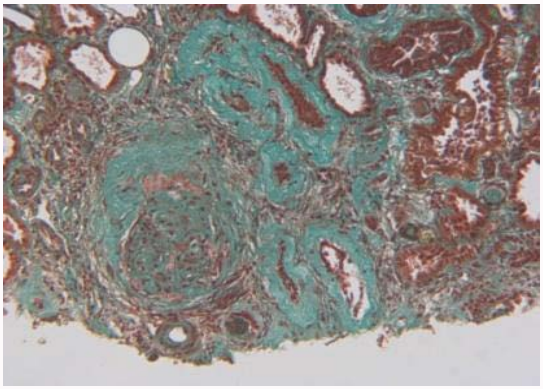

Figure 1: Glomerular fibrosis. Masson's trichrome stain highlighting collagen deposition. Magnification 100X.

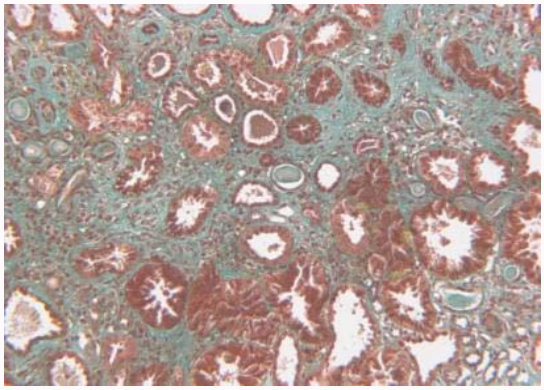

Figure 2: Tubulo-interstitial fibrosis. Masson's trichrome stain highlighting collagen deposition. Magnification 100X.

repulsion not less important in the glomerular filtration process is the slit diaphragm [10]. In the past few years, much has been learned about the molecular architecture of this structure. During development, podocytes are initially connected via tight-junction and gap-junction components. Gradually, these components are replaced by the neuronal-junction components, nephrin, podocin and Neph1. These proteins form a zipper-like structure that is the hallmark of the mature slit diaphragm [11]. Podocyte injury is not the only cause of glomerular diseases, but a stable podocyte architecture with interdigitating foot processes connected by highly specialized filtration slits is essential for the maintenance and proper function of the glomerular filtration barrier. Experimental and clinical studies have indicated a pivotal role of podocyte injury in the development and progression of glomerular diseases [12].

The classical view of a tight glomerular filtration barrier was challenged a couple of years ago, when intravital 2-photon microscopy was used to reassess the glomerular sieving coefficient of albumin in live rats [13]. The quantity of albumin that passes through the glomerular filtration barrier is a controversial point. Tenten et al. [14] suggested that large amounts of albumin (in the range of 200 g/day when translated into human subjects) pass through the glomerular barrier and are subsequently retrieved by the proximal tubule and transferred via transcytosis to the circulation as intact protein. On the other hand, using the same intravital imaging approach, other groups have reported a glomerular sieving coefficient of albumin which was much lower [15]. These differences are probably due to the conditions of the animal and the collection of out-of-focus fluorescence in Bowman's space [13]. These data suggest a role also for tubular cells in the pathogenesis of proteinuric diseases.

Glomerulopathies

Glomeruli may be injured by different mechanisms and in the course of a number of systemic diseases. Diseases that present with proteinuria can be divided into three categories: diseases that are caused by abnormal glomerular cell function (e.g., Minimal Change Disease, Focal Segmental Glomerulosclerosis (FSGS), Diffuse Mesangial Sclerosis), diseases with antibody- mediated mechanisms (e.g., lupus nephritis, membranoproliferative glomerulonephritis, membranous nephropathy, IgA nephropathy) and diseases that are associated with metabolic disorders (e.g., diabetic nephropathy, obesity related glomerulopathy) [16].

A large number of inflammatory and degenerative glomerular diseases are prone to progress to chronic kidney disease displaying different histopathological features and time courses of progression [17]. Progression of fibrosis at glomerular level starts with the loss of the separation between the tuft and the Bowman's capsule. In degenerative conditions, this process leads to focal segmental glomerulosclerosis, in inflammatory conditions to crescentic glomerulonephritis. In both, the outcome with respect to progression is very similar [18]. Moreover, metabolic disorders such as diabetes and obesity, can also induce FSGS and fibrosis at glomerular level [19,20] (Figure 1).

Albuminuria has been shown to be also an independent risk factor for the progression of renal disease and protein overload was demonstrated to cause interstitial inflammation and fibrosis both in human and animals [21-23]. The concept of albumin-induced renal inflammation and scarring at tubular interstitial level has been intensively explored using both clinical and experimental approaches. The term "tubulointerstitial fibrosis" identifies extensive scar formation encountered in autopsies/biopsies of fibrosis development, including early stages of focal nephron degeneration/atrophy locally associated with interstitial matrix production [17] (Figure 2). Interstitial fibrosis is known to be of major importance in the deterioration of renal function and the well-known strong relationship to proteinuria suggests a role for filtered proteins in the development of this condition. In fact, the progression of the interstitial fibrosis may be perpetuated by filtered proteins through activation of tubular cells [24]. Transforming Growth Factor (TGF)-β1 is a key molecule in the fibrogenic cascade leading to CKD. It has been demonstrated that high concentrations of albumin induced TGF-β1 gene resulted in enhanced release of the cytokine in the supernatant of proximal tubular cells in culture [24].

Current thinking suggests that virtually all cases of the diseases caused by abnormal glomerular cell function begin with podocyte damage or dysfunction. For this reason, these diseases have been termed podocytopathies [16]. Podocytopathies are the most common group of glomerular disorders leading to proteinuria [25,26]. There is an evidence for a potential role for immune- regulation in podocytes damage since these cells express cytokine and chemokine receptors that were demonstrated to be target of immune stimuli both in vivo and in vitro [10].

In the next paragraph, we go in deep in the analysis of genes involved in hereditary glomerulopathies.

Hereditary Podocytopathies: Podocytopathies can also be consequences of genetic events (genetic mutations and deletions) in

podocytes genes. The genes involved in podocytes injury are numerous and code for structural elements of the slit diaphragm, for podocyte polarity and cytoskeleton, for proteins and enzymes associated with the glomerular basement membrane, for mitochondrial and lysosomal proteins, for nuclear proteins necessary for normal podocyte function and development, and for molecules involved in metabolic and signaling pathways (Table 1). Glomerular basement membrane gene defects usually result in insidious plasma protein leak whereas defects in slit diaphragm genes cause precipitous leak and nephrotic syndrome [10] (Table 1).

How Kidney Rescue Proteins

Tubular protein uptake

The nephron is composed of many different segments that work together to filter plasma, generating urine essentially devoid of large molecular weight proteins and regulating electrolyte balance through tubular secretion and re-absorption. The protein filtration process begins at the glomerulus and proceeds at proximal tubular level with the involvement of the endocytic machinery located at the brush border membrane [27].

Proximal tubular epithelial cells (PTECs) are tall and have a dense brush border of microvilli that greatly expand the area available for absorption. At the bases of the microvilli, the cell membrane is pitted extensively and displays numerous apical canaliculi that lead into the cytoplasm. This network of pits and tubules is underlined by vesicles representing various stages of endocytosis and is related to resorption of proteins from the ultrafiltrate [28]. The proximal tubule is appointee to retrieve low molecular weight (LMW) proteins and albumin from the glomerular filtrate. Defects in the receptors that mediate the uptake of these filtered ligands or saturation of the clearance pathway (e.g. in diabetes), lead to LMW proteinuria (LMWP). Prolonged LMWP (also termed tubular proteinuria because it does not involve glomerular dysfunction) causes further deterioration in kidney function and leads to renal failure [29].

In the kidney, reabsorption of filtered proteins occurs principally by receptor-mediated endocytosis in the proximal tubule. The apical endocytic apparatus is very elaborate consisting of coated pits, coated vesicles, endosomes and lysosomes responsible for membrane and receptor recycling from endosomes to the apical plasma membrane [30]. The most extensively studied and best characterized process at tubular level is clathrin-mediated endocytosis, which involves the internalization of cell-surface receptors and soluble molecules from the extracellular fluid in clathrin-coated vesicles that bud off from the plasma membrane [31,32]. Protein endocytosis at tubular level needs an active receptor-mediated pathway that principally involves megalin (LRP2), cubilin (CUBN), amnionless (AMN), disabled-2 (Dab2), the low density lipoprotein receptor adaptor protein 1 (ARH) and the Cl^-/H^+ antiporter ClC-5 [33] (Figure 3). Receptor-mediated endocytosis requires the coordinated functioning of numerous proteins and signal transduction molecules. In particular, megalin and cubilin play a central role in the process [33]. These receptors bind a variety of filtered ligands with varying affinities mediating delivery of their ligands to the lysosomes of the proximal tubule, while they undergo recycling themselves [34,30]. Upon endocytic uptake, progress to the lysosomes requires endosomal acidification to dissociate proteins from the receptors, permitting their degradation in lysosomes by the action of specific enzymes [35]. Although proteins are degraded in lysosomes, vitamins and trace elements are returned to the circulation by transport across the basolateral

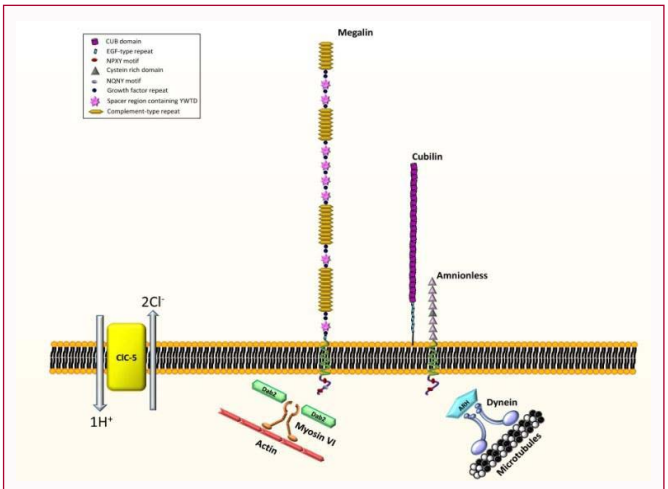

Figure 3: Protein uptake system at tubular level. Megalin and the Cubilin-AMN complex (CUBAM) are the main actors in protein uptake mechanism since both Megalin and Cubilin present albumin binding sites in their structure. ARH and Dab2 are involved in the vesicle trafficking binding to the NPXY motif present in both Megalin and AMN but interacting with different adaptor proteins (ARH binds to microtubules *via* Dynein while Dab2 binds to actin filaments *via* Myosin VI). Although a small proportion of ClC-5 can be detected in the apical brush-border membrane of renal proximal tubular cells, its importance in the endocytic process was well described.

membrane. In addition, reabsorbed substances may be metabolized within the proximal tubule cells as illustrated by the important renal activation of endocytosed 25-OH- vitamin D3 [30]. In vitro studies have established a number of pathways by which albumin and other filtered proteins may activate cellular pathways in proximal tubule cells leading to apoptosis, endoplasmic reticulum stress, interstitial inflammation and fibrosis, and possibly epithelial-mesenchymal transformation, eventually leading to interstitial fibrosis and accelerated nephron loss [36].

In the following sections, we outline properties and functions of the components involved in protein uptake in proximal tubular epithelial cells.

Megalin: Megalin (gp330) was first identified as a rat Heymann nephritis antigen (37) (Figure 3). Cloning and sequencing of the megalin encoding gene LRP2 uncovered a huge, glycosylated protein (600 kDa, 4,655 amino acids) with similarities to endocytic receptors of the LDL receptor family [30]. Megalin plays a particular key role in the proximal tubular uptake of glomerular-filtered albumin and other LMW proteins. Consistent with this function, Lrp2 knockout (KO) mice develop LMWP and albuminuria. Lrp2 KO mice have important ultrastructural changes in the endosomal compartments of proximal tubular epithelial cells, including the absence of apical dense tubules, which correspond to the apical recycling compartment, and other endocytic structures, such as clathrin-coated pits and vesicles [38].

Cubilin: Cubilin, which was first identified as the receptor for intrinsic factor-vitamin B12, is co- expressed with megalin on apical tubular epithelial cells and in podocytes [33,39,40] (Figure 3). This giant protein (glycosylated 460 kDa) shares no homology with other known receptors [41]. Cubilin ligands may be divided into ligands that bind only to cubilin and ligands that bind to both megalin and cubilin. Whereas some vitamin carriers such as the retinol binding protein apparently bind exclusively to megalin, others such as vitamin D-binding protein bind with similar affinity to both megalin and

Table 1: Genes associated with podocytes damage *in vivo* and/or *in vitro*.

Gene	Protein	Clinical signs	Ref.
SD associated and adaptor proteins			
CD2AP	CD2-associated protein	Focal segmental glomerulosclerosis type 3	[116]
E2F3	E2F transcription factor 3	Early onset nephrotic syndrome	[10]
FYN	Fyn proto-oncogene	Focal segmental glomerulosclerosis	[117]
GNE	Glucosamine uridine diphospho–N- acetylglucosamine 2-epimerase/ N-acetylmannosamine kinase	Severe glomerular proteinuria	[118]
NEPH1	Kin of IRRE-like 1	Proteinuria and perinatal lethality	[119]
NPHS1	Nephrin	Finnish type nephrotic syndrome	[120]
NPHS2	Podocin	Steroid-resistant nephrotic syndrome type 2	[121]
NXF5	Nuclear RNA export factor 5	Nephrotic syndrome with co- segregating heart block disorder	[10]
PLCE1	Phospholipase C ε 1	Steroid-resistant nephrotic syndrome type 3	[122]
PTPRO/GLEPP1	Protein-tyrosine phosphatase receptor or/Glomerular epithelial protein 1	Childhood-onset nephrotic syndrome	[123]
SYNPO	Synaptopodin	Focal segmental glomerulosclerosis	[117]
TRPC6	Transient receptor potential cation channel, homolog of 6	Focal segmental glomerulosclerosis type 2	[124]
ZHX1, ZHX2 and 3	Zinc fingers and homeoboxes 1/2/3	Nephrotic syndrome	[125]
GBM			
CD151	CD151	Nephrotic syndrome	[126]
COL4A3, COL4A4 and COL4A5	Type IV collagen α3, α4 and α5	Alport's syndrome Goodpasture's syndrome	[127]
DDR1	Discoidin domain receptor 1	GBM thickening and proteinuria	[128]
ITGA3	Integrin subunit alpha 3	Epidermiolysis bullosa and pyloris atresia with nephrotic syndrome	[10]
ITGB1	Integrin β1	Proteinuria and kidney failure	[129]
ITGB4	Integrin β4	Congenital focal segmental glomerulosclerosis	[130]
LAMB2	Laminin beta 2	Pierson syndrome	[131]
LMNA	Lamin A/C	Familial partial lipodystrophy with nephrotic syndrome	[10]
RAP1GAP	RAP1 GTPase-activating protein	Focal segmental glomerulosclerosis	[132]
TLN1	Talin 1	Nephrotic syndrome	[133]
Podocyte polarity			
aPCKλ/ι	A typical protein kinase Clambda/iota	Nephrotic syndrome	[134]
CDC42	Cdc42	Congenital nephropathy	[135]
VANGL2	Van Gogh-like [planar cell polarity] protein 2	Neural tube defects	[136]
Cytoskeleton			
ACTN4	Alpha-Actinin 4	Focal segmental glomerulosclerosis type 1	[137]
ACTN4	α-actinin 4	Focal segmental glomerulosclerosis	[138]
ANLN	Anilin	Focal segmental glomerulosclerosis	[139]
ARHGAP24	RhoA-activated Rac1 GTPase-activating protein 24	Focal segmental glomerulosclerosis	[140]
ARHGDIA	Rho guanine nucleotide dissociation inhibitor-α	Nephrotic syndrome	[141]
CFL1	Cofilin-1	Proteinuria	[142]
CLTA4/CD152	cytotoxic T-lymphocyte associated protein 4	Sporadic nephrotic syndrome	[10]
GPC5	Glypican 5	Nephrotic syndrome	[143]
INF2	Inverted formin 2	Focal segmental glomerulosclerosis	[144]
KANK1, KANK2 and KANK4	Kidney ankyrin repeat- containing protein	Nephrotic syndrome	[145]
MYH9	Myosin heavy chain 9	Adult onset nephrotic syndrome	[10]
MYO1E	Myosin 1e	Focal segmental glomerulosclerosis	[146]
PDSS2	Decaprenyl diphosphate synthase, subunit 2	Leigh syndrome [associated with nephrotic syndrome]	[147]
PODXL	Podocalyxin like	Early or adult onset nephrotic syndrome	[10]
RHPN1	Rhophilin-1	Focal segmental glomerulosclerosis	[148]

TTC21B	Tetratricopeptide repeat domain 21B	Nephrotic syndrome with tubulointerstitial involvement	[10]
Signaling pathway			
CMIP	C-Maf-inducing protein	Nephhrotic syndrome	[149]
CTNNB1	β-catenin	Focal segmental glomerulosclerosis	[150]
ILK	Integrin-linked kinase	Progressive glomerulosclerosis	[151]
NOTCH-IC	Notch's intracellular domain	Glomerulosclerosis	[152]
PI3KC2α	Class II phosphoinositide 3- kinase C2 α	Minimal-change nephropathy and focal segmental glomerulosclerosis	[153]
PIK3C3/VPS34	Class III phosphoinositide 3- kinase/Vacuolar protein sorting 34	Glomerulosclerosis	[154]
VHLH/HIF1A	Hypoxia-inducible factor 1 α	GBM thickening and proteinuria	[155]
WNT	Wingless-type MMTV integration site family 1	Focal segmental glomerulosclerosis	[150]
YAP	Yes-associated protein	Focal segmental glomerulosclerosis	[156]
Nuclear proteins			
LMX1B	LIM homeobox transcription factor 1 beta	Nail–patella syndrome	[157]
NFAT	Nuclear factor of activated T cells	Glomerulosclerosis	[158]
PAX2	Paired box gene 2	Adult-onset focal segmental glomerulosclerosis	[159]
SMARCAL1	SWI/SNF-related, matrix associated, actin-dependent regulator of chromatin, subfamily a-like protein 1	Schimke immuno-osseous dysplasia [associated with nephrotic syndrome]	[160]
WDR73	WD repeat domain 73	Galloway-Morwat Syndome	[10]
WT1	Wilms tumor 1 gene	Denis-Drash syndrome Frasier syndrome	[161] [162]
Mitochondrial			
ACDK4	aarF domain containing kinase 4	Nephrotic syndrome, steroid- resistant	[163]
ADCK4/NPHS9	Alpha-actinin 4	Focal segmental glomerulosclerosis	[164]
COQ2	Coenzyme Q2	COQ2 deficiency [associated with nephrotic syndrome]	[165]
COQ6	Coenzyme Q6	Nephrotic syndrome with sensorineural defects	[166]
CYP11B2	Cytochrome P450 family 11 subfamily B member 2	C-344 SNP risk factor for IgA nephropathy, nephrotic syndrome and proliferative glomerulopathy	[10]
MTTL1	Mitochondrial tRNA leucine 1	Steroid-resistant nephrotic syndrome. Focal segmental glomerulosclerosis	[167]
tRNAAsn	Mitochondrial tRNA Asparagine	Multiorgan failure and nephrotic syndrome	[10]
tRNAIle	Mitochondrial tRNA Isoleucine	Deafness, nephrotic syndrome, epilepsy and dilated cardiomyopathy	[10]
tRNATyr	Mitochondrial tRNA Tyrosine	Mitochondrial cardiomyopathy and nephrotic syndrome	[10]
ZMPSTE24	Zinc metallopeptidase STE24	Mandibuloacral dysplasia with nephrotic syndrome	[10]
Metabolic or lysosomal			
ALG1	Chitobiosyldiphosphodolichol beta-mannosyltransferase	Congenital disorder of glycosylation	[10]
PMM2	Phosphomannomutase 2	Congenital disorder of glycosylation	[10]
SCARB2/LIMP2	Scavenger receptor class B, member 2	AMRF syndrome [Action myoclonus-renal failure syndrome]	[168]

cubilin, and albumin probably with highest affinity to cubilin [30].

Since constitutive knockout of Lrp2 or Cubn caused perinatal death in the KO mice, several conditional KO mice were obtained. In vivo studies on the role of cubilin and megalin in the progression of renal disease using Cubn or Lrp2 knockout mice have produced somewhat conflicting results with no conclusive evidence for a role of the receptors in the progression of the disease due to: 1. differences between mice and humans, possibly related to a difference in expression levels or individual lifespan and observation time; 2. residual expression in the various conditional gene knockout mouse models; or 3. downstream effects following loss of expression in non-renal organs [41]. A role of megalin in the regulation of apoptosis has also been proposed [42], but an involvement of megalin and cubilin in protein overload-induced tubulopathy and interstitial inflammation and fibrosis is, however, not definite [43].

Amnionless: Amnionless (AMN) exists in at least five different sizes ranging from 38–50 kDa [41]. Cubilin and AMN co-localize closely in the kidneys, intestine and yolk sac of mice and AMN is essential during biosynthesis and trafficking of cubilin [44] (Figure 3). Cubilin forms a functional complex with AMN named CUBAM, which is translocated to the plasma membrane and displays megalin-independent activity [33]. Moreover, cubilin is dependent on AMN for its normal translocation from the endoplasmic reticulum (ER) to the membrane as well as for consequent endocytosis. The association to cubilin occurs through the epidermal growth-factor- like repeats in cubilin [41]. It is difficult to exclude whether AMN contributes to cubilin endocytosis also when cubilin is associated to megalin, since studies of cubilin without AMN are difficult to perform due to the

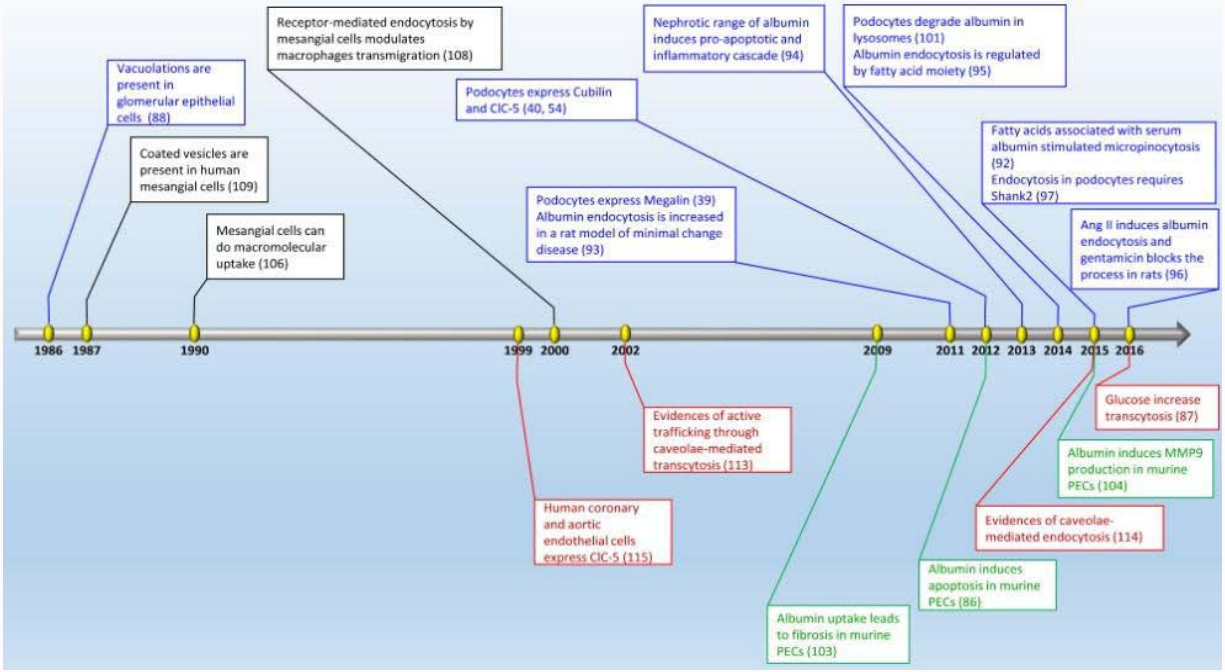

Figure 4: Glomerular protein handling: scientific-historical timeline. Timeline summarizes major events that comprise the history of knowledge in glomerular protein handling. Cell types are characterized with different colors: Blue: podocytes; Black: mesangial cells; Red: endothelial cells; Green: parietal epithelial cells (PECs).

interdependent apical sorting of AMN and cubilin [30].

Disabled-2: Disabled-2 (Dab2, also known as DOC-2 or p96) was described as a member of cargo-specific adaptor proteins as it binds to megalin, clathrin, clathrin adaptor 2 (AP-2), and phosphoinositides [45] (Figure 3). The Dab2 gene is alternatively spliced to produce two protein products, one of which, p96, binds to clathrin and the clathrin adaptor AP2, and localizes to clathrin-coated pits, whereas the other, p67, does not [46]. Dab2 is also implicated in signal transduction events in cell proliferation and differentiation during development. $Dab2^{-/-}$ mutant embryos arrest before gastrulation and fail to grow due to significant apoptosis [45]. Moreover, Dab2 associates with both the type I and type II TGFβ receptors in vivo, suggesting an essential role in the TGFβ signaling pathway [47]. In the kidney, Dab2 acts as an adaptor protein in the megalin-mediated endocytosis of filtered proteins [48]. Dab-2 interacts with myosin VI, the motor protein responsible for trafficking of vesicles through the cortical actin barrier, which underline the role of Dab-2 in receptor internalization and the steps immediately after it [49]. The adapter protein Dab2 and megalin mutually regulate each other's localization in PTECs. The expression of Dab2 in PTECs appears to be dependent on megalin or factors associated with megalin, while knocking-out of the Dab2 gene decreases the level and alters the subcellular distribution of megalin in PTECs [50].

ARH: Dab2 is not the only adaptor protein involved in megalin endocytosis. The low density lipoprotein receptor adaptor protein 1 (LDLRAP1 or ARH) is required for megalin trafficking from early endosomes to the endocytic recycling compartment while Dab2 mediates megalin internalization [51] (Figure 3). The difference between the functions of ARH and Dab-2 can most likely be explained by the differences in the proteins they interact with. ARH interacts with the motor protein dynein, which facilitates trafficking of vesicles along microtubules to the pericentriolar region [52]. The megalin–ARH complex is taken up by clathrin-mediated endocytosis and delivered to early endosomes, where ARH recruits dynein and facilitates vesicular transport of megalin along microtubules toward the endocytic recycling compartment. Megalin recycles from this compartment back to the plasma membrane via the slow recycling pathway. In the absence of ARH, there is a switch to fast recycling of megalin-containing vesicles to the plasma membrane directly from early endosomes [51]. Dab2 and ARH are not only crucial for megalin trafficking, but also for the endocytosis of the CUBAM complex, since AMN provides the two signals that potentially allow the endocytic process via both adaptor proteins [53].

ClC-5: The Cl^-/H^+ antiporter ClC-5 is primarily expressed in proximal tubular cells, in cortical collecting duct α intercalated cells and in the thick ascending limb of Henle's loop. Recently, this channel was demonstrated to be expressed also at glomerular level, in particular in podocytes [54]. In proximal tubular cells, it is predominantly located in intracellular subapical endosomes, which are involved in the endocytic reabsorption of LMW proteins and albumin that have passed the glomerular filter (Figure 3). ClC-5 is expressed on early endosomes, where it co-localizes with the V-type H^+-ATPase and endocytosed protein, leading to an efficient intraluminal acidification [55-58]. Two independent groups demonstrated that ClC-5 functions as a Cl^-/H^+ antiporter when activated by positive voltages [59,60]. A small portion of the ClC- 5 channels is also located on the cell surface, where it is thought to mediate plasma membrane chloride currents [55-57,61], or to participate in the macromolecular complexes at plasma membrane level deputed to LMW proteins and albumin endocytosis [62].

Virtually all the components of the macromolecular complex may lose their function and cause a complete or partial renal Fanconi syndrome. Renal Fanconi is a syndrome of inadequate reabsorption in the proximal tubules. It can present with various heterogeneous manifestations depending upon the causes underlying the disease (e.g. gene mutations, diabetes, infections or heavy metals toxicity)

[63]. Collagen vascular disorders and interstitial nephritis can be seen and it is also not uncommon a presentation with nephrolithiasis, nephrocalcinosis and LMWP [63]. Although proximal tubulopathies are consequences of injury or diseases not primarily involving the kidney, they may also be due to genetic mutations in proteins of the uptake process. In next paragraph we discuss the principal proximal tubulopathies of genetic origin.

Hereditary tubulopathies: The rare, autosomal recessive disorder Donnai–Barrow syndrome (DBS) is caused by mutations in LRP2 gene [64] (Table 2). The syndrome is characterized by proteinuria, a distinct facial appearance, along with functional defects such as high-grade myopia, sensorineural hearing loss, developmental delay, and in some cases agenesis of the corpus callosum, congenital diaphragmatic hernia, and omphalocele or umbilical hernia [41]. Normal renal function was reported in most DBS patients; however, a progressive decline in estimated glomerular filtration rate associated with evidence of focal glomerulosclerosis have been reported [65]. A rare autosomal recessive inherited defect in cubilin function has been described as Imerslund- Grasbeck syndrome (IGS) or megaloblastic anemia 1 caused by mutations in either CUBN or AMN gene [66,67]. The condition is characterized by B12 deficiency due to impaired intestinal absorption and proteinuria. The LMWP in most IGS patients is consistent with defective proximal tubule reabsorption and with observations in cubilin-deficient mice. Recent studies have characterized proximal tubule endocytic function in patients with CUBN or AMN mutations presenting LMWP, displaying a defective expression or apical targeting of cubilin [41]. Recently, intermittent nephrotic-range proteinuria without evident megaloblastic anemia was reported in a pair of siblings harboring a deletion in the CUBN gene [68].

Mutations in the CLCN5 gene are the main cause of Dent's Disease [69-71], an X-linked renal disorder characterized by LMWP and variable presence of hypercalciuria, nephrocalcinosis and/or nephrolithiasis [72-74]. Two ClC-5 KO mouse models reproduced the human proteinuria and revealed defective endocytosis by the proximal tubule [69]. Furthermore, Dent's disease patients display a significant reduction of cubilin and megalin expression in proximal tubular epithelial cells, probably as a consequence of a disturbed intracellular trafficking of the receptors [44]. Growing number of reports described patients carrying CLCN5 mutations with nephrotic-range proteinuria, histological findings of Focal Segmental and/or global Glomerulosclerosis (FSGS) and episodic evidence of isolated proximal tubular dysfunction [75-80].

Lowe syndrome is a multisystem disorder caused by mutations in the OCRL gene which encodes OCRL-1, an inositol polyphosphate 5-phosphatase [81]. The classic form of the oculocerebrorenal syndrome of Lowe is characterized by the triad of congenital cataracts, severe intellectual impairment, and renal tubular dysfunction with slowly progressive renal failure [82]. LMWP is a cardinal finding in Lowe syndrome and is observed in all patients. This condition reflects impaired megalin–cubilin receptor-mediated endocytosis in the proximal tubule [83].

Glomerular Protein Handling

Several studies have been done regarding the filtration barrier provided by the components of the slit diaphragm, but little attention has been paid to whether resident glomerular cells (podocytes, mesangial, parietal and endothelial cells) have the potential to handle plasma proteins [7,84-87].

The first evidence of the endocytic process at glomerular level was described by Yoshikawa et al. [88] in 1986 that reported the presence of vacuolations of the epithelial glomerular cells in children with nephrotic syndrome and focal segmental glomerulosclerosis. Subsequently, Eyre et al. [89] described and quantified an albumin endocytic function in podocytes both in vitro and in vivo, and Koop et al. [90] identified protein droplets in podocyte cell bodies and major processes in Dahl salt-sensitive rats. In puromycin aminonucleoside nephrotic rats, it has been demonstrated that albumin is filtered via other pathways beyond the slit diaphragm, which include endocytosis by endothelial cells, uptake by podocytes and entrapment in the paramesangium [91]. In Figure 4 we describe a timeline of the scientific-historical events that leaded to an increase of knowledge of the protein handling processes carried out by resident glomerular cells. In the following sections, we describe findings on protein uptake in podocytes, parietal epithelial, mesangial and endothelial cells.

Podocytes

Numerous studies using electron microscopy have shown increased numbers of protein- containing vesicles in podocytes under nephrotic conditions in which there is significant leakage of proteins across the GBM [92]. In addition, a study by Kinugasa et al. [93] observed an increased endocytosis of albumin by podocytes in vivo in a rat model of minimal change disease and that albuminuria was decreased after treatment of proteinuric animals with an antibody that blocks transcytosis.

It was demonstrated that the presence of free fatty acids (FFAs) associated with serum albumin stimulated macropinocytosis in cultured podocytes. The Authors suggested that the response to FFAs may function in the development of nephrotic syndrome by amplifying the effects of proteinuria [92]. Furthermore, albumin exposure at levels comparable to what is found in the urine of patients with nephrotic syndrome, increases cell death, pro-inflammatory cytokines and pro-apoptotic pathways in a cultured podocyte-like cell line [94].

Evidences are emerging that podocytes are able to endocytose proteins such as albumin using kinetics consistent with a receptor-mediated process. Pawluczyk et al. [95] demonstrated that receptor-mediated endocytosis of albumin by podocytes was regulated by the fatty acid moiety, although some of the detrimental effects were induced independently of it. Previously, it was demonstrated that human podocytes express megalin and cubilin both in vivo and in vitro [39,40]. In addition, our group revealed that ClC-5 was expressed in human glomeruli of normal and proteinuric kidneys, in particular in podocytes [54]. Intriguingly, ClC-5 was found to be overexpressed in glomeruli of diabetic nephropathy and membranous glomerulonephritis patients both at mRNA and protein level suggesting a role for this protein in albumin endocytosis by podocytes [54]. Recently, it was confirmed the presence of albumin-containing vesicles, which co-localized with megalin, in podocin-positive cells of Ang II–infused rat kidneys. Additionally, the podocyte endocytosis of albumin was markedly reduced in the presence of gentamicin, a competitive inhibitor of megalin-dependent endocytosis [96].

Shank2, a scaffolding protein that binds proteins involved in modulating actin dynamics and endocytosis regulation, was also demonstrated to be required in albumin endocytosis by podocytes [97]. In facts, knockdown of SHANK2 in cultured human podocytes

decreased albumin uptake. The Authors also demonstrated that Shank2 knockout mice had significantly decreased expression and altered localization of caveolin-1 in podocytes suggesting that disruption of albumin endocytosis in Shank2 knockouts is mediated via caveolin-1 since caveolin-1 is well-known to be required for albumin endocytosis in cultured podocytes [97].

Podocytes both degrade and transcytose albumin. Studies in other cell type have found that both clathrin-mediated and caveolae-mediated endocytosis can deliver albumin to the lysosomal compartment [98-100]. Some data suggested the involvement of lysosomes in the processing of endocytosed albumin in podocytes. In fact, it has been found that rat podocytes may degrade albumin in lysosome-like acidified vesicles as it was already demonstrated in cultured human urine-derived podocyte-like epithelial cells [96,101]. The Authors disclosed that lysosomes were involved in the processing of endocytosed albumin in podocytes, and lysosomal dysfunction could contribute to podocyte injury and glomerulosclerosis in albuminuric diseases. Moreover, they suggested that modifiers of lysosomal activity may have therapeutic potential in slowing the progression of glomerulosclerosis by enhancing the ability of podocytes to process and degrade albumin [101].

Evidences of a preferential uptake by podocytes at the apical membrane, which faces the urinary space, would suggest that albumin uptake in these cells mirrors that by proximal tubules and serves to recover albumin that leaks across the glomerular filtration barrier and enters the urinary space [102]. Same Authors suggested the hypothesis that podocytes were actively engaged in clearing large serum proteins from the GBM-podocyte interface since they demonstrated podocytes' ability to perform polarized uptake of albumin from the basal membrane along with the capacity of these cells to release full length albumin back into the extracellular space [102].

Parietal Epithelial Cells

Podocytes are not the only cell type involved in albumin uptake at glomerular level. Parietal Epithelial Cells (PECs) are positioned between the main glomerular filtration barrier and the proximal tubule and they are considered to be in a prime position to potentially act as a sensor for kidney health [86]. Recently, the Shankland group proposed the hypothesis that an important function of PECs was to restrict protein in the glomerular ultrafiltrate to the confines of Bowman's space, thereby preventing protein from passing into the extraglomerular space [103]. Moreover, they described a model in which whenever there was an increased permeability of the glomerular filtration barrier (due to injury to the endothelial cell, GBM, and/or podocyte), the increased filtered proteins were handled by various mechanisms, including not only the uptake by proximal tubular cells but also by podocytes and PECs. This mechanism leaded to an inflammatory response, recognized as periglomerular inflammation and ultimately fibrosis [103]. In 2012 the same group showed that PECs were able to internalize albumin in normal and overload conditions. Furthermore, they observed an increase of the apoptotic process in the presence of an excess of protein uptake, process which was ameliorated by increasing ERK1/2 phosphorylation [86].

Recently, albumin was identified as a signaling molecule that can stimulate MMP-9 production by activated glomerular parietal cells, indicating that MMP-9 may play an important role in PEC migration and podocyte dysfunction during the development and progression of diabetic nephropathy [104]. Nevertheless, the mechanisms involved in albumin handling and whether this eventually leads to proliferation, or trans-differentiation remains to be delineated [105].

Mesangial Cells

Mesangial Cells (MCs) are well-known to be the glomerular structural cells for their role in synthesis and assembly of the mesangial matrix, which in turn regulates the viscoelastic and hydraulic properties of the mesangium [106,107]. Subsequently, these cells have become increasingly recognized as multifunctional cells capable of mediating glomerular disease.

Although it is clear that glomerular resident macrophages play a major role in handling macromolecules and immune complexes, there are also evidences that cultured MCs have specific uptake mechanisms for macromolecules and immune complexes, exhibiting macrophage like qualities [107,108]. The macromolecular uptake by cultured MCs was studied by use of transmission electron microscopy, identifying a mechanism involving a typical coated vesicle mechanism with delivery of the gold particles to endosomes and eventually phagolysosomes. Moreover, the pretreatment with cytochalasin B (a mycotoxin which inhibited network formation by actin filaments) virtually prevented endocytosis of fresh serum particles, indicating active participation of the cytoskeleton [109].

Glomerular Endothelial Cells

Glomerular Endothelial Cells (GECs) are highly specialized cells with regions of attenuated cytoplasm punctuated by numerous fenestrae [110]. GECs are the first layers of the glomerular filtration barrier and the key regulators of glomerular microvascular permeability even though podocytes are absent [111]. Historically, GECs were considered as passive actors in the proteinuria pathogenic process due to the close association between endothelial dysfunction and microalbuminuria [112]. It was already demonstrated the presence of an active trafficking of vesicles containing albumin from the luminal to abluminal side of the endothelial cells, predominantly through caveolae-mediated transcytosis [113]. Furthermore, it was demonstrated the presence of a caveolae-mediated endocytosis in GECs, with a significant upregulation of albumin transcytosis by high glucose concentration but not by mannitol, excluding the potential effect of osmotic pressure [114,87]. Notably, it was already reported the expression of the ClC-5 exchanger in human coronary and aortic endothelial cells [115], but its presence in GECs is not yet demonstrated.

Conclusion

Proteinuria has been recognized as an independent risk factor for both renal failure and cardiovascular disease. In this review we have reported some evidences regarding the mechanisms by which tubular and glomerular cells can lose and rescue proteins. We focused our attention on the protein uptake machinery principally studied at proximal tubular level (LRP2, Cubilin, AMN, Dab2, ARH and ClC-5) and on recent discoveries about protein handling in the glomerulus. The knowledge scenario on protein uptake by the glomerular compartment had a burst in the last few years thanks to the possibility to have available immortalized culture of different glomerular cell types.

Taken together, these studies underline the ability of glomerular cells to endocytose albumin and suggest mechanistic insight into cellular mechanisms of protein handling. These evidences indicate a new role at glomerular level for the well-known tubular uptake

machinery in the progression of the renal disease, supporting the idea of a partnership between tubular and glomerular cells in albumin uptake, via the same way of internalization.

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