

Reduced Proximal Tubular Expression of Protein Endocytic Receptors in Proteinuria is Associated with Urinary Receptor Shedding

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Abstract

Background

Filtered proteins, including albumin, are reabsorbed in the proximal tubule mediated by megalin, cubilin and the neonatal Fc receptor. Proteinuria is an important renal biomarker linked to poor prognosis but expression of these key receptors is not well studied.

Methods

Megalín expression was determined at protein and mRNA levels in kidneys from proteinuric patients, and the expression of megalín, cubilin and neonatal Fc receptor were examined in the kidneys of mice with protein overload proteinuria. The presence of receptors in the urine of proteinuric and control mice was also studied.

Results

In nephrotic patients, megalín expression is reduced whilst mRNA is increased. In proteinuric mice megalín, cubilin and the neonatal Fc receptor protein are all reduced in proximal tubules. Megalín and neonatal Fc receptor mRNA are increased in proteinuric mice, whereas that for cubilin was reduced. In proteinuric mice increased urinary excretion of each of these endocytic receptors was observed.

Conclusions

It is concluded that in proteinuria, expression of all the key protein re-absorptive receptors is significantly reduced in the proximal tubule in association with increased turnover and urinary shedding.

Running Head: Urinary protein endocytic receptors in proteinuria.

Key Words: Proteinuria, endocytosis, megalín, cubilin, neonatal Fc receptor

Introduction

Proteinuria is a critical and adverse prognostic feature in renal disease and reducing proteinuria is an important therapeutic target to prevent progressive chronic kidney disease (1,2). The concept of proteinuric nephropathy, whereby excess filtered proteins exert a multitude of adverse effects on proximal tubular epithelial cells (PTECs) contributing to progressive renal injury, is now well described (1,3). Filtered proteins, including albumin, are largely reabsorbed in the proximal tubule by receptor mediated endocytosis. Megalin and cubilin, two large transmembrane receptors are expressed at the PTEC apical membrane to form a receptor complex responsible for endocytosis of filtered proteins (4,5,6,7) where cubilin is primarily responsible for ligand binding and megalin for subsequent internalisation of the ligand-megalin/cubilin complex (8). Amnionless is a chaperone protein which facilitates cubilin export from the endoplasmic reticulum to the plasma membrane where it remains expressed in a functional complex with cubilin (7,9). Recent evidence indicates that other receptors also have a role in the proximal tubule (PT) handling of albumin functioning alongside the megalin/cubilin complex. Most notably, the neonatal Fc receptor (FcRn) mediates albumin retrieval from tubular fluid by transcytosis across PTEC (10).

Re-absorption of proteins from glomerular filtrate by PTEC is not simply a constitutive process, but rather a dynamic regulated function that can increase or decrease under various conditions (11). In rats infused with endothelin PTEC albumin uptake is increased in response to increased glomerular sieving (12). Conversely, in acutely albumin overloaded rats with proteinuria, increased whole-body albumin is associated with reduced PTEC albumin uptake (13). In podocyte-injured rats with glomerular albuminuria PTEC albumin uptake is significantly increased (13).

Given the importance of proteinuria in the development of progressive chronic kidney disease, an understanding of the regulatory mechanisms underpinning variable PTEC endocytosis rates may open avenues for new therapeutic approaches. In the gallbladder, expression of megalin and cubilin is directly regulated by bile acids acting through the farnesoid X receptor (14). However, surprisingly little is known about the regulation of endocytic receptor expression and function in the PT in proteinuria. In cell culture models chronic exposure of cells to albumin reduces PTEC albumin binding sites and consequently endocytosis (15). In diabetic rats megalin PTEC expression is reduced (16), and in microalbuminuric diabetics megalin is detected in the urine (17,18). Megalin is also found in greater quantities in the urine of patients with IgA nephropathy and membranous nephropathy compared to controls (19). In diabetic mice, kidney megalin mRNA expression is inversely correlated with albuminuria and renal TGF β 1 levels (20).

To examine expression levels of components of the protein endocytic apparatus in health and disease we performed a comprehensive assessment of megalin, cubilin, and FcRn expression in kidneys of both healthy and proteinuric mice, and in humans with proteinuric renal diseases.

Methods

Laboratory Chemicals, Antibodies and PCR Reagents

Except where indicated, all general laboratory chemicals were obtained from Sigma-Aldrich (Gillingham, UK). Primary antibodies and the working dilutions used are given in Table 1. All qPCR reactions were performed by TaqMan gene expression assay (Thermo Fisher Scientific, Paisley, UK) using the probes listed in Table 2.

Patient Samples

Renal biopsy samples were surplus to the requirements of the clinical diagnostic pathway and obtained from patients presenting with nephrotic syndrome due to minimal change disease (MCD) (n=9), membranous nephropathy (MN) (n=10) or focal segmental glomerulosclerosis (FSGS) (n=9). All patients were aged 18yr or over, with an MDRD eGFR ≥ 60 ml/min/1.72m² and a urine PCR ≥ 350 mg/mmol creatinine. Patient details are provided in Table 3. Two patients in the MCD group had childhood nephrotic syndrome and the biopsy material used in this study was from their first diagnostic biopsy. No patients had received immunosuppressive treatment in the 12 months prior to biopsy. None were diabetic and none were receiving renin angiotensin system modifying agents. All biopsies had been performed in the 30 months preceding the study and had been stored for similar lengths of time under identical conditions in fully accredited NHS clinical laboratories. Normal tissue was obtained from the unaffected pole of nephrectomy specimens (n=11) well clear of the tumour margin from age-matched renal cell carcinoma patients with normal eGFR. Use of patient biopsy material was approved by the North Leicestershire Research Ethics Committee.

For quantification of megalin staining, 6 representative fields from each slide were photographed at x100 and x200 magnification under light microscopy. Each field was

studied independently by six blinded individuals. Scores were determined by the intensity of brown staining where minimal staining (exemplified by a negative control) was scored 0, and maximal staining (exemplified by healthy renal tissue) was scored 5. A total of 234 cortical fields were scored.

Protein Overload Proteinuria (POP) in Mice

All animal procedures were subject to institutional ethical review, and approved under UK Home Office Project Licence PPL 60/4438. Male BALB/c mice, 8 weeks old, weighing 25-30g, were purchased from Charles River Laboratories (Harlow, UK) and housed 5 mice/cage in an environment controlled at $22\pm 2^{\circ}\text{C}$, relative humidity 60% and a 12/12hr light/dark cycle. Mice were fed standard diet and allowed *ad libitum* access to water.

After acclimatisation for 1 week, mice were subjected to left unilateral nephrectomy through a flank incision and allowed to recover for 7 days. Overload proteinuria was induced as described in mice (21). Briefly, nephrectomised animals were divided into two groups. One group received intraperitoneal (IP) injections of low-endotoxin bovine serum albumin (BSA) (Thermo Fisher Scientific) for 16 days. BSA was prepared as a 40% solution in saline, filtered and administered to animals beginning at 2mg/g body weight (BW) on day 1, increasing thereafter by daily increments of 2mg/g BW to a maximum dose of 15mg/g BW on day 6 which then continued until day 16. The second group received IP injections of identical volumes of saline and served as non-proteinuric controls.

At the end of the study, mice were placed in individual metabolic cages to collect 24hr urine samples. Urine samples were centrifuged 13,000 rpm for 5 min at 4°C to remove cellular material, the pellets were discarded and the supernatants aliquoted and stored at -80°C . For Western blotting and ELISA, urine samples were thawed, centrifuged at 17,000 g for 15 min at 4°C and the resulting supernatants further centrifuged at 200,000g for 1hr at 4°C to

remove all particulate matter. Urinary creatinine concentrations were then measured using the QuantiChromCreatinine Assay Kit (BioAssay Systems, Hayward, CA, USA).

Immunohistochemistry

Four micron sections from formalin-fixed, paraffin embedded samples of human kidney biopsies or from tissue blocks from experimental mice were de-waxed in xylene, rehydrated in graded alcohols (100%, 90% and 70%) and treated with 30% hydrogen peroxide for 10 min to block endogenous peroxidase activity. To retrieve masked epitopes, sections were incubated in pre-heated sodium citrate (pH 6.0) in a microwave oven for 12min. Sections were then allowed to cool at room temperature for 25 min in sodium citrate buffer. To prevent non-specific antibody binding, sections were blocked by incubation with either 0.5% BSA, 3% milk powder, 10% normal goat serum in phosphate buffered saline (PBS), pH 7.4, for 60 min at room temperature, or for cubilin, with Animal-Free Blocking Reagent (Vector Laboratories, Peterborough, UK) for 60 min at room temperature. Endogenous biotin and avidin binding was then blocked using an Avidin/Biotin Blocking Kit (Vector Laboratories). Sections were then incubated with primary antibodies (Table 1) diluted in 10% swine serum in PBS overnight at 4°C. After three washes in PBS, sections were incubated with biotinylated secondary antibodies diluted 1:200 in PBS. Sections were then incubated with streptavidin/horseradish peroxidase reagent for 25 min followed by DAB Peroxidase (HRP) Substrate reagent (Vector Laboratories) for 3-10 min. Finally sections were counterstained with hematoxylin, mounted and visualised using an Olympus Cytology microscope equipped with spectral image acquisition software.

Western Blotting

Renal cortex was homogenised in RIPA lysis buffer (Santa Cruz Biotechnology, Heidelberg, Germany) containing protease inhibitors at 4°C. Lysates were then centrifuged at 12,000

rpm for 30 min at 4°C. Protein concentrations in the resulting supernatants were measured (Bio-Rad DC protein assay kit, Bio-Rad Laboratories, Hemel Hempstead UK), samples were diluted in Laemmli sample buffer and denatured by heating at 95°C for 5 min. Urine samples were prepared as described above and also diluted in Laemmli sample buffer.

Equal amounts of protein were separated on 4% gels (megalin), 4-20% gradient gels (cubilin) or 10% gels (FcRn) and transferred onto PVDF membranes, blocked with 2.5% non-fat milk in TTBS and probed with the primary antibodies (Table 1). After application of relevant secondary antibodies diluted in TTBS with 2.5% semi-skimmed milk, bound antibodies were detected using Amersham ECL Western Blotting Detection Reagent (Scientific-Lab Supplies Ltd, UK). All blots were performed in triplicate for samples derived from ≥ 3 individual animals.

Densitometric analysis was performed using ImageJ software. The intensity of protein bands was measured in samples from the various experimental conditions and the intensity of the target protein was then divided by the intensity of either the β -actin or DPP-4 loading control for that blot. This target protein:loading control ratio was used to express relative target protein abundance in the various experiments .

Quantitative PCR (qPCR)

For human kidney tissue, total RNA was isolated from formalin-fixed paraffin embedded sections using the miRNeasy FFPE Kit (Qiagen Ltd, Manchester, UK) according to the manufacturer's instructions. Generally, total RNA was isolated from 25-30 slide-mounted, 10 micron thick sections per needle biopsy from patient or normal control kidney specimens. Total RNA was isolated from fresh mouse kidney cortex using RNeasy Mini Plus Kit (Qiagen Ltd) according to manufacturer's instructions.

Total RNA was reverse transcribed to cDNA using the Promega Reverse Transcription System kit (Promega, Southampton, UK) according to the manufacturer's instructions, and quantified by NanoDrop spectrophotometer 1000 with NanoDrop1000 software, v3.7.1. The qPCR was performed by TaqMan gene expression assay in an Applied Biosystems 7500 Fast Real Time PCR system (Thermo Fisher Scientific). All reactions were conducted in triplicate for each sample derived from ≥ 4 individual animals. qPCR data were analyzed using Applied Biosystems 7500 Software v2.0.6. Normalised Ct values (delta Ct values) were calculated using GAPDH as a reference gene. Delta Ct values were calculated as Ct (target) - Ct (normalising gene) and the relative expression ratio calculated as $2^{\Delta(\text{Ct-treated} - \text{Ct-untreated})}$ ($\Delta\Delta\text{Ct}$).

Urine ELISA

Excretion of megalin, cubilin and FcRn in mouse urine was measured by ELISA using the mouse LRP2 ELISA kit (Catalogue no. MBS068632, MyBioSource, San Diego, CA), the mouse Cubilin ELISA kit (Catalogue no. CSB-EL006213MO-5, Generon Ltd, Maidenhead, UK) or the mouse FcRn Large Subunit p51 ELISA Kit (Catalogue no. MBS937874, MyBioSource). Protein receptor excretion was determined in urine samples, normalised to creatinine concentration from each of ≥ 4 control and protein overload mice, in triplicate, at appropriate dilutions according to the manufacturer's instructions in 96 well plates.

Unless otherwise indicated all data are presented as mean \pm SEM of at least $n=3$ experiments. Statistical comparisons between the control human kidneys and those from proteinuric nephropathies were performed by ANOVA and Tukey's test for multiple comparisons. Comparisons between control and proteinuric groups of animals were performed by unpaired t-test.

Results

Initially megalin expression in the kidneys of proteinuric patients with glomerular disease compared to normal controls was studied. Of the 39 patients providing kidney tissue, 22 were male. In normal control kidney sections megalin staining was intense in the sub-apical region of the PTECs as previously described (6). No clear evidence of glomerular or distal tubular expression of megalin was observed. Conversely, in the sections from proteinuric patients megalin in PTEC apical membranes was clearly reduced (Fig. 1A). This was true for MCD, MN and FSGS. By semi-quantitative analysis using an arbitrary scale, healthy control biopsies yielded a megalin expression score of 4.56 ± 0.032 , compared to FSGS (2.31 ± 0.06), MCD (2.29 ± 0.07) and MN (3.14 ± 0.06) (Fig. 1B). These differences were statistically significant for each nephrotic condition compared to controls. To determine the effect of proteinuria on megalin gene expression, mRNA was extracted from fixed, mounted sections of kidney tissue, reverse transcribed and subjected to qPCR. Megalin mRNA was significantly increased by 8.72 ± 3.27 fold in the kidneys of nephrotic MCD patients with compared to control kidneys (Fig. 1C). Unfortunately insufficient tissue was available to study the mRNA expression of megalin in other nephropathies, or the expression of cubilin and FcRn. Therefore to fully profile expression of megalin, cubilin and FcRn a mouse model of POP was used. Mice with POP exhibited a significant increase in serum total protein concentration of 39.71 ± 2.71 mg/ml, compared to 23.89 ± 0.78 mg/ml in controls (Fig. 2B). Mice with POP also demonstrated a modestly increased serum creatinine to 40.22 ± 1.57 μ mol/L compared to 25.34 ± 1.75 μ mol/L in controls (Fig. 2A). In proteinuric mice urine PCR was 132.00 ± 12.87 mg/mg, whereas in control mice urine PCR was 43.43 ± 1.39 mg/mg (Fig. 2C). By IHC megalin, cubilin and FcRn were all expressed at the apical membranes of PTECs in normal mice (Fig. 3). Some glomerular staining of FcRn was evident in normal mice. In

proteinuric mice with POP expression of megalin, cubilin and FcRn appeared substantially reduced in PTEC apical membranes. Glomerular FcRn appeared unchanged in proteinuric animals but was not further evaluated in this study. Expression of dipeptidyl peptidase-4 (DPP-4) was used as a control protein as in previous studies of proximal tubular injury (21). The expression of DPP-4 in proximal tubules was unchanged in proteinuria (Fig 3).

To complement the IHC, kidney cortex from control and proteinuric mice was subject to Western blotting (Fig 4). These studies confirmed significantly reduced protein levels of megalin, cubilin and FcRn in proteinuric mice compared to controls when normalised to both β -actin and DPP-4.

Despite the reduced expression of megalin and FcRn at the protein level in proteinuric kidneys, gene expression of both megalin (2.80 ± 0.11 fold) and FcRn (2.27 ± 0.18 fold) was significantly increased in proteinuric mouse kidney cortex (Fig 5). Conversely, cubilin gene expression was significantly suppressed (0.26 ± 0.12 fold) in the kidneys of proteinuric mice. Finally, urinary excretion of megalin, cubilin and FcRn was evaluated (Fig 6). By ELISA the urine of proteinuric mice contained significantly greater amounts of megalin (proteinuric mice urinary [megalin] 204.40 ± 13.42 ng/mg creatinine compared to 7.2 ± 2.81 ng/mg creatinine for controls, $p < 0.0001$), cubilin (proteinuric mice urinary [cubilin] 2.00 ± 0.30 ng/mg creatinine for controls compared to 0.35 ± 0.08 ng/mg creatinine for controls, $p < 0.01$) and FcRn (proteinuric mice urinary [FcRn] 281.50 ± 22.29 pg/mg creatinine compared to 52.22 ± 14.22 pg/mg creatinine for controls, $p < 0.0001$) than the urine of control mice. To exclude interference of urinary protein with the ELISAs, all standard curves were performed in the presence of albumin. Standard curves were identical with or without exogenous added albumin (data not shown). Excess of both megalin and cubilin were also found in the urine of proteinuric animals by Western blotting (Fig 6 D,E). It was not possible to identify

FcRn in the urine of either group by Western blotting, most likely due to being below the limit of detection by this method.

Discussion

These results provide a comprehensive analysis of proximal tubular protein endocytic receptor expression in proteinuria and reveal clearly reduced levels of megalin, cubilin and FcRn at the apical membranes of PTECs in proteinuric mice. Phenotypically the POP model described herein is similar to that previously described in mice, with a small rise in serum creatinine and an early significant rise in urinary protein excretion (22,23,24). Overall in POP the PTEC turnover of megalin and FcRn is increased, with elevated levels of their mRNAs accompanying reduced amounts of membrane associated protein, whereas conversely, cubilin mRNA levels are reduced alongside reduced cubilin membrane protein. Most strikingly all three receptors are present in greatly elevated quantities in the urine of proteinuric mice. Thus in the case of megalin and FcRn, the predominant reason for depressed membrane protein levels majority is urinary receptor shedding, whereas reduced cubilin receptor expression in proteinuria can be explained by reduced gene transcription combined with urinary receptor shedding.

The finding of significantly reduced megalin receptor levels in proteinuric human MCD kidneys alongside increased megalin gene expression is completely consistent with the findings in the mouse model. The relative balance between cortex and medulla in patient biopsies was considered as a potential cause of sampling bias driving the megalin gene expression findings in MCD kidneys. However all patient biopsy samples contained good numbers of glomeruli and sufficient cortical material to make an adequate clinical diagnosis. Furthermore the control samples were taken from blocks of tissue from the uninvolved pole of cancer nephrectomy specimens and consisted predominantly of cortex. This would tend to favour higher levels of megalin mRNA in controls, the opposite of what is herein described. In addition, we cannot completely exclude differences in tissue preservation due

to obligate differences in the clinical tissue diagnostic pathway between kidney needle biopsies and larger tissue blocks from cancer nephrectomy samples as a cause of differences observed, particularly in mRNA levels, between disease and control samples in the human studies. However, the similarity of results in mice where tissue handling between groups offers reassurance of the validity of the results in human samples.

Previous investigators reported urinary loss of tubular protein endocytic receptors in proteinuric patients and animal models of renal disease. In type 2 diabetes, urinary excretion of the extracellular ectodomain of megalin is associated with early diabetic nephropathy, whereas full-length megalin in the urine is linked progressive diabetic nephropathy and falling eGFR (17,18). In patients with type 1 diabetes the development of cubilinuria precedes microalbuminuria (25), and in a mouse model of type 1 diabetes both megalin and cubilin are down regulated in the PT (25). Megalin and cubilin expression in the PT is reduced in diabetic Goto-Kakizaki rats but normalised by the sulphonylurea, gliquidone, which also reduces proteinuria in these animals (26).

In IgA nephropathy urinary megalin loss correlates with proteinuria and severity of glomerular injury (19), although in these studies tubular expression of megalin protein was maintained. A similar pattern of megalin excretion is also observed in nephrotic patients with MN (19). In a proteinuric dog model of Alport disease both megalin and cubilin appear in the urine alongside other proteins (27).

These observations suggest that increased urinary megalin and/or cubilin excretion may be common to proteinuria-induced tubular injury (1,3) in several renal diseases. However not all PT disorders are associated with loss these receptors. In the renal Fanconi syndrome caused by nephropathic cystinosis, expression of megalin and cubilin is preserved in PT segments despite clear evidence of PT dysfunction (28), and in both Dent's disease and

Lowe syndrome megalin is either reduced or absent from the urine (29) compared to unaffected individuals.

Hathaway *et al* (20) studied megalin expression in the kidneys of Akita diabetic mice genetically modified to over-express TGF β 1, and in contrast to the current study found reduced megalin mRNA in the kidneys. This reduction in gene expression would likely translate into reduced megalin receptor expression but significant differences exist between the Akita mouse model and that presently described. The Akita mice were aged 40 weeks, diabetic with long-standing proteinuria and genetically modified to exhibit up to a 300% increase in renal TGF β 1 (20), whereas the currently described POP mice were approximately 11 weeks old at sacrifice, non-diabetic with a short duration of proteinuria. It is conceivable that increased megalin mRNA expression in early proteinuria may eventually down-regulate in later stage disease.

The FcRn has been described localised to the apical membrane of PTECs (30), and has been ascribed a role in the transcytosis of albumin and immunoglobulins by podocytes and in the PT (10, 31). Mice deficient in FcRn display significant albuminuria. There are no data on modulation of kidney FcRn expression in proteinuria, nor on urinary excretion of FcRn.

The mechanisms of differential endocytic receptor gene regulation in proteinuric kidneys are uncertain. Expression of both megalin and cubilin is positively regulated at both gene and protein levels in PTECs by PPAR- α and - γ (32,33). In proteinuric rats, Cabezas *et al* (32) reported down-regulation of megalin mRNA and protein in the PT that was reversed by agonists of PPAR- α and - γ (32). However in other studies PPAR- γ activation is described in PTECs in proteinuria (34), thus providing a potential explanation for the up-regulation of megalin gene expression in observed in kidneys from proteinuric patients and POP mice, but not for down regulation of cubilin gene expression. Although various inflammatory

mediators acting through different transcription factors may up- and down-regulate FcRn in various tissues (35) there is currently no information regarding regulation of kidney FcRn gene expression. Nonetheless, and notwithstanding the mechanisms underlying the differential gene expression observed in these studies, the net effect of the observations is a reduction of receptors in the PT.

Indeed the loss of these protein endocytic receptors into the urine must contribute to their accompanying reduced expression in the PTEC apical membranes. Like other large transmembrane proteins, megalin undergoes regulated intramembrane proteolysis (RIP) (36,37). By this process megalin is subject to metalloproteinase-mediated ectodomain shedding regulated by protein kinase-C. RIP releases the megalin ectodomain into the tubular lumen and leaves an intracellular domain of megalin that serves as a substrate for γ -secretase which in turn releases a soluble intracellular megalin domain that is potentially phosphorylated and possessive of intrinsic signalling capacity (37,38,39,40). Thus RIP is a compelling explanation for the megalinuria and reduction of PTEC megalin protein observed in the current studies. As an entirely extracellular protein cubilin cannot be subject to RIP, but as a consequence of its close physical association with megalin may potentially be pulled free from the PTEC apical membranes as a consequence of megalin ectodomain shedding. The mechanism of FcRn loss into the urine is unclear.

The relative contributions of glomerular permselectivity and tubular reabsorption to the regulation of urinary protein excretion remains an intensely controversial topic (41,42,43). Observations of compromised tubular re-absorption of glomerular filtered proteins contributing significantly to proteinuria (41,43) have challenged the orthodox view that increased urine protein excretion results largely from impaired glomerular permselectivity (42). Although it is not possible to determine whether the loss of PTEC protein receptors

seen in the current studies is a primary or secondary event in proteinuria, these data support the concept that dynamic changes in PTEC function impact on urinary protein excretion. A full understanding of these findings would be enhanced by future studies of the PTEC endolysosomal compartment that provides an essential network supporting macromolecular transport by these epithelial cells (43).

In summary, we have demonstrated shedding of the major PT receptors for filtered proteins in the urine with resulting reduced receptors in the apical membranes of PTECs in proteinuria. These results are broadly consistent with the limited information available in the literature, and at least for megalin are consistent between proteinuric humans and mice. Whether this is an adaptive or maladaptive response of PTECs remains unclear but the mechanism merits further investigation. Nonetheless the results underline the complexity of this system and the potential for therapeutic manipulation in disease.

Author Contributions

The study was conceived by NJB, JB and RB. HF, NB, RC, MC all performed key experiments.

All authors provided input into data analysis. NJB wrote the first draft of manuscript and all authors subsequently provided editions to develop the finally submitted manuscript version.

Conflict of Interest Statement and Transparency Declarations

The results presented in this paper have not been published previously in whole or part, except in abstract format. The authors have no declarations or conflicts of interest.

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| Primary Antibody | Supplier | IHC Dilution | WB Dilution |
|--|--|--------------|-------------|
| Rabbit anti-human/mouse LRP-2 polyclonal antibody | Proteintech (Manchester, UK) Cat. No. 9700-1-AP | 1:100 | 1:1000 |
| Rabbit anti-human/mouse FcRn polyclonal antibody | Insight Biotechnology Ltd (Wembley, UK) Cat. No. bs-13151R | 1:100 | 1:1000 |
| Goat anti-mouse/rat cubilin(A-20) polyclonal antibody | Santa Cruz Biotechnology, Inc (Heidelberg, Germany) Cat. No. sc-20609 | 1:800 | 1:1000 |
| Goat anti-mouse dipeptidyl peptidase-4 polyclonal antibody | R&D Systems (Abingdon, UK) Cat. No. AF954 | 1:50 | 1:1000 |

Table 1. Primary antibodies, source and dilutions used.

| Target | Assay ID |
|-------------------|----------------|
| Mouse Megalin/LRP | Mm01328171_m1 |
| Human Megalin/LRP | Hs 00189742_m1 |
| Mouse Cublin | Mm01325077_m1 |
| Mouse FcRn | Mm00438885_g1 |
| Mouse GAPDH | Mm99999915_g1 |
| Human GAPDH | Hs 03929097_g1 |

Table 2. TaqMan gene expression assay ID numbers used in qPCR studies.

| | Age | Male/Female | eGFR ml/min/1.73m ² | Urine PCR mg/mmol | Glomeruli (n) |
|---------------------------------------|-----|-------------|-----------------------------------|----------------------|------------------|
| Minimal Change Disease | 41 | Female | 73 | 400 | 18 |
| | 28 | Female | >90 | >1000 | 7 |
| | 25 | Male | >90 | >1000 | 16 |
| | 38 | Male | >90 | 601 | 30 |
| | 19 | Male | >90 | 364 | 13 |
| | 75 | Male | 60 | 618 | 14 |
| | 20 | Male | >90 | 800 | 18 |
| | 18 | Female | >90 | 500 | 18 |
| | 76 | Male | 72 | >1000 | 12 |
| Focal Segmental Glomerulosclerosis | 82 | Female | 61 | >1000 | 15 |
| | 51 | Female | 60 | 720 | 26 |
| | 63 | Male | 62 | 500 | 9 |
| | 53 | Male | 71 | >1000 | 9 |
| | 50 | Female | 63 | >1000 | 7 |
| | 64 | Female | 62 | >1000 | 14 |
| | 32 | Female | 89 | >1000 | 21 |
| | 63 | Male | 81 | >1000 | 6 |
| | 72 | Male | 62 | 822 | 16 |
| Membranous Nephropathy | 73 | Male | 90 | >1000 | 38 |
| | 48 | Male | 90 | 805 | 10 |
| | 71 | Male | 72 | 384 | 9 |
| | 41 | Male | 82 | 590 | 10 |
| | 41 | Male | 90 | 618 | 8 |
| | 47 | Female | 64 | >1000 | 16 |
| | 24 | Female | 90 | 714 | 14 |
| | 70 | Male | 90 | 612 | 18 |
| | 73 | Female | 65 | 985 | 19 |
| | 33 | Female | 90 | 450 | 23 |

Table 3. Clinical characteristics of patients providing renal biopsy samples

Figure Legends

Figure 1. Expression of megalin protein and mRNA in the proximal tubule of human patients with nephrotic syndrome.

A: Representative photomicrographs showing renal expression of megalin in nephrotic conditions by IHC. In all photomicrographs scale bars = 10 μ M. B: semi-quantitative evaluation of IHC analysis of renal megalin expression. * $p < 0.0001$, $n=10$, except FSGS where $n=9$. C: qPCR analysis of megalin mRNA in control and MCD kidneys. Data are mean \pm SD, $n=4$, * $p < 0.05$.

Figure 2. Biochemical parameters in control and POP mice.

A: mouse serum creatinine levels. * $p = 0.0002$, $n=5$. B: mouse serum protein concentrations. * $p = 0.0005$, $n=5$. C: mouse urine protein:creatinine ratios. * $p = 0.0001$, $n=5$.

Figure 3. Representative photomicrographs showing IHC analysis of mouse kidney tissue for megalin, FcRn, cubilin and DPP-4.

Kidney sections were stained for megalin, cubilin, FcRn and DPP-4 as described in Methods. Negative control sections are from non-proteinuric animals processed without the inclusion of primary antibodies. In all photomicrographs scale bars = 10 μ M.

Figure 4. Analysis of protein receptor expression in mouse kidneys by Western blotting.

Kidney cortex from control and proteinuric mice was solubilised and subject Western blotting for A: megalin, B: cubilin and C: FcRn. Representative blots are shown with the middle and lower panels of each group depicting membranes stripped and re-probed for β -

actin and DPP-4 respectively as housekeeping control proteins. Each lane represents a kidney sample from different animals. Histograms represent desitometric quantification of the protein of interest expressed as a ratio to β -actin or DPP-4. For megalin blots $*p < 0.05$, for cubilin blots $*p = 0.001$ and for FcRn blots $*p < 0.05$. All blots were performed at least in triplicate.

Figure 5. Fold change in mRNA expression for megalin, cubilin and FcRn in mouse kidneys.

mRNA was extracted from kidney cortex and subject to qPCR. The relative expression ratio of the gene of interest compared to GAPDH was calculated using $\Delta\Delta C_t$ analysis for A: megalin, $***p < 0.0001$, B: cubilin $*p < 0.01$, and C: FcRn $**p < 0.001$. All qPCR reactions were performed in triplicate for each animal sample.

Figure 6. Excretion of protein endocytic receptors in mouse urine.

Urine samples from control and proteinuric mice containing equal amounts of creatinine were examined by ELISA for A: megalin, $*p < 0.001$, B: cubilin, $*p < 0.01$ and C: FcRn, $*p < 0.001$. Receptor levels were measured in triplicate in samples from multiple animals. Panels D and E represent Western blotting of urine samples from control and proteinuric animals, normalised for creatinine concentration, for megalin and cubilin respectively.