Increase of Total Nephron Albumin Filtration and Reabsorption in Diabetic Nephropathy

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ABSTRACT
The amount of albumin filtered through the glomeruli and reabsorbed at the proximal tubules in normal and in diabetic kidneys is debated. The megalin/cubilin complex mediates protein reabsorption, but genetic knockout of megalin is perinatally lethal. To overcome current technical problems, we generated a drug-inducible megalin–knockout mouse line, megalin(lox/lox);Ndrg1-CreERT2 (iMegKO), in which megalin expression can be shut off at any time by administration of tamoxifen (Tam). Tam administration in adult iMegKO mice decreased the expression of renal megalin protein by 92% compared with that in wild-type C57BL/6J mice and almost completely abrogated renal reabsorption of intravenously injected retinol–binding protein. Furthermore, urinary albumin excretion increased to 175 mg/d (0.46 mg albumin/mg creatinine) in Tam-treated iMegKO mice, suggesting that this was the amount of total nephron albumin filtration. By comparing Tam-treated, streptozotocin-induced diabetic iMegKO mice with Tam-treated nondiabetic iMegKO mice, we estimated that the development of diabetes led to a 1.9-fold increase in total nephron albumin filtration, a 1.8-fold increase in reabsorption, and a significant reduction in reabsorption efficiency (86% efficiency versus 96% efficiency in nondiabetic mice). Insulin treatment normalized these abnormalities. Akita;iMegKO mice, another model of type 1 diabetes, showed equivalent results. Finally, nondiabetic iMegKO mice had a glomerular sieving coefficient of albumin of $1.7 \times 10^{-2}$, which approximately doubled in diabetic iMegKO mice. This study reveals actual values and changes of albumin filtration and reabsorption in early diabetic nephropathy in mice, bringing new insights to our understanding of renal albumin dynamics associated with the hyperfiltration status of diabetic nephropathy.


Albuminuría or proteinuria is one of the most important biomarkers to diagnose CKD and to predict its prognosis.1–3 The extent of albuminuría defines the stages of diabetic nephropathy.4,5 Not only damage in glomerular filtration barrier6 but also impairment in tubular reabsorption cause albuminuría.7 The glomerular sieving coefficient (GSC) of albumin among superficial nephrons given by multiphoton microscopy was 50-times higher than that by micropuncture,8,9 generating an intense controversy lasting until now.7,10 Diabetic nephropathy, especially in its early phase, is characterized with marked glomerular
hypertension and hyperfiltration, in which glomerular filtration of creatinine and urinary albumin excretion are increased. It has been almost taken for granted that glomerular albumin filtration should be increased in this morbid disorder. Surprisingly, we cannot find recent reports providing evidence for that. Indeed, researchers using multiphoton microscopy or micropuncture have commonly reported that glomerular albumin filtration is not increased and tubular albumin reabsorption is decreased in streptozotocin (STZ)-induced type 1 diabetic rats, explaining the cause of increased albuminuria.12,13

Albumin and other proteins filtered through glomeruli are reabsorbed by the megalin/cubilin complex expressed on the brush border membrane (BBM) of proximal tubules.14 A fraction of albumin transferred to intracellular acidic endosomes is transported toward the basolateral membrane by the neonatal Fc receptor, recycling intact albumin back into circulation.15 Disruption of the megalin (or Lrp2) gene is sufficient to block uptake of albumin into proximal tubules.14,16 Most systemic (or conventional) megalin knockout (KO) mice die perinatally due to developmental defects in the lung and forebrain.17 Two to five percent of mutants survive until adulthood14,17 but are unhealthy, because they also lack reabsorption of vitamins and sex steroids,18,19 suggesting that they may not be suitable for analysis of adult renal disorders.

In this study, to precisely measure the amount of albumin filtered through glomeruli in healthy and diabetic conditions, we established a novel mouse line in which the megalin gene can be silenced after normal growth or even after development of diabetes.20,21 In these mice, we can measure the overall albumin filtration among the whole glomeruli (including juxtamedullary glomeruli) in the absence of anesthesia use, overcoming major shortcomings in current techniques.

RESULTS

Effects of megalin Gene Deletion upon Reabsorption of Low Molecular Weight Proteins in Mice

To enable timely deletion of the megalin gene, we generated megalin(lox/lox);Ndrg1-CreERT22 mice.20,21 These drug-inducible megalin knockout (iMegKO) mice were injected intraperitoneally (ip) with either a low- or high-dosage of Tam (150 mg/g body wt for 3 or 5 days, respectively) at 6 weeks of age and euthanized 5 weeks later.

Renal megalin protein expression in control mice was observed throughout the proximal tubules (S1 to S3 segments, Figure 1A). In iMegKO mice with low-dose Tam treatment (iMegKO-Low), megalin expression was markedly reduced in cortical proximal tubules (S1 and S2) but maintained in medullary proximal tubules (S3) in a patchy pattern (Figure 1B). Only a trace amount of megalin protein was expressed in the kidneys of mice given a high dose (iMegKO-High, Figure 1C). Tubular uptake of low molecular weight proteins was examined by intravenous injection of fluorescence (Alexa546)-labeled retinol binding protein (RBP, 1 µg/g body wt, whose molecular mass is 21 kDa),22 given 15 minutes before analysis. In control mice, RBP filtered by glomeruli was efficiently reabsorbed from the urine at S1 and S2 (Figure 1D). In iMegKO-Low mice, RBP reabsorption was mostly eliminated from S1–S2 and found at S3 in a mosaic manner (Figure 1E). RBP uptake was barely observed in iMegKO-High mice (Figure 1F). In megalin(lox/lox);apoE-Cre mice, megalin expression was partially eliminated throughout S1–S3 in a mosaic manner (Figure 1G).16 In megalin(+/-) mice, megalin expression was diffusely decreased (Figure 1H). In megalin(lox/lox);apoE-Cre and megalin(+/-) mice, RBP signals were observed at similar nephron segments as megalin expression (Figure 1, I and J). Rearrangement of the megalin gene was observed in DNA extracted from the kidneys of iMegKO mice given low-dose Tam but not in DNA from the tail (Figure 1K). Compared with wild-type mice, megalin mRNA expression was reduced by 72 ± 5% and 92 ± 2% in iMegKO-Low and -High animals, respectively (P < 0.01 versus wild-type, Figure 2A). In megalin(lox/lox);apoE-Cre and megalin(+/-) mice, renal megalin mRNA expression was reduced approximately to half of wild-type mice (P < 0.01, respectively). Moreover, Western blot analysis of renal BBM extracts indicated that megalin protein expression was reduced by 74 ± 7% and 92 ± 2% in iMegKO-Low and -High animals in comparison with wild-type mice, respectively (P < 0.05 versus wild-type, Figure 2, B and C).

At high magnification, megalin protein expression and RBP signals colocalized at the apical surface of S1–S2 proximal tubules (Figure 3). At S3, colocalization of megalin and RBP was observed in iMegKO-Low, megalin(lox/lox);apoE-Cre, and megalin(+/-) mice, but very few RBP signals were found at S3 in control and iMegKO-High mice. In iMegKO-Low mice, RBP signals were observed not only in aquaporin 1–expressing proximal tubules but also in nephron segments expressing neither aquaporin 1 nor Tamm Horsfall protein (a marker of the thick ascending limb of Henle). Furthermore, RBP signals in these mice were also found at the apical surface of distal nephron segments expressing calbindin-D28k (a marker of convoluted distal tubules) or aquaporin 2 (a marker of collecting ducts), in agreement with a recent work elucidating expression of a megalin-independent receptor with albumin binding activity along these nephron segments.23 When a similar amount of Alexa546-labeled BSA (1 µg/g) was intravenously injected into wild-type mice, we could observe faint fluorescence signals only at high magnification (Supplemental Figure 1), indicating that a very small fraction of albumin in circulation is filtered at normal glomeruli. Of note, injection of a larger amount of fluorescent albumin leads to a prominent accumulation of signals along the apical surface of proximal tubules.13

These findings indicate that we could successfully suppress megalin expression and reabsorption activity in adult mouse kidneys with high-dose Tam. When megalin was deleted selectively in S1–S2 segments by low-dose Tam, RBP was conveyed downstream and reabsorption at S3 and distal nephrons became detectable.
Urinary Excretion of Albumin and Neutrophil Gelatinase–Associated Lipocalin in megalin-Disrupted Mice

Urinary excretion of albumin (66 kDa in size) in iMegKO-High mice measured by ELISA was 175 ± 626 mg/d, which was 16-fold higher than that of wild-type mice (11 ± 6 mg/d, P < 0.01, Figure 4A). When urinary albumin-to-creatinine ratios were calculated, the difference was also 16-fold (460 ± 47 versus 28 ± 1 µg/mg, P < 0.01), which are consistent to values reported for megalin(lox/lox);Wnt4-EGFP-Cre mice. Neutrophil gelatinase–associated lipocalin (NGAL or Lcn2, a 25 kDa low molecular weight protein) is an emerging biomarker of acute tubular injury in humans and mice.25–29 Urinary NGAL excretion in iMegKO-High mice was 800-fold higher than that of wild-type mice (0.05 ± 0.01 µg/d, P < 0.01, Figure 4B). As urinary NGAL/creatinine ratio, the difference was 900-fold (100 ± 12 versus 0.11 ± 0.02 µg/mg, P < 0.01). In a time course study, urinary albumin excretion reached a plateau within 2 weeks and was sustained for, at least, 2 more weeks at similar levels between iMegKO-Low and -High animals (Figure 4C). Protein staining of urinary samples from control and iMegKO mice separated by gel electrophoresis showed that intensities of protein bands corresponding to...
full-length albumin matched well with the concentrations obtained by ELISA (Figure 4D).

Morphologically, wild-type and Tam-treated iMeg-KO mice were not distinguishable by periodic acid–Schiff staining of kidney tissues (not shown). By transmission electron microscope, foot process and slit membrane of podocytes, glomerular basement membrane, and fenestrated endothelial cells (which are important components of glomerular filtration barrier) appeared normal in iMegKO mice (Figure 4, E and F, Figure 5, A and B) as previously reported, showing that glomerular ultrastructure was maintained in mutant mice. On the other hand, in proximal tubules, the numbers of clathrin-coated pits, endosomes, and recycling vesicles were much reduced in iMegKO mice compared with controls (Figure 5, C and D) as previously reported, showing that the presence of glomerular filtration barrier.
megalin is required to maintain reabsorption apparatus in proximal tubules.20

These findings indicate that urinary albumin and NGAL excretion is markedly increased in iMegKO mice after Tam treatment. Furthermore, low- and high-dose treatment caused similar increase of these urinary molecules (Figure 4, A and C), indicating that the majority of filtered proteins from glomeruli are taken up by S1–S2 proximal tubules and the contribution of S3 and distal nephrons as a reabsorption reservoir is relatively small.

**Estimation of Total Nephron Albumin Filtration, Reabsorption, and GSC in iMegKO Mice**

Because inhibition of tubular reabsorption activity by low- or high-dose Tam in iMegKO mice appeared to result in similar maximum levels of urinary albumin excretion, we
Propose to name these values as ‘total nephron albumin filtration.’ Furthermore, ‘total nephron albumin reabsorption’ can be calculated by subtraction of urinary albumin excretion before Tam from that after Tam (Figure 6). In this setting, reabsorption ratio or efficiency is defined as the estimated reabsorption value divided by the estimated filtered value. GSC is determined as urinary albumin-to-creatinine ratio divided by serum albumin-to-creatinine ratio in iMegKO-Low mice (Table 1).33

**Figure 4.** Urine albumin and NGAL levels are markedly elevated in iMegKO mice. (A) iMegKO mice given a low or high dose of Tam similarly exhibited a more than ten-fold increase in daily urinary albumin excretion. It was also significantly increased in Megalin (lox/lox); apoE-Cre mice. n=5–7. **P<0.01 versus Tam-untreated wild-type. N.S., not significant. (B) Daily urinary NGAL excretion showed results proportional to albumin excretion (n=5–7). (C) Time course of urinary albumin excretion of iMegKO mice after Tam or vehicle administration (control) indicated that it reached a plateau within 2 weeks and was sustained for 2 more weeks, at similar levels between low and high dose Tam treatment (n=4–5). *P<0.05 and **P<0.01 versus pretreatment (Pre). w, weeks. (D) Coomassie Brilliant Blue staining of mutant mouse urine. Each number described above the gel indicates urinary albumin content in each lane calculated from Albuwell ELISA. Black triangle indicates full-length albumin, whose staining intensity in comparison with BSA standards appears to meet well with ELISA measurement. White triangle, major urinary protein. (E) Transmission electron microscopy photo of glomerulus in high-dose Tam-treated iMegKO mice. Bar, 2 μm. (F) Higher magnification of (E), indicating preservation of normal structure. Bar, 500 nm.
iMegKO mice. Just before Tam treatment (at 8 weeks after STZ injection), urinary albumin excretion was increased by 6.8-times in STZ;iMegKO mice (50.9 ± 8.5 mg/d) compared with vehicle-injected (non-STZ);iMegKO mice (7.5 ± 1.5 mg/d, P<0.01, Figure 6A). In creatinine normalized evaluation, urinary albumin-to-creatinine ratio was changed from non-nephropathy level (27.3 ± 4.1 mg/mg) to microalbuminuric level (70.6 ± 10.4 mg/mg, 2.6-fold, P<0.01) by STZ in iMegKO mice without Tam, which corresponds to a standard model of STZ-induced diabetic nephropathy usually investigated.34

Two weeks after low-dose Tam treatment, urinary albumin excretion (i.e., total nephron albumin filtration) in STZ;iMegKO (390 ± 33 μg/d) was 1.9-fold higher than non-STZ;iMegKO (202 ± 28 μg/d, P<0.01, Figure 6A). When renal handling of IgG or NGAL was examined, induction of diabetes mildly increased estimated total nephron filtration of these molecules but not significantly (Figure 6, B and C). Tubular albumin reabsorption was 1.8-fold higher (340 ± 34 versus 194 ± 28 μg/d, P<0.05, Figure 6D) and tubular albumin reabsorption ratio was significantly smaller in STZ;iMegKO mice (86 ± 3% versus 96 ± 1%, P<0.01, Figure 6E) compared with non-STZ;iMegKO mice.

As another model of type 1 diabetes, we crossbred iMegKO mice with Akita mice35 to generate triple mutant Ins2Akita/+; megalin(lox/lox);Ndrg1-CreER T2 mice. Two weeks after Tam treatment (at 14 weeks of age), Akita;iMegKO-Low mice exhibited comparable amounts of total nephron albumin filtration and tubular reabsorption similar to or slightly more compared with STZ;iMegKO-Low mice (Figure 6, A and D). By transmission electron microscopy, the total area of endosomes per cell was significantly increased in S1 cortical proximal tubules of STZ mice (Supplemental Figure 2), supporting a concept that tubular albumin reabsorption is enhanced in these mice (Figure 6D). Moreover, we speculate that markedly increased luminal concentration of filtered protein (ligand concentration) and involvement of larger (i.e., more downstream) areas in S1–S2 proximal tubules for uptake may also contribute to increased albumin reabsorption at proximal tubules in diabetic nephropathy. On the other hand, the size and number of clathrin-coated pits in podocytes were not altered in Akita mice (Supplemental Figure 3).

After 7 days of insulin infusion, ad libitum-fed serum glucose levels in STZ;iMegKO-Low (changed from 619 ± 84 to 148 ± 22 mg/dl, n=5, P<0.01) or Akita;iMegKO-Low mice (from 759 ± 62 to 154 ± 36 mg/dl, n=5, P<0.01) were significantly reduced, reaching nondiabetic control levels (159 ± 11 mg/dl, n=7). Moreover, by insulin, total nephron albumin filtration (and therefore albumin reabsorption also) was normalized to nondiabetic levels (Supplemental Figure 4), indicating that hyperglycemia (and/or insulin deficiency) was the critical but reversible cause of changes in renal albumin handling in two diabetic models studied in this study. Compared with non-STZ;control mice, renal megalin mRNA expression

Figure 5. Transmission electron microscope elucidates marked reduction of endocytic apparatus in megalin mutant mice. (A, B) High-power fields of glomerular filtration barrier and (C, D) endocytic apparatus in proximal tubules. Megalin(lox/lox);Ndrg1-CreER T2 mice were treated with vehicle (A, C) or low-dose Tam (B, D). (B) Podocyte foot process, slit membrane, glomerular basement membrane, and fenestrated endothelium appeared normal in megalin mutant mice. (D) The numbers of recycling vesicles (arrowheads), clathrin-coated pits (arrows), and endosomes E were much reduced in megalin mutant mice. Bar, 500 nm.
levels were reduced to 61±4% in STZ;control mice, and to 4±1% in STZ;iMegKO-Low mice (P<0.01, respectively, Figure 6F). Consistently, megalin protein expression was reduced to 76±6% in STZ;control mice and to 260% in STZ;iMegKO-Low mice (P<0.05 versus non-STZ control, Supplemental Figure 5). Creatinine clearance was increased by 1.5-fold by induction of diabetes in iMegKO-Low mice (P<0.05, Supplemental Figure 4).

Finally, we compared the estimated GSC of albumin, IgG, and NGAL among diabetic and nondiabetic mice. The GSC of albumin in nondiabetic control mice was 1.7±0.2×10^{-5}, and this was increased by 2.2-fold in STZ diabetic mice (P<0.05, Table 1). Because GSC values calculated in this work are normalized with creatinine concentrations, these findings indicate that albumin passage is much more enhanced than creatinine passage in STZ mice. On the other hand, presence of 

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<td>Non-STZ</td>
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Values are expressed as mean±SEM. Alb, albumin.

*P<0.05 between STZ and non-STZ animals (n=5, respectively).
diabetes increased the GSC of IgG to a smaller extent (1.5-fold), suggesting that some size selectivity of glomerular filtration barrier may exist in diabetic nephropathy as experienced in minimal change nephrotic syndrome patients.\textsuperscript{36} NGAL was almost freely filtered at glomeruli (GSC=0.3), both in normal and diabetic conditions.

DISCUSSION

In this study, by the use of novel, drug-inducible, proximal tubule-specific megalin conditional KO mice, we have shown that total nephron albumin filtration and reabsorption in the whole kidneys of normal mice were both estimated as approximately 200 $\mu g/d$, which were much larger than urinary albumin excretion measured in a standard way (approximately 10 $\mu g/d$). These filtration and reabsorption values were doubled in the early phase of STZ-induced or Akita-inherited type 1 diabetic mice, and could be reversed by insulin treatment. Furthermore, we report here that the GSC of albumin in normal mice was $1.7 \pm 0.2 \times 10^{-5}$, and this was also doubled in STZ diabetic nephropathy, when creatinine clearance was elevated by 1.5-fold.

**Megalin Gene Expression Level and Reabsorption Activity**

Although megalin(lox/lox);apoE-Cre and megalin(+/−) mice showed similarly reduced megalin mRNA expression levels in the whole kidney preparation by half (Figure 2A), the former had about 3-fold higher urinary albumin levels than the latter. Furthermore, induction of diabetes by STZ reduced megalin gene expression to 61%, but the total nephron albumin reabsorption was rather increased. These findings suggest that chimeric presence of megalin-deficient cells, especially in S1–S2 proximal tubules, exerts a stronger effect upon overall reabsorption activity than diffusible reduction of megalin mRNA expression.

**Quantitation of Reabsorption Activity**

In previous works, for evaluation of tubular reabsorption activity, fixed amounts of labeled-albumin or NGAL were given to diabetic and control animals, and fluorescence or immunogenicity of exogenous protein was quantitated, which allowed estimation of the reabsorption ratio or efficiency of exogenous protein was quantitated, which allowed estimation of the reabsorption ratio or efficiency, but not absolute reabsorption amount.\textsuperscript{12,13,27,37} These studies elucidated that the reabsorption efficiency was significantly reduced in STZ animals. Here, we show that reabsorption of endogenous albumin was doubled in STZ mice and reabsorption efficiency was reduced at the same time. There is another point to be discussed. Previous studies relied upon the assumption that the amount of protein accumulated in endosomes and lysosomes of proximal tubules is mainly determined by the speed of reabsorption and transit time of proteins in proximal tubules, and stability of immunogenicity or fluorescence intensity are not altered in diabetic conditions. However, to cope with protein overload from the tubular lumen in diabetic conditions, exocytosis of proximal tubules has to be markedly upregulated, and we speculate there is a possibility that transit time may be reduced in diabetic conditions, which might lead to underestimation of reabsorption.

**Comparison of GSC with Previous Reports**

The GSC of albumin in this study is the smallest value among reports published so far. Russo et al. proposed a GSC value of 0.034 in normal rats by multiphoton microscopy.\textsuperscript{8} Continuous efforts have been made to minimize background signals from free fluorescence dye, out-of-focus signals, inappropriate BP, and body temperature during assay in multiphoton microscopy, and Schießl et al. recently reported a value of $4.4 \times 10^{-4}$ in rats.\textsuperscript{38}

Tojo et al. utilized micropuncture and reported $6.2 \times 10^{-4}$ as GSC in rats.\textsuperscript{9} In rats with STZ-induced diabetic nephropathy, a slight reduction in single nephron albumin filtration at superficial nephrons under anesthesia has been reported both by micropuncture and multiphoton microscopy.\textsuperscript{12,13} These findings may not be necessarily contradictory to our findings which showed doubling of total nephron albumin filtration in diabetic mice. Rather, it is because we cannot rule out the possibility that albumin hyperfiltration may develop selectively at juxtamedullary glomeruli and steal phenomena might be occurring at superficial glomeruli. Indeed, juxtamedullary glomeruli are especially sensitive to hemodynamic stress.\textsuperscript{39,40} Comparisons with other works are discussed in the Supplemental Material.

A pathophysiologic role of megalin-dependent reabsorption activity in proteinuric disorders has been studied. It is still not clear whether protein reabsorption in proximal tubules is protective or detrimental at a cellular level and for the whole kidney.\textsuperscript{16,41}

Measuring the sum of albumin filtration in the total nephrons is a strength and weakness of this study. For the specific assessment of albumin filtration in juxtamedullary nephrons, Cre-driving promoters specifically activated in such location will be required. As an experimental design, we could examine the effects of megalin ablation only after Tam administration, thus potential direct effects of Tam to glomerular filtration and tubular reabsorption require attention. In this study, reabsorption of RBP in iMegKO-High mice was almost completely inhibited compared with that in control mice (Figure 1, D and F), but we did not examine whether albumin reabsorption is also suppressed to a similar extent. Therefore, the potential presence of an albumin-specific receptor in the kidney which does not bind RBP cannot be formally denied. Langelueddecke et al. recently proposed a novel, megalin-independent mechanism of protein reabsorption at distal nephron, but the binding characteristics of the receptor were not specific to albumin.\textsuperscript{25}

In conclusion, we measured total nephron glomerular filtration and tubular reabsorption of albumin by novel drug-inducible megalin knockout mice in adulthood. We revealed
that both albumin filtration and reabsorption were increased in two models of insulin-deficient diabetes. This study may provide a theoretic rationale for early treatment intervention to normalize glomerular abnormality of hyperfiltration (and glomerular hypertension) by blood glucose level-lowering and BP-lowering reagents.

CONCISE METHODS

Experimental Animals
In megalin(lox/lox) mice, exons 72–74 were flanked with loxP sites, which allowed removal of the transmembrane region of megalin protein.20 These mice were bred with either apoE-Cre or Ndrg1-CreERT2 mice to establish conditional knockout animals. Human apoE promoter directs Cre expression in the kidney, but not in the liver or other organs.42 Ndrg1 gene is most abundantly expressed in renal proximal tubules and also expressed in the nervous system at lower levels.21,43 To generate the megalin null allele, tissue nonspecific alkaline phosphatase (TNAP or Alpl)-Cre knockin mice were used, which were created by inserting the IRES-Cre cassette immediately after exon 6 of the Alpl gene according to a previous report.44 Akita type 1 diabetic mice (Ins2Akita/Akita; purchased from Japan SLC, Hamamatsu, Japan)35 were bred with megalin(lox/lox);Ndrg1-CreERT2 mice. All mice were backcrossed to C57BL/6j genetic background (>6 generations), maintained on a 12-h light/12-h dark cycle and provided with water and standard chow.

To delete the megalin gene in adulthood, 150 mg/kg body wt of Tam (Sigma-Aldrich, St Louis, MO) in sunflower seed oil (10 μL/g body wt, Sigma-Aldrich) was injected ip to mice for 3 (low dosage) or 5 (high dosage) days.53 Tam was given consecutively to nondiabetic mice and every other day to diabetic mice. We found that Tam treatment tended to increase urine volume, urinary albumin-to-creatinine ratio, and creatinine clearance (by approximately 50%) in wild-type mice, but not significantly (Supplemental Table 2). An individual mouse was housed in a metabolic cage, and a urine sample was collected for 24 hours in a noncoated, standard glass bottle at room temperature without the use of proteinase inhibitor. For genotyping of mice, DNA was extracted from the kidney or tail. The following three primers were used for PCR amplification of megalin alleles (Figure 1K): 5‘-CGG TTT TCT GTG AGG GTC TTC C-3’, 5‘-ATC GGA ACA AGA ACT AGG GGT CA-3’, and 5‘-TCT ATG CAA GCT CCT CCC ACC T-3’.

Renal BBM Isolation and Western Blot Analysis
Western blot using renal BBM was performed as described previously with some modification.47,48 Extracts from renal BBM were prepared at 4°C by homogenizing whole kidneys in extraction buffer (containing 150 mM D-mannitol, 2.5 mM ethylene glycol tetraacetic acid, 6 mM Tris-HCl, 12 mM MgCl2, 0.5 mM phenylmethysulfonyl fluoride at pH 7.1). Isolated BBM (10 μg/lane) was dissolved in Laemmli buffer, separated using nonreducing SDS-PAGE, and transferred onto polyvinylidene fluoride membranes. The membranes were incubated overnight at 4°C with antimegalin or anti-β-actin antibody (Supplemental Table 1). The immunoblots were processed using peroxidase-conjugated anti-IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), ECL Prime, and ImageQuant LAS 4000 system (GE Healthcare, Waukesha, WI). Densitometry was performed with ImageJ software (National Institutes of Health, MD) and normalized by β-actin.

Measurement of Urinary and Serum Albumin, NGAL, IgG, and Creatinine
Urinary and serum levels of murine albumin were determined by Albunex ELISA (Exocell, Philadelphia, PA; main text) or Lbis ELISA (Shibayagi, Gunma, Japan; Supplemental Figure 6). NGAL (BioPorto Diagnostics, Hellerup, Denmark) and IgG (Bethyl Laboratories, Montgomery, TX) were also measured with ELISA. Creatinine levels were measured by an enzymatic method (Oriental Yeast, Co. Ltd., Tokyo, Japan).46 Serum albumin concentrations were determined by an enzymatic method (SRL, Inc., Tokyo, Japan), which gave values consistent with ones obtained by Albunex ELISA. For Coomassie Brilliant Blue staining (R-250; Nacalai Tesque, Kyoto, Japan) of urine, a similar amount (1/2,000 of daily urine) was loaded in each lane and separated by SDS-PAGE.

Quantitative RT-PCR
Total RNA was extracted from mouse kidneys with TRIzol reagent (Invitrogen, Carlsbad, CA) and cDNA in each sample was synthesized by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Gene expression levels were examined by Premix Ex Taq (Takara, Kyoto, Japan) and StepOnePlus Real Time PCR System (Applied Biosystems). Cre-nondisrupted, intact megalin cDNA was specifically amplified with the following primer/probe set: forward primer, 5‘-TGCCCAGCTGCAAGCT-3’; reverse primer, 5‘-CACACCAGTGCTCATGTGACCA-3’; and probe, 5‘-FAM-AGCCCTGTATCCTAAAGTGCACCCCGT-TAMRA-3’. Expression levels of megalin were normalized by glyceraldehyde-3-phosphate dehydrogenase levels, whose primer/probe set was purchased from Applied Biosystems. A standard curve was made by serial dilution of cDNA from kidneys of untreated wild-type mice.
Injection and Detection of RBP and Albumin
To investigate renal reabsorption of glomerular-filtered proteins, human RBP and BSA (fraction V; Sigma-Aldrich) were labeled with Alexa546 fluorescent dye (Life Technologies, Carlsbad, CA). Mice were anesthetized with diethyl ether and intravenously injected with fluorescent proteins (1 μg/g body wt, dissolved in 0.2 mL of phosphate-buffered saline) via tail vein. Kidneys were harvested 15 minutes after injection, fixed with 4% paraformaldehyde at 4°C for 30 minutes, and incubated overnight with 30% sucrose at 4°C. The next day, tissues were frozen in Tissue-Tek optimal cutting temperature compound (Sakura Finetek Inc., Torrance, CA) and stored at −80°C until analysis.

Immunofluorescence Study of Megalin and Nephron Segment Markers
Kidneys were sliced with a cryostat (CM1850; Leica Biosystems, Wetzlar, Germany) at 10 μm thickness, and incubated with 10% normal donkey serum and primary antibodies at 4°C overnight (Supplemental Table 1). Primary antibodies were visualized with FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and examined using a fluorescence microscope (IX81-PAFM; Olympus, Tokyo, Japan).

Electron Microscopy
Freshly isolated kidney blocks were fixed with 2.5% glutaraldehyde (Wako Pure Chemicals, Osaka, Japan) and 4% paraformaldehyde, and then treated with 1% osmium tetroxide (Nacalai Tesque). Tissue examination was performed using a transmission electron microscope (H-7650; Hitachi, Yokohama, Japan).49

Statistical Analyses
Results are expressed as mean±SEM and analyzed by two-tailed t test or one-way ANOVA with Bonferroni post hoc test, except for Figure 4C (Holm post hoc test). Statistical significance was defined as P<0.05.

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