Increase of Total Nephron Albumin Filtration and Reabsorption in Diabetic Nephropathy

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ABSTRACT

There is a hot debate concerning actual amount of albumin filtered through glomeruli and reabsorbed at proximal tubules in normal kidneys and diabetic conditions. To overcome current technical problems, we generated a drug-inducible megalin knockout mouse line, *megalin(lox/lox);Ndrg1-CreER^{T2}* (or iMegKO), whose protein reabsorption can be shut off anytime by tamoxifen (Tam). After Tam administration, renal megalin protein expression was reduced by 92% compared to wild-type C57BL/6J mice, and renal reabsorption of intravenously-injected retinol binding protein was almost completely abrogated. Urinary albumin excretion increased to 175 μg/day (0.460 mg/mg-creatinine), suggesting that this was the amount of total nephron albumin filtration. Glomerular sieving coefficient of albumin was 1.7 x 10^{-5}. By comparing streptozotocin-induced, Tam-treated, diabetic STZ;iMegKO mice with non-STZ;iMegKO mice, we estimated that daily albumin filtration was increased by 1.9-fold, reabsorption was increased by 1.8-fold, and reabsorption efficiency was reduced to 86% by development of diabetes (versus 96% in control). Such abnormalities were well normalized after insulin treatment. Another type 1 diabetic model of Akita;iMegKO mice showed equivalent results. This study reveals actual values and changes of albumin filtration and reabsorption in early diabetic nephropathy, bringing new insights into our understanding of renal albumin dynamics in hyperfiltration status of diabetic nephropathy.

INTRODUCTION

Albuminuria or proteinuria is one of the most important biomarkers to diagnose chronic kidney disease and to predict its prognosis.[1-3] The extent of albuminuria defines the stages of diabetic nephropathy.[4, 5] Not only damage in glomerular filtration barrier[6] but also impairment in tubular reabsorption cause albuminuria.[7] 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.[11] 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 megalin/cubilin complex expressed on brush border membrane of proximal tubules.[14] A fraction of albumin transferred to intracellular acidic endosomes is transported towards basolateral membrane by neonatal Fc receptor, recycling intact albumin back into circulation.[15] Disruption of *megalin (or Lrp2)* gene is sufficient to block uptake of albumin into proximal tubules.[14, 16] Most of systemic (or conventional) *megalin* knockout (KO) mice die perinatally due to developmental defects in the lung and forebrain.[17] Two to five percents of mutants survive until adulthood[14, 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 *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 *megalin* gene, we generated *megalin(lox/lox);Ndrg1-CreER*^{T2} mice.[20, 21] These drug-inducible *megalin* knockout (iMegKO) mice were injected intraperitoneally with either low- or high-dose of Tam (150 mg/g body weight for 3 or 5 days, respectively) at 6 weeks of age and sacrificed 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 trace amount of megalin protein was expressed in the kidneys of mice given high dose (iMegKO-High, Figure 1C). Tubular uptake of low molecular weight (LMW) proteins was examined by intravenous injection of fluorescence (Alexa546)-labeled retinol binding protein (RBP, 1 μg/g body weight, whose molecular weight is 21 kDa),[22] given 15 min 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 (Figures 1I, 1J). Rearrangement of *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 to wild-type mice, *megalin* mRNA expression was reduced by 72±5% and 92±2% in iMegKO-Low and -High animals, respectively (P<0.01 vs 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 brush border membrane 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 vs wild-type, Figures 2B, 2C).

At high magnification, megalin protein expression and RBP signals colocalized well 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 thick ascending limb of Henle). Furthermore, RBP signals in these mice were also found at 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 similar amount of Alexa546-labeled bovine serum albumin (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 very small fraction of albumin in circulation is filtered at normal glomeruli. Of note, injection of larger amount of fluorescent albumin leads to prominent accumulation of signals along 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 to 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 (65 kDa in size) in iMegKO-High mice measured by enzyme-linked immunosorbent assay (ELISA) was 175±26 μg/day, which was 16-fold higher than that of wild-type mice (11±2 μg/day, $P<0.01$, Figure 4A). When urinary albumin/creatinine ratios were calculated, the difference was also 16-fold (460±47 vs. 28±1 μg/mg, $P<0.01$), which are consistent to values reported for *megalin(lox/lox);Wnt4-EGFP-Cre* mice.[24] Neutrophil gelatinase-associated lipocalin (NGAL or Lcn2, a 25 kDa LMW protein) is an emerging biomarker of acute tubular injury in humans and mice.[25-29] Urinary NGAL excretion in iMegKO-High mice was 41±9 μg/day, which was 800-fold higher than that of wild-type mice (0.05±0.01 μg/day, $P<0.01$, Figure 4B). As urinary NGAL/creatinine ratio, the difference was 900-fold (100±12 vs. 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)[30] appeared normal in iMegKO mice (Figure 4E, 4F, and Figures 5A, 5B) as previously reported,[31] 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 to controls (Figures 5C, 5D) as previously reported,[31, 32] showing that presence of 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 (Figures 4A-4C), indicating that majority of filtered proteins from glomeruli are taken up by S1-S2 proximal tubules and contribution of S3 and distal nephrons as reabsorption reservoir is relatively small.

Estimation of total nephron albumin filtration, reabsorption and GSC in iMegKO mice

Since 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 estimated reabsorption value divided by estimated filtered value. GSC is determined as urinary albumin/creatinine ratio divided by serum albumin/creatinine ratio in iMegKO-Low mice (Table 1).[33]

Induction of diabetes and alteration of total nephron albumin filtration, reabsorption and GSC

We examined the changes in total nephron albumin filtration and reabsorption by injection of streptozotocin (STZ) to 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 μg/day) compared with vehicle-injected (non-STZ);iMegKO

mice (7.5±1.5 μg/day, P<0.01, Figure 6A). In creatinine normalized evaluation, urinary albumin/creatinine ratio was changed from non-nephropathy level (27.3±4.1 μg/mg) to microalbuminuric level[5] (70.6±10.4 μg/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/day) was 1.9-fold higher than non-STZ;iMegKO (202±28 μg/day, P<0.01, Figure 6A). When renal handling of immunoglobulin G (IgG) or NGAL was examined, induction of diabetes mildly increased estimated total nephron filtration of these molecules but not significantly (Figures 6B, 6C). Tubular albumin reabsorption was 1.8-fold higher (340±34 vs 194±28 μg/day, P<0.05, Figure 6D) and tubular albumin reabsorption ratio was significantly smaller in STZ;iMegKO mice (86±3% vs 96±1%, P<0.01, Figure 6E) compared to non-STZ;iMegKO mice.

As another model of type 1 diabetes, we crossbred iMegKO mice with Akita mice[35] to generate triple mutant *Ins2*$^{Akita/+}$*;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 to STZ;iMegKO-Low mice (Figures 6A, 6D). 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 non-diabetic control levels (159±11 mg/dL, n=7). Moreover, by insulin, total nephron albumin filtration (and therefore albumin reabsorption also) was normalized to non-diabetic 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 to non-STZ;control mice, renal *megalin* mRNA expression 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±10% in STZ;control mice and to 2±0% in STZ;iMegKO-Low mice (P<0.05 vs. 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 estimated GSC of albumin, IgG and NGAL among diabetic and non-diabetic mice. GSC of albumin in non-diabetic control mice was 1.7±0.2 x 10^{-5}, and this was increased by 2.2-fold in STZ diabetic mice (P<0.05, Table 1). Since 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 diabetes increased GSC of IgG in 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.[36] NGAL was almost freely filtered at glomeruli (GSC>0.3), both in normal and diabetic conditions.

DISCUSSION

In the present study, by 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 μg/day, which were much larger than urinary albumin excretion measured in a standard way (approximately 10 μg/day). These filtration and reabsorption values were doubled in early phase of STZ-induced or Akita-inherited type 1 diabetic mice, and could be reversed by insulin treatment. Furthermore, we report here that GSC of albumin in normal mice was $1.7\pm0.2 \times 10^{-5}$, 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 impact upon overall reabsorption activity than diffuse 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 reabsorption ratio or efficiency, but not absolute reabsorption amount.[12, 13, 27, 37] Those studied elucidated that 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 assumption that the amount of proteins 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.[8] Continuous efforts have been made to minimize background signals from free fluorescence dye, out-of-focus signals, inappropriate blood pressure and body temperature during assay in multiphoton microscopy, and Schiessl et al. recently reported a value of 4.4×10^{-4} in rats.[38]

Tojo et al. utilized micropuncture and reported 6.2×10^{-4} as GSC in rats.[9] In rats with STZ-induced diabetic nephropathy, slight reduction in single nephron albumin filtration at superficial nephrons under anesthesia has been reported both by micropuncture and multiphoton microscopy.[12, 13] These findings may not be necessarily contradictory to our findings which showed doubling of total nephron albumin filtration in diabetic mice. It is because we cannot rule out a 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.[39, 40] Comparison with other works are discussed in Supplemental Discussion.

A pathophysiological 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.[16, 41]

Measuring sum of albumin filtration in the total nephrons is a strength and weakness of this study. For 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 to that in control mice (Figures 1D and 1F), but we did not examine whether albumin reabsorption is also suppressed to similar extent. Therefore, 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.[23]

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. The present study may provide theoretical rationale for early treatment intervention to normalize glomerular abnormality of hyperfiltration (and glomerular hypertension) by blood glucose level-lowering and blood pressure-lowering reagents.

CONCISE METHODS

Experimental animals

In *megalin(lox/lox)* mice, exons 72 to 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-CreER*^{T2} 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 *megalin* null allele, tissue non-specific alkaline phosphatase *(TNAP or Alpl)-Cre* knockin mice were used, which were created by inserting IRES-Cre cassette immediately after exon 6 of *Alpl* gene according to a previous report.[44] Akita type 1 diabetic mice (*Ins2*$^{Akita/+}$; purchased from Japan SLC, Hamamatsu, Japan)[35] were bred with *megalin(lox/lox);Ndrg1-CreER*^{T2} 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 *megalin* gene in adulthood, 150 mg/kg body weight of Tamoxifen (Tam, Sigma-Aldrich, St Louis, MO, USA) in sunflower seed oil (10 μL/g body weight, Sigma-Aldrich) was intraperitoneally injected to mice for 3 (low dose) or 5 days (high dose).[45] Tam was given consecutively to non-diabetic mice and every other day to diabetic mice. We found that Tam treatment tended to increase urine volume, urinary albumin/creatinine ratio and creatinine clearance (approximately by 50%) in wild-type mice, but not significantly (Supplemental Table 2). Individual mouse was housed in a metabolic cage, and urine sample was collected for 24 hours in a non-coated, standard glass bottle at room temperature without use of proteinase inhibitor. For genotyping of mice, DNA was extracted from kidney or tail. The following 3 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', 5'-TCT ATG CAA GCT CCT CCC ACC T-3'.

Organ and blood collection was performed under sodium pentobarbital anesthesia (50 mg/kg ip, Dainippon Sumitomo Pharma, Osaka, Japan) and all efforts were made to minimize suffering. This study was carried out in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals. The protocol was approved by Animal Research Committee of Kyoto University (Permit Number: Med Kyo 12330).

Induction of diabetes and treatment with insulin

Streptozotocin (100 mg/kg/day for 3 consecutive days, intraperitoneally, Sigma-Aldrich) or vehicle (citrate buffer, pH 4.0) was injected to 6-8 week-old

megalin(lox/lox);Ndrg1-CreER^{T2} and control mice, and low-dose Tam was injected 8 weeks later. Akita mice were treated with low-dose Tam at 14 weeks of age. Urine samples were collected with metabolic cages before and 2 weeks after Tam treatment. For insulin treatment, osmotic pump (model 2001, ALZET Osmotic Pumps, Cupertino, CA, USA) filled with human regular insulin (2 U/kg/hour, Eli Lilly Japan, Kobe, Japan) was subcutaneously implanted for 7 days.

Measurement of urinary and serum albumin, NGAL, IgG and creatinine

Urinary and serum levels of murine albumin were determined by Albuwell ELISA (Exocell, Philadelphia, PA, USA; main text) or Lbis ELISA (Shibayagi, Gunma, Japan; Supplemental Figure 6). NGAL (BioPorto Diagnostics, Hellerup, Denmark) and IgG (Bethyl Laboratories, Montgomery, TX, USA) 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 Albuwell ELISA. For Coomassie Brilliant Blue staining (R-250, Nacalai Tesque, Kyoto, Japan) of urine, similar amount (1/2,000 of daily urine) was loaded in each lane and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Quantitative reverse-transcription PCR

Total RNA was extracted from mouse kidneys with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA in each sample was synthesized by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Gene expression levels were examined by Premix Ex Taq (Takara Bio, Otsu, 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'-TGCCCAAGCTGCCAAGCT-3'; reverse primer, 5'-CACACCGATGTCCATGTTCACA-3'; and probe, 5'-FAM-AGCCCCTGATCTGAAAGTCACCCCGT-TAMRA-3'. Expression levels of *megalin* were normalized by *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) levels, whose primer/probe set was purchased from Applied Biosystems. Standard curve was made by serial dilution of cDNA from kidneys of untreated wild-type mice.

Renal brush border membrane (BBM) isolation and Western blot analysis

Western blot using renal BBM was performed as described previously with some modification.[48,49] 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 $MgC1_2$, 0.5 mM phenylmethylsulfonyl fluoride at pH 7.1). Isolated BBM (10 μg/lane) was dissolved in Laemmli buffer, separated using non-reducing SDS-PAGE, and transferred onto PVDF membranes. The membranes were incubated overnight at 4°C with anti-megalin or anti-β-actin antibody (Supplemental Table 1). The immunoblots were processed using peroxidase-conjugated anti-IgG antibody (Jackson ImmunoResearch, PA, USA), ECL Prime and ImageQuant LAS 4000 system (GE Healthcare, UK). Densitometry was performed with ImageJ software (National Institutes of Health, MD, USA) and normalized by β-actin.

Injection and detection of retinol binding protein (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, USA). Mice were anesthetized with diethyl ether and intravenously injected with fluorescent proteins (1 μg/g body weight, 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. Next day, tissues were frozen in Tissue-Tek O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan) and stored at -80°C until analysis.

Immunofluorescence study of megalin and nephron segment markers

Kidneys were sliced with cryostat (CM1850; Leica Biosystems, Wetzlar, Germany) at 10 μm thickness, 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, West Grove, PA, USA) and examined by a fluorescence microscope (IX81-PAFM; Olympus, Tokyo, Japan).

Electron microscopy

Freshly isolated kidney blocks were fixed with 2.5% glutaraldehyde (Wako Pure Chemical Industries, Osaka, Japan) and 4% paraformaldehyde, and then treated with 1% osmium tetroxide (Nacalai Tesque). Tissue examination was performed by a transmission electron microscope (H-7650; Hitachi, Tokyo, Japan).[47]

Statistical analysis

Results are expressed as mean±SEM and analyzed by 2-tailed Student's t test or

one-way ANOVA with Bonferroni's post hoc test, except for Figure 4C (Holm's post hoc test). Statistical significance was defined as $P<0.05$.

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DISCLOSURES

None.

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Table 1. GSC of albumin, IgG and NGAL in STZ-diabetic and non-diabetic, megalin-deficient mice

	GSC (Alb)	GSC (IgG)	GSC (NGAL)
non-STZ	$1.74\pm0.16 \times 10^{-5}$	$1.42\pm0.10 \times 10^{-5}$	0.39±0.08
STZ	$3.80\pm0.47 \times 10^{-5}$	$2.09\pm0.27 \times 10^{-5}$	0.55±0.11
Fold-difference	2.18[a]	1.46	1.40

[a] $P<0.05$ between STZ and non-STZ animals ($n=5$, respectively).

Figure Legend

Figure 1. Efficiency of *megalin* gene disruption in *megalin* mutant mouse kidneys.

(A) Megalin protein expression was abundantly observed in control mice, which were wild-type and tamoxifen (Tam)-untreated *Megalin(lox/+);Ndrg1-CreER^{T2}* mice. Bar, 500 μm. (B) Low-dose Tam treatment of inducible-megalin KO (iMegKO) mice resulted in deletion of megalin expression in the cortex. (C) Renal megalin expression was almost completely removed by high-dose Tam treatment. (D) Abundant reabsorption of Alexa546-labeled retinol binding protein (RBP) was found in the control kidney, 15 min after intravenous injection. (E) By low-dose Tam treatment, RBP signals in the cortex were diminished and shifted to outer medulla in iMegKO mice. (F) Very few RBP signals were found after high-dose treatment. (G) Megalin protein was expressed in a sparse manner in *Megalin(lox/lox);apoE-Cre* mice. (H) Megalin expression was diffusely reduced in *Megalin(+/-)* mice. (I) RBP signals were sparsely observed in *Megalin(lox/lox);apoE-Cre* mice, in a similar pattern as megalin expression. (J) Reduced RBP signals were also observed in *Megalin(+/-)* mice. (K) *Megalin* gene rearrangements were examined in DNA extracted from whole kidney (lanes 1-3) or tail tissue (lane 4) of low-dose Tam treated mice. *Megalin* gene disruption specifically occurred in the mutant kidney. WT, wild-type (116 bp); Flox, floxed (210 bp); Dis, disrupted allele (320 bp).

Figure 2. Renal megalin mRNA and protein expression in control and *megalin* mutant mice after tamoxifen treatment.

(A) Whole kidney *megalin* mRNA expression levels (n=5-7). Significant reduction was found in *Megalin(+/-)*, *Megalin(lox/lox);apoE-Cre* and low-dose Tam-injected *Megalin(lox/lox);Ndrg1-CreER^{T2}* mice vs Tam-untreated wild-type (**P<0.01, respectively). Only 8% of *megalin* mRNA expression remained after high-dose treatment (**P<0.01). Two lines of single mutant mice treated with Tam, *Megalin(lox/lox);Cre(-)* and *Megalin(+/+);Ndrg1-CreER^{T2}*, served as negative controls. (B) Renal megalin protein expression in brush border membrane preparation (arrowhead, approximately 600 kDa). To generate a standard curve for quantitation, kidney sample from *Megalin(lox/lox);Ndrg1-CreER^{T2}* mouse with high-dose Tam was mixed with sample from wild-type mouse. (C) Quantitative analysis of megalin protein expression in renal brush border membranes normalized by β-actin (n=4-6). Significant reduction was found in *Megalin(lox/lox);apoE-Cre* and low-dose Tam-injected *Megalin(lox/lox);Ndrg1-CreER^{T2}* mice

vs Tam-untreated wild-type (*P<0.05, respectively). Only 8% of megalin protein expression remained after high-dose treatment (*P<0.05).

Figure 3. Colocalization of megalin and RBP at high power field.

(A) Cortex and (B) outer stripe. Bar, 20 μm. Megalin expression and RBP signals were colocalized at apical surface of proximal tubules in control (wild-type) mice and *Meg(+/-) mice.* When *megalin* gene was disrupted by *apoE-Cre*, both signals were deleted at proximal tubules in a mosaic manner. Arrowheads indicate boundaries between megalin-expressing and non-expressing proximal tubules. In control mice, RBP reabsorption was completed in S1-S2. In low-dose Tam-treated iMegKO mice (iMegKO-Low), it was predominantly observed in S3 expressing megalin. Very little RBP signals were found in mutants given high-dose Tam (iMegKO-High). (C) In iMegKO-Low mice, RBP signals were mainly observed in aquaporin 1 (AQP1)-expressing proximal tubules. As shown by arrows, however, RBP signals were also found in (C) AQP1-negative nephron segments and in (D) segments not expressing Tam Horsfall protein (THP, a marker of thick ascending limb of Henle). Furthermore, RBP was found along apical surface of (E) calbindin-D28k-expressing distal convoluted tubules and (F) aquaporin 2 (AQP2)-expressing collecting ducts. OS, outer stripe; IS, inner stripe.

Figure 4. Urine albumin and NGAL levels in iMegKO mice.

(A) iMegKO mice given low or high dose of Tam similarly exhibited more than 10-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 vs 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 vs pre-treatment (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.

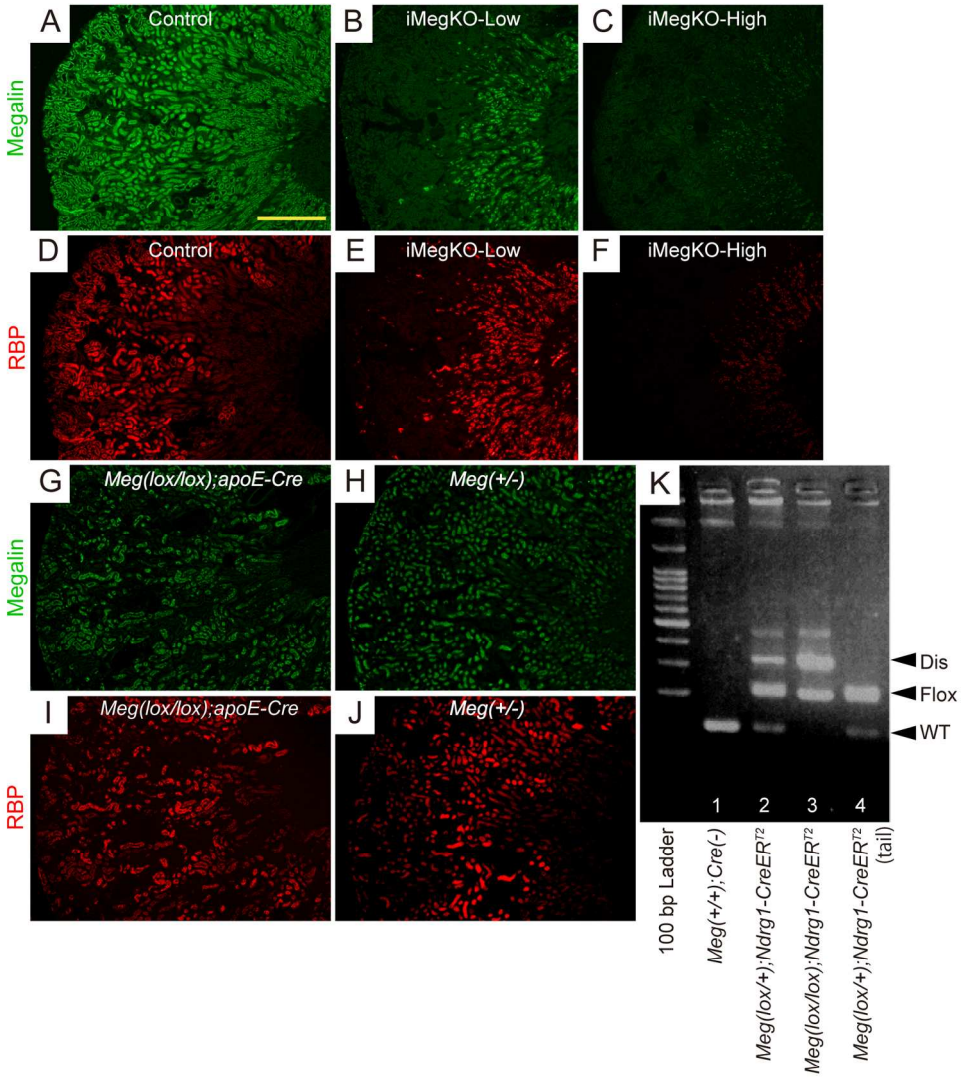
Figure 5. Transmission electron microscope photos of control and *megalin* mutant mice.

(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.

Figure 6. Urinary excretion of albumin, NGAL and IgG and estimated albumin reabsorption in control and *megalin* mutant mice under diabetic and non-diabetic conditions.

(A) Daily urinary albumin excretion. Tam-untreated *Meg(lox/lox);Cre(-)* mice were studied as control. Total nephron albumin filtration is estimated by urinary albumin excretion in iMegKO-mice given low-dose Tam. Subtraction of albumin excretion before Tam from that after Tam corresponds to estimated tubular albumin reabsorption (hatched box in A and D). Total nephron albumin filtration and reabsorption were significantly increased in STZ and

Akita diabetic mice compared to non-STZ mice (P<0.05, respectively). (E) Albumin reabsorption ratio calculated as albumin reabsorption divided by albumin filtration was reduced in diabetic mice. On the other hand, daily urinary excretion of (B) NGAL or (C) IgG was not significantly increased in diabetic mice. (F) *Megalin* mRNA expression levels in whole kidney were significantly reduced in STZ;megalin intact (control) mice, and even more in STZ;iMegKO mice. n=5-11.



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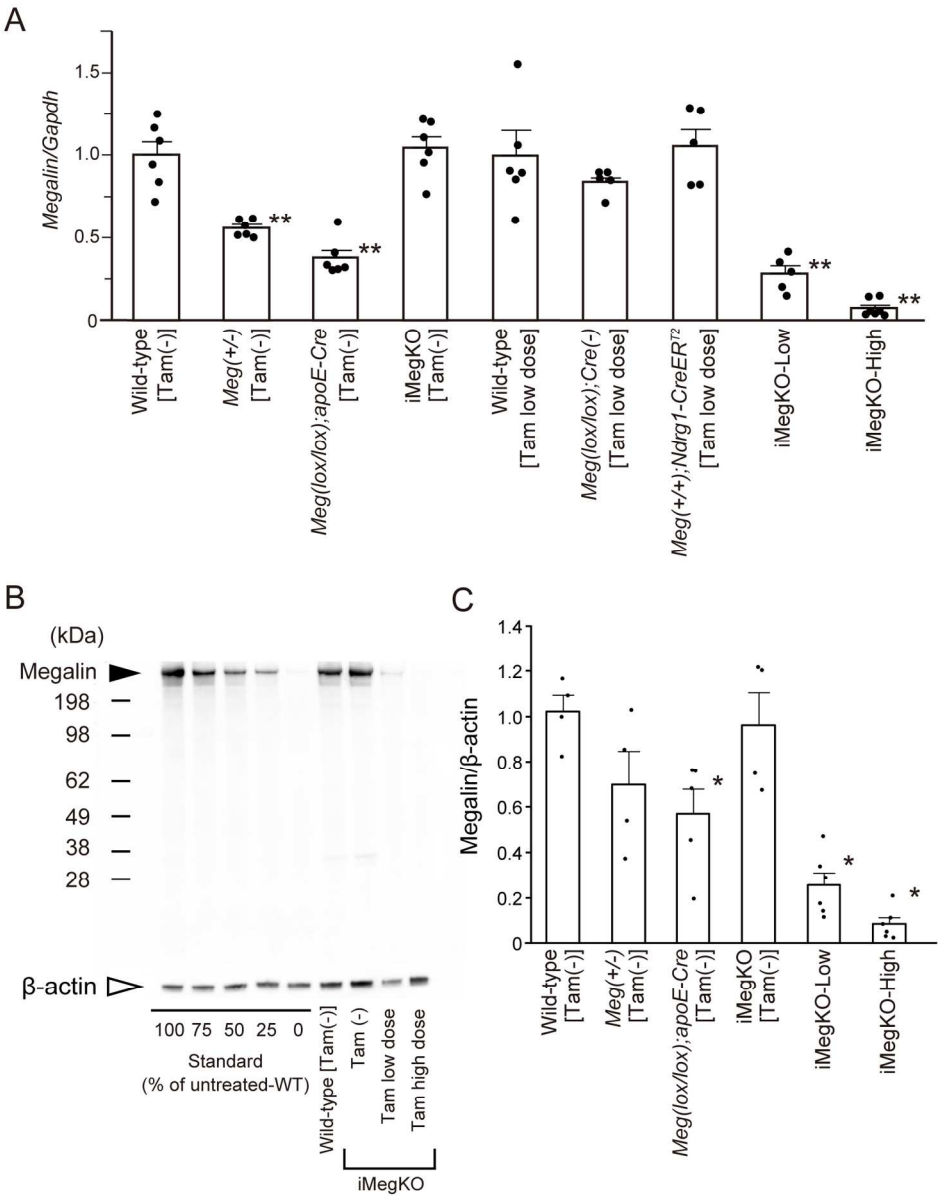

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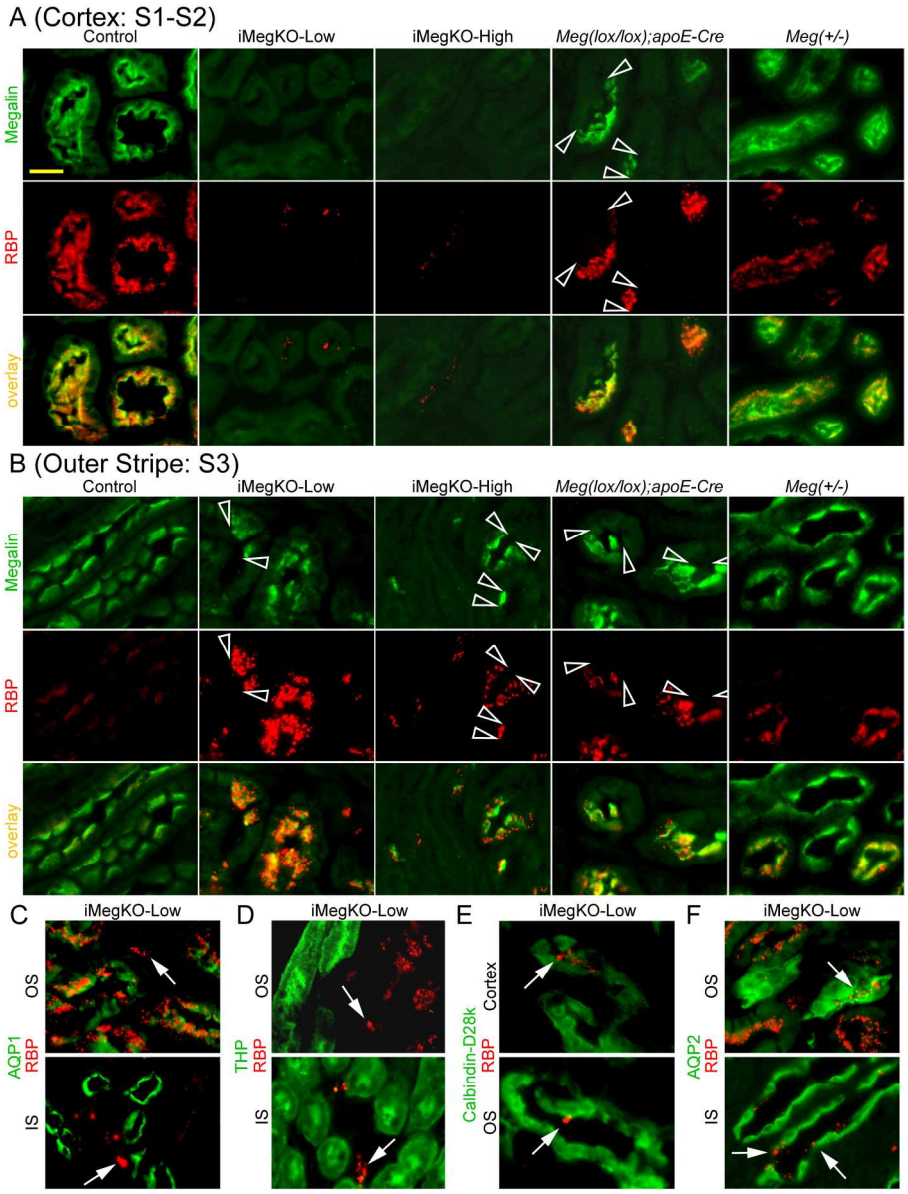

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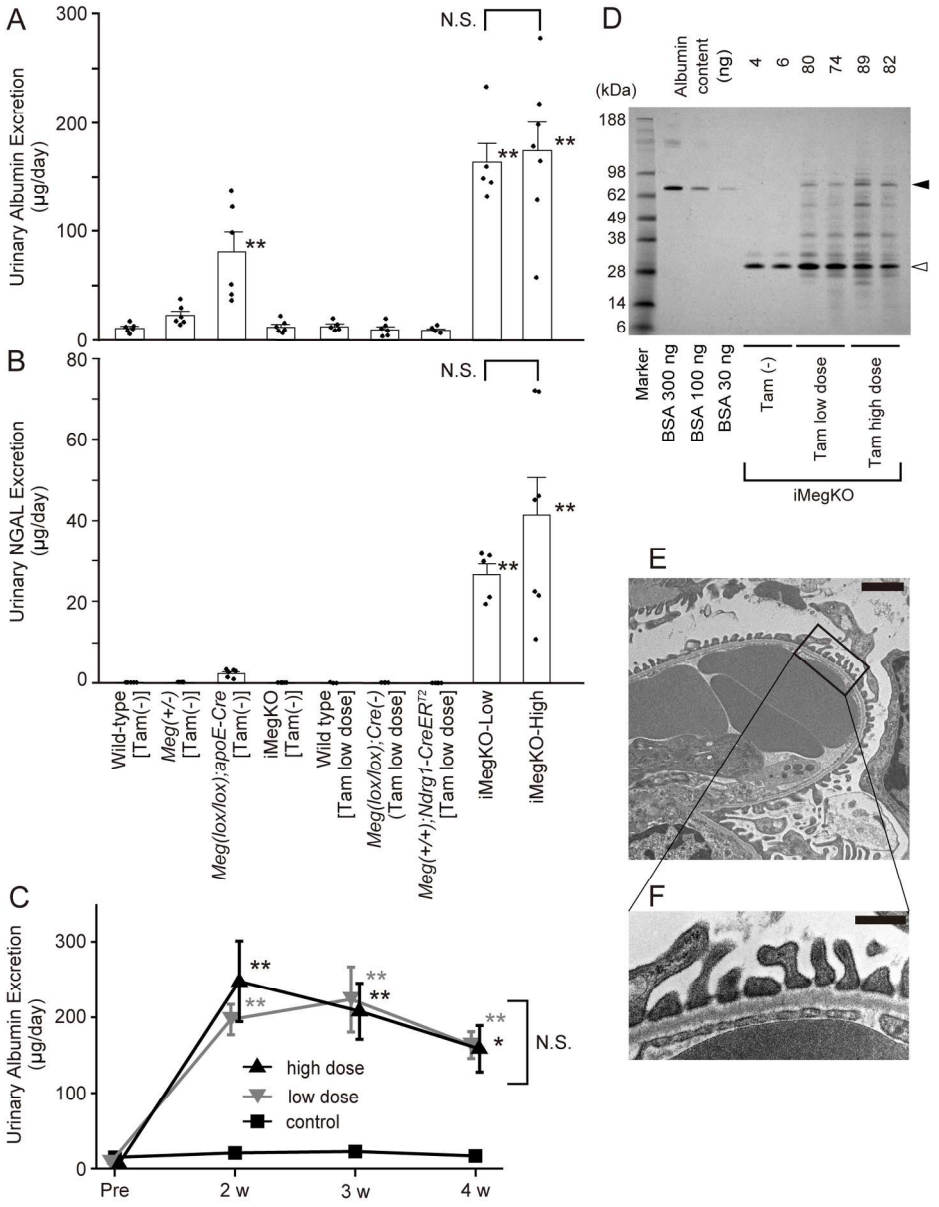

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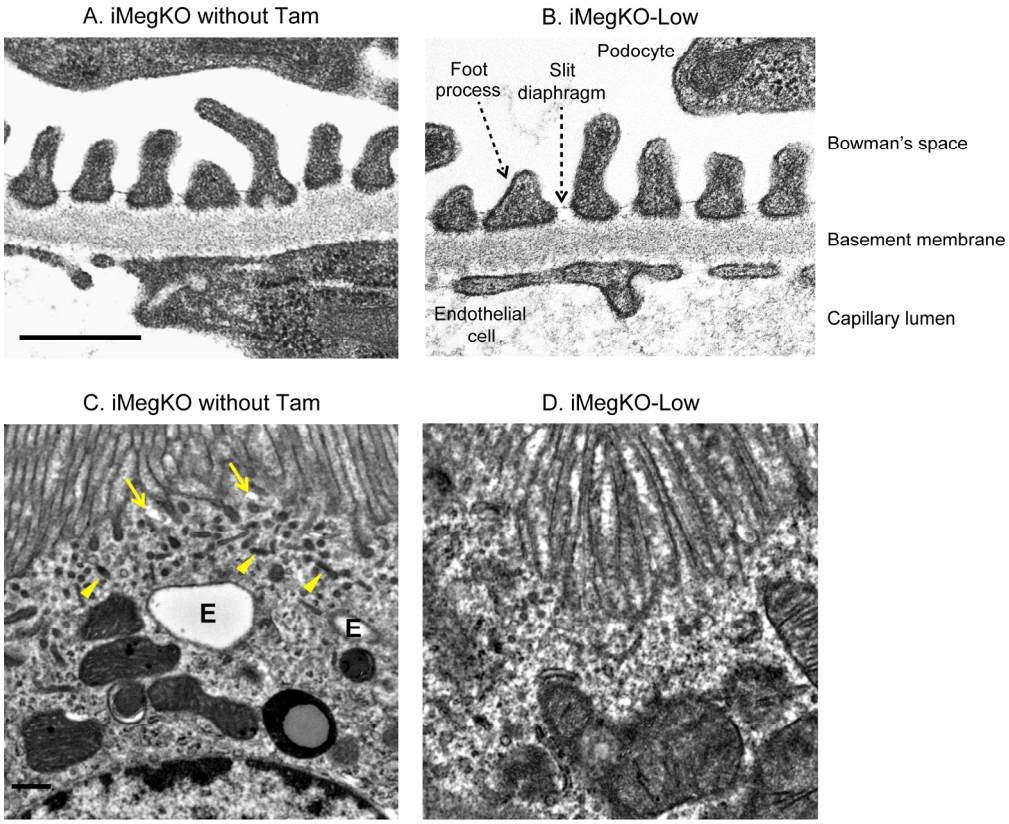

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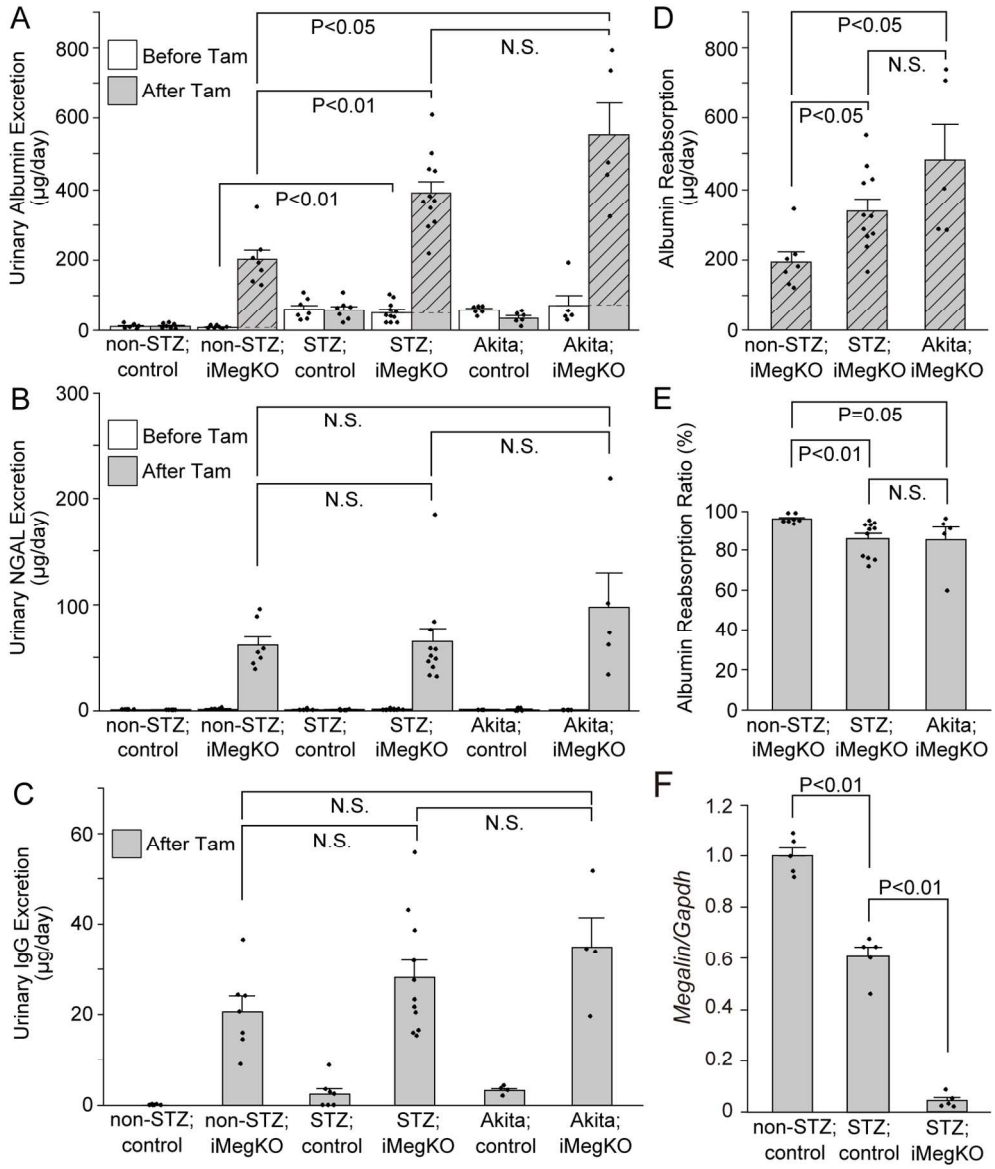

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SUPPLEMENTAL DATA

Increase of Total Nephron Albumin Filtration and Reabsorption in Diabetic Nephropathy

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SUPPLEMENTAL DISCUSSION

Albumin measurement

In this study, we tried to measure albumin levels as precisely as possible. We first used Albuwell ELISA kit, which is recommended for use in analysis of murine diabetic nephropathy by Animal Models of Diabetic Complications Consortium (Figures 3A-3C and 5).[1] These values were consistent with the findings obtained by protein staining of full-length albumin (Figure 3D). Next, we utilized a second kit, Lbis ELISA. Urinary albumin levels measured by these two kits gave highly proportional values in a broad range of distribution (r=0.97 by Pearson correlation, Supplemental Figure 6), confirming the accuracy of quantitation. Of note, Lbis gave aprroximately 40% of values compared to Albuwell. Osicka et al. have reported that full-length albumin consists of several forms which exhibit different immunogenicity among various assays.[2] We decided to use Albuwell concentrations for further analysis.[1]

Comprehensive comparison of GSC from various reports (more information added compared to the discussion section in the main text)

Using drug-inducible reabsorption disruption system, we estimated that GSC of albumin is 1.7×10^{-5} in normal mice and it is increased by 2.2-fold with development of diabetes (Table 1). Tojo et al. reported that 26% of filtered albumin is reabsorbed at distal nephron in rats.[3] If this is taken into account, GSC of albumin in normal mice is recalculated as 2.3×10^{-5}.

Russo et al. proposed a GSC value of 0.034 in normal rats by multiphoton microscopy.[4] Continuous efforts have been made to minimize background signals and Schiessl et al. recently reported a value of 4.4×10^{-4} in rats.[5]

Tojo et al. utilized micropuncture and reported 6.2×10^{-4} as GSC in rats.[6] In rats with STZ-induced diabetic nephropathy, slight reduction in single nephron albumin filtration at superficial nephrons has been reported both by micropuncture and multiphoton microscopy.[7, 8]

Bertolatus and Hunsicker injected ^{131}I-labeled BSA into rats and added the amounts of radioactivity recovered from the kidney and urine.[9] They reported GSC of 6×10^{-4}, but this value may contain albumin fragments generated outside of the kidney and filtered at glomeruli.[10]

Tucker et al. estimated total nephron albumin filtration in rats by measuring urinary albumin after inhibiting tubular reabsorption with continuous lysine infusion, but it was difficult to prove that the amount of lysine used was sufficient to completely

block reabsorption.[11]

Norden et al. analyzed blood and urine from 5 patients with Dent's disease and reported their average GSC of albumin as 7.7 x 10^{-5}, which gave the closest GSC to our findings.[12] Dent's disease is an X-linked disorder with defective proximal tubule reabsorption, which is caused by mutation in *CLCN5* ion channel.[13] Drawbacks of this approach included that it was difficult to evaluate the extent of reabsorption defect at proximal tubules in individual cases (without renal biopsy and immunostaining of reabsorbed proteins) and it was impossible to assess relative reabsorption capacity in distal nephrons. Furthermore, Storm et al. recently reported *megalin* deficient cases.[14] In those patients, GSC of albumin can be calculated as approximately 1.5 or 2.5 x 10^{-5}, (if we assume serum creatinine and albumin were 1.0 mg/dL and 4.0 g/dL, respectively), whose GSC values are very similar to murine GSC values obtained here. In the present study, by comparing low- and high-dose Tam-treated iMegKO mice, we could show, at least in mice, that inhibition of reabsorption at S1-S2 by low-dose Tam is sufficient for almost maximal inhibition of renal albumin reabsorption (obtained by high-dose Tam) and contribution of S3 and distal nephrons for reabsorption is small.

Gene engineering methods making *megalin* mutant mice have been also used to study renal albumin handling. Motoyoshi et al. reported that 40% of proximal tubule cells in *megalin(lox/lox);apoE-Cre* mice express residual megalin protein in a mosaic manner.[15] Amsellem et al. revealed that most of *megalin(lox/lox);MORE* mice died rapidly after birth with 95% mortality.[16] Weyer et al. showed that urinary albumin/creatinine in control and *megalin(lox/lox);Wnt4-Cre* mice (in C57BL/6+129/Sv mixed genetic background) were approximately 50 and 500 μg/mg, respectively.[17] These values are consistent to our results with *megalin(lox/lox);Ndrg1-CreER*^{T2} mice but the authors did not evaluate GSC of albumin in diabetic nephropathy. Christensen et al. reported that albumin GSC of *megalin* knockout mice was 1.6 x 10^{-4}, but experimental details were not described and urinary albumin excretion of 0.2 mg/day in control mice was quite high.[18]

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Supplemental Table 1. List of antibodies used in this work

Antigen	Antibody species	Company	Catalogue number
Megalin	Goat	Santa Cruz Biotechnology, Santa Cruz, CA, USA	sc-16478
Aquaporin 1	Rabbit	Chemicon, Temecula, CA, USA	AB3065
Aquaporin 2	Goat	Santa Cruz Biotechnology, Santa Cruz, CA, USA	sc-9882
Tamm Horsfall protein	Rabbit	Biomedical Technologies, Stoughton, MA, USA	BT590
Calbindin-D28k	Mouse	Sigma-Aldrich, St. Louis, MO, USA	C9848
β-actin	Mouse	Sigma-Aldrich, St. Louis, MO, USA	A5441

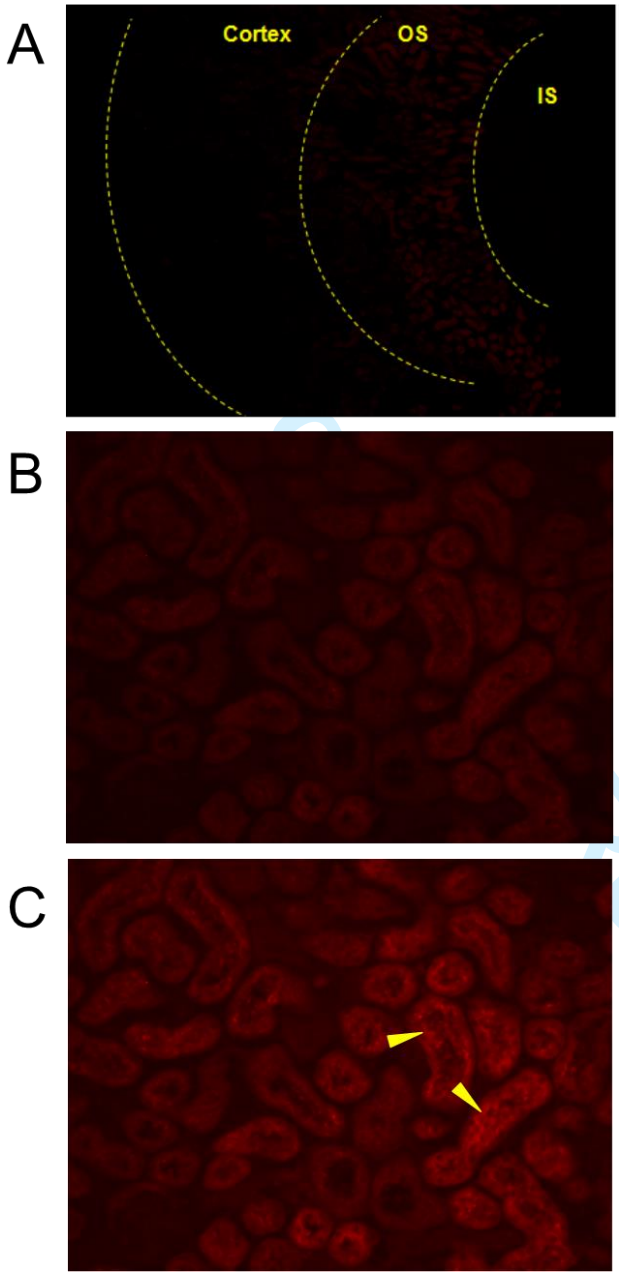
Supplemental Table 2. Effects of STZ and Tam upon blood and urinary parameters

			UV (mL/day)	uCr (mg/day)	UACR (μg/mgCr)	UAE (μg/day)	sCr (mg/dL)	sAlb (g/dL)	BW (g)	Ccr (mL/day/g)
STZ(-)	Cre(-)	Tam(-)	0.86±0.11	0.40±0.04	21.7±1.5	8.6±0.9	0.14±0.01^{x}	2.9±0.1	32.6±0.5^{x}	9.2±1.0
		Tam(+)	1.28±0.25	0.35±0.06	32.3±5.9	11.3±2.9	0.11±0.01	2.9±0.1	28.2±0.6	13.7±3.0
	iMegKO	Tam(+)	2.29±0.31^{a}	0.51±0.03	403.3±60.4^{a,x}	201.8±27.9^{a,x}	0.12±0.01	2.7±0.1	28.4±1.2^{a}	17.3±1.7^{a}
STZ(+)	Cre(-)	Tam(-)	24.20±2.70^{a}	0.84±0.03^{a}	111.7±25.3^{a}	89.9±17.1^{a}	0.16±0.01	2.2±0.1^{a}	24.0±0.8^{a}	22.6±1.0^{a}
		Tam(+)	30.62±2.87^{a}	0.80±0.05^{a}	74.4±15.4^{a}	57.4±10.8^{a}	0.17±0.03	2.1±0.2^{a}	23.0±2.2^{a}	22.4±2.5^{a}
	iMegKO	Tam(+)	26.09±4.29^{a}	0.62±0.06^{a}	672.2±57.4^{a,c,y}	390.4±32.6^{a,c,y}	0.13±0.01^{b}	2.3±0.2^{a}	20.8±1.3^{a}	25.1±2.0^{a,c}

STZ treatment significantly increased urine volume, urinary creatinine and albumin excretion and creatinine clearance, which have been described as hyperfiltration status.[11] STZ also significantly decreased serum albumin and body weight. Tam treatment tended to increase urine volume, urinary albumin/creatinine ratio and creatinine clearance (approximately by 50%) in non-STZ (STZ(-);Cre(-)) megalin intact mice, but not significantly. These effects of Tam were not observed in STZ;Cre(-) mice. *n*=5-11.

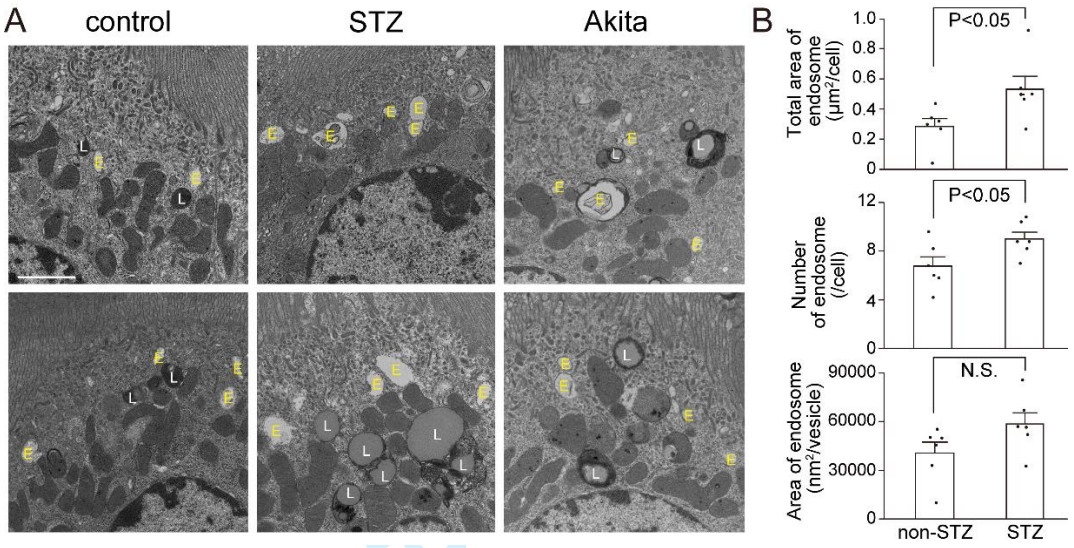
^{a}P<0.05 vs STZ(-);Cre(-);Tam(-). ^{b}P<0.05 vs STZ(+);Cre(-);Tam(-). ^{c}P<0.05 vs STZ(-);iMegKO;Tam(+). ^{X}P<0.05 vs STZ(-);Cre(-);Tam(+). ^{Y}P<0.05 vs STZ(+);Cre(-);Tam(+).

STZ, streptozotocin, Tam, tamoxifen, UV, urine volume, uCr, urinary creatinine excretion, UACR, urinary albumin-creatinine ratio, UAE, urinary albumin excretion, sCr, serum Cr level, sAlb, serum albumin level, BW, body weight, Ccr, creatinine clearance.



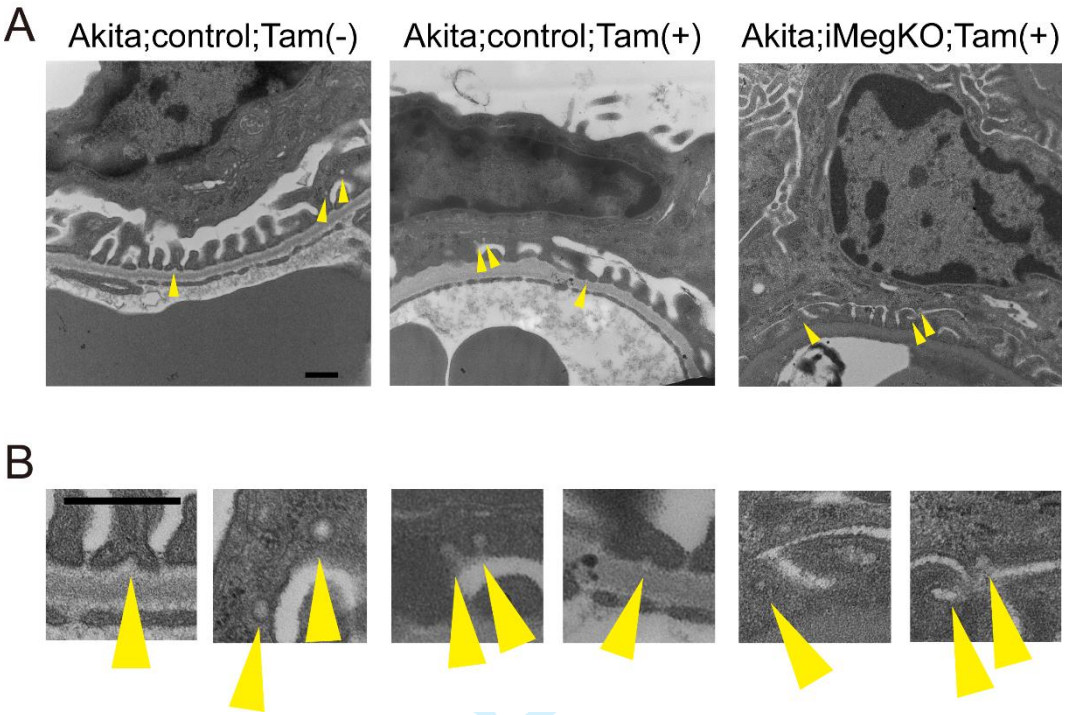
Supplemental Figure 1. Weak signals of exogenous bovine serum albumin in proximal tubules of wild-type mice.

(A) Low power field (Magnification 4x). Alexa546-labeled bovine serum albumin was intravenously injected 15 min earlier. (B) High power field (20x). (C) Longer exposure of (B). OS, outer stripe. IS, inner stripe. Arrowheads, Alexa546 signals.



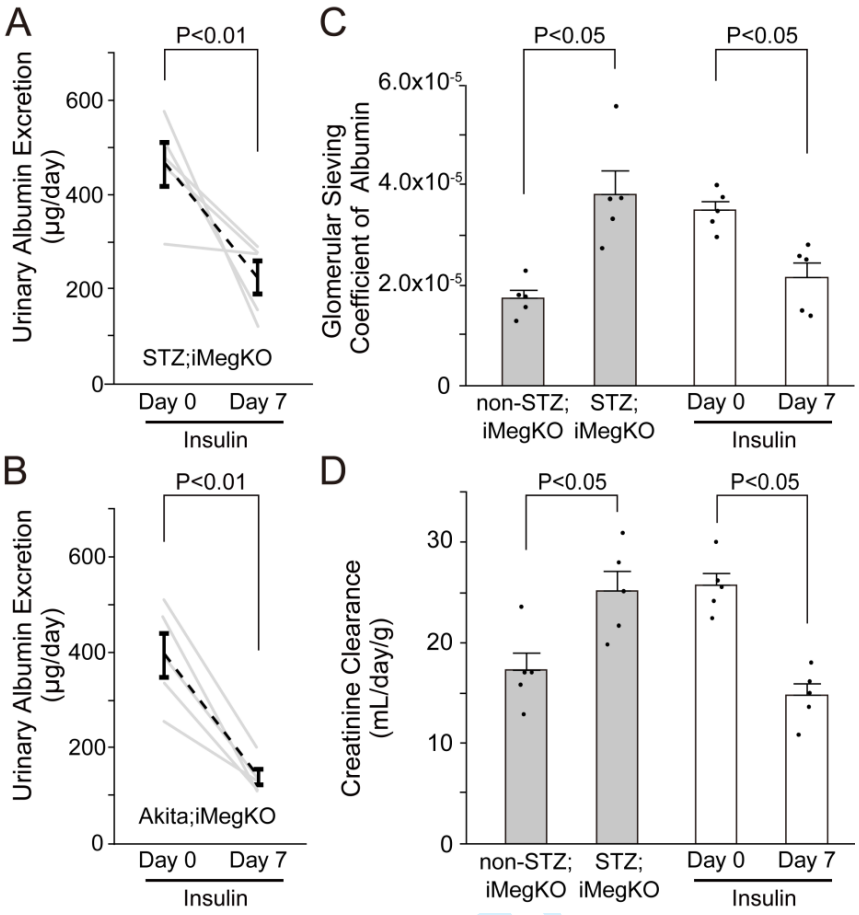
Supplemental Figure 2. Endocytosis-related apparatus in proximal tubules of diabetic mice.

(A) Transmission electron microscopy pictures of S1 cortical proximal tubules in wild-type (control), STZ-treated wild-type (STZ) and Akita;Cre(-) mice (Akita) without Tam treatment. S1 segments were identified as described previously.[19] In S1 cells of STZ and Akita mice, the numbers and sizes of endosomes (E) and lysosomes (L) tended to be larger compared with those of control mice. Bar, 2 μm. (B) For quantitative image analysis, 5 fields including one cell per field at a magnification of 3,000x were randomly scanned in wild-type (non-STZ) and STZ mice (n=6). Morphometric measurement was performed using ImageJ software. In STZ mice, the number of endosomes in each cell was increased by 1.3-fold (P<0.05), the size of endosomes was increased by 1.4-fold (not significant) and the total areas of endosomes in each single S1 cell were enlarged by 1.9-fold (P<0.05), suggesting that reabsorption activity was enhanced in S1 segments



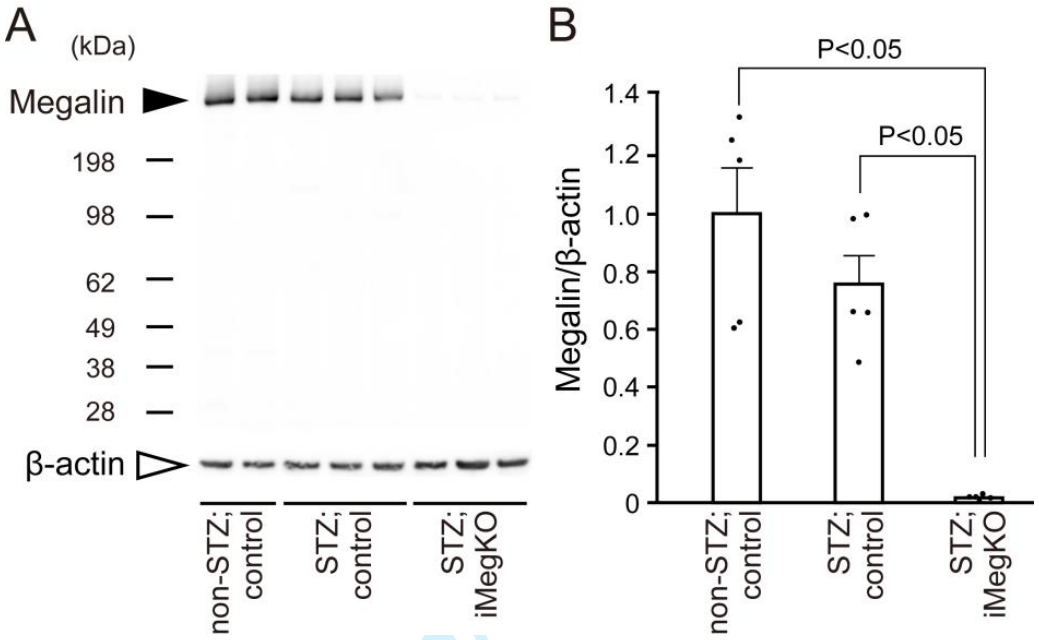
Supplemental Figure 3. Clathrin-coated pits in podocytes of Akita mice.

(A) Transmission electron microscopy pictures of podocytes in Akita;Cre(-) mice with or without low-dose Tam treatment, and Akita;iMegKO mice with low-dose Tam. Bar 500 nm. (B) Higher magnification of clathrin-coated pits as shown by arrowheads in (A), indicating preservation of the normal size and number of clathrin-coated pits in podocytes of Akita:iMegKO mice after Tam treatment. Bar, 500 nm.



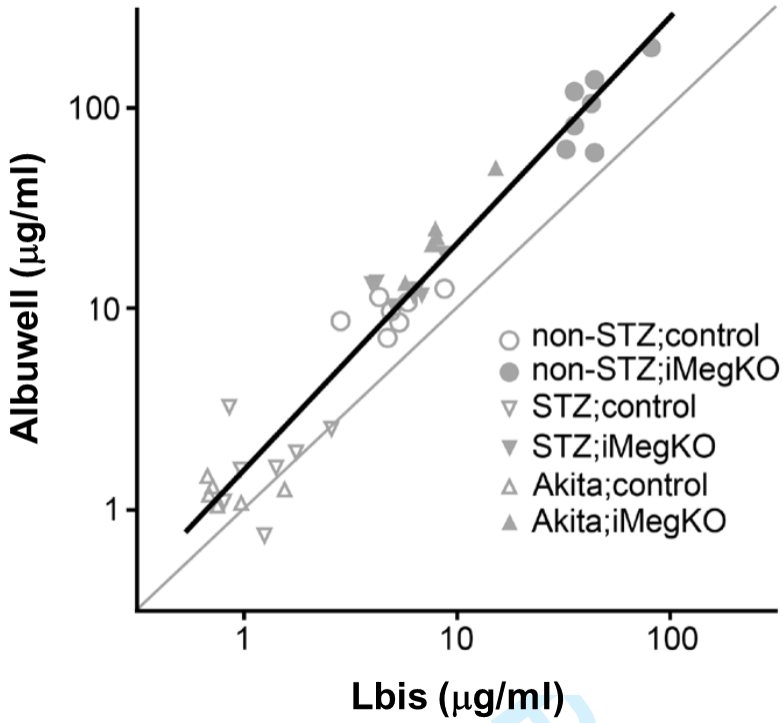
Supplemental Figure 4. Effects of insulin upon urinary albumin excretion, GSC of albumin and creatinine clearance in diabetic iMegKO mice

Insulin treatment normalized increased albumin excretion (or albumin filtration) in (A) STZ;iMegKO and (B) Akita;iMegKO mice (P<0.01, n=5, respectively). (C) GSC of albumin and (D) creatinine clearance (Ccr) were significantly increased in iMegKO mice by STZ (n=5). To examine effects of insulin upon GSC, Ccr and proximal tubular reabsorption in STZ;iMegKO mice, the serum concentrations of albumin and creatinine before and after insulin treatment are required but those values were not available to us. For that purpose, we made assumption that those values are equal to those of STZ;iMegKO and non-STZ;iMegKO mice, respectively, obtained from different experiments. Under these conditions, in average, GSC (-38%), proximal tubule albumin reabsorption (-53%), total nephron albumin filtration (urinary albumin excretion, -51%) and Ccr (-43%) in STZ;iMegKO mice were all significantly reduced by insulin (P<0.05, n=5, respectively) and became close to values in non-STZ;iMegKO mice, suggesting that hyperglycemia and glomerular hyperfiltration are the fundamental abnormalities occurring in STZ diabetic mice.



Supplemental Figure 5. Renal megalin protein expression in control and *megalin* mutant mice at diabetic conditions.

(A) Renal megalin protein expression in brush border membrane preparation (black arrowhead, approximately 600 kDa). β–actin expression serves as internal control (white arrowhead, approximately 42 kDa). (B) Quantitative analysis of renal megalin protein expression (*n*=5). Induction of diabetes by STZ reduced megalin protein expression by 24% in control mice (not significantly). Furthermore, only 2% of megalin protein expression remained after low-dose Tam treatment in STZ;iMegKO mice.



Supplemental Figure 6. Comparison of urinary albumin measurements using Albuwell and Lbis ELISA kits.

Bold black line indicates linear regression line. r=0.97, P<0.001 by Pearson correlation.