RESEARCH ARTICLE

Podocyte-specific deletion of tubular sclerosis complex 2 promotes focal segmental glomerulosclerosis and progressive renal failure

Wakiko Iwata1,2, Hiroyuki Unoki-Kubota1,* Hideki Kato3, Akira Shimizu4, Michihiro Matsumoto5, Toshiyuki Imasawa6, Arisa Igarashi7, Kenji Matsumoto7, Tetsuo Noda8, Yasuo Terauchi2, Masaomi Nangaku3, Masato Kasuga9, Yasushi Kaburagi1*

1 Department of Diabetic Complications, Diabetes Research Center, Research Institute, National Center for Global Health and Medicine, Tokyo, Japan, 2 Department of Endocrinology and Metabolism, Yokohama City University Graduate School of Medicine, Yokohama, Kanagawa, Japan, 3 Division of Nephrology and Endocrinology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan, 4 Department of Analytic Human Pathology, Nippon Medical School, Tokyo, Japan, 5 Department of Molecular Metabolic Regulation, Diabetes Research Center, Research Institute, National Center for Global Health and Medicine, Tokyo, Japan, 6 Kidney Center, National Hospital Organization Chiba-Higashi National Hospital, Chiba, Japan, 7 Department of Allergy and Clinical Immunology, National Research Institute for Child Health and Development, Tokyo, Japan, 8 Cancer Institute of Japanese Foundation for Cancer Research, Tokyo, Japan, 9 National Center for Global Health and Medicine, Tokyo, Japan

* kaburagi@ri.ncgm.go.jp (YK); hkubota@ri.ncgm.go.jp (HU)

Abstract

Obesity can initiate and accelerate the progression of kidney diseases. However, it remains unclear how obesity affects renal dysfunction. Here, we show that a newly generated podocyte-specific tubular sclerosis complex 2 (Tsc2) knockout mouse model (Tsc2Δpodocyte) develops proteinuria and dies due to end-stage renal dysfunction by 10 weeks of age. Tsc2Δpodocyte mice exhibit an increased glomerular size and focal segmental glomerulosclerosis, including podocyte foot process effacement, mesangial sclerosis and proteinaceous casts. Podocytes isolated from Tsc2Δpodocyte mice show nuclear factor, erythroid derived 2-like 2-mediated increased oxidative stress response on microarray analysis and their autophagic activity is lowered through the mammalian target of rapamycin (mTOR)—unc-51-like kinase 1 pathway. Rapamycin attenuated podocyte dysfunction and extends survival in Tsc2Δpodocyte mice. Additionally, mTOR complex 1 (mTORC1) activity is increased in podocytes of renal biopsy specimens obtained from obese patients with chronic kidney disease. Our work shows that mTORC1 hyperactivation in podocytes leads to severe renal dysfunction and that inhibition of mTORC1 activity in podocytes could be a key therapeutic target for obesity-related kidney diseases.

Introduction

The prevalence of obesity is increasing worldwide and contributes to many health problems, including type 2 diabetes mellitus (T2DM), cardiovascular disease and several types of cancer
Nphs2-Cre transgenic mice was supported in part by NIH1P30DK114857-01A1 (to Dr. Susan Quaggin, Northwestern University, USA). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Abbreviations: 4EBP1, eukaryotic translation initiation factor 4E-binding protein 1; ACR, albumin-to-creatinine ratio; BCAA, branched-chain amino acids; BUN, blood urea nitrogen; CKD, chronic kidney disease; FSGS, focal segmental glomerulosclerosis; HDL-C, HDL-cholesterol; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; NRF2, nuclear factor erythroid derived 2, like 2; PAS, periodic acid-Schiff; pS6, phospho-S6 ribosomal protein; SCr, serum creatinine; T2DM, type 2 diabetes mellitus; TC, total cholesterol; TSC1, tuberous sclerosis complex 1; TSC2, tuberous sclerosis complex 2; ULK1, unc-51-like kinase 1; WT1, Wilms tumor 1.

In this study, we hypothesize that mTORC1 activity might contribute to obesity-related renal functional decline. Accordingly, we generated podocyte-specific Tsc2 knockout mice (Tsc2<sup>lopodocyte</sup>), in which Tsc2 is specifically depleted by Podocin-Cre [16]. Deletion of the Tsc2 gene in podocytes increases glomerular size and the characteristics of focal segmental glomerulosclerosis (FSGS) and causes end-stage renal dysfunction concomitant with impaired autophagy in podocytes. Assessment of the involvement of mTORC1 in human kidney biopsy specimens demonstrated that mTORC1 signalling was surprisingly activated in podocytes from obese patients with CKD.

Materials and methods

Animals

Male obese db/db mice and their nonobese controls (db/m) were obtained from CLEA Japan Inc. (Tokyo, Japan). Mice with exons 3 and 4 of the Tsc2 gene flanked by two loxP sequences have previously been reported [17]. Heterozygous Nphs2-Cre transgenic mice were provided by Dr. Susan E Quaggin (Feinberg Cardiovascular Research Institute, Northwestern University, Chicago, IL) [16]. For generation of homozygous floxed Tsc2 mice on an ICR background, which are more sensitive to glomerular diseases, mice were backcrossed for more than ten generations (Jcl:ICR; CLEA Japan Inc.). Offspring from the backcrossed Tsc2<sup>flox/wt</sup> mice were crossed with Nphs2-Cre<sup>−/−</sup> mice to generate mice heterozygous for the Tsc2-floxed allele (genotype: Tsc2<sup>flox/wt</sup>, Nphs2-Cre<sup>+/−</sup>). These mice were bred with Tsc2<sup>flox/flox</sup> mice to inactivate both Tsc2 alleles by Cre-mediated excision, thereby creating conditional knockout mice in which the Tsc2 gene was specifically disrupted in podocytes (Tsc2<sup>−lopodocyte</sup>, genotype: Tsc2<sup>flox/flox</sup>, Nphs2-Cre<sup>+/−</sup>). PCR was used for Tsc2 loxP and Nphs2-Cre genotyping. Tsc2<sup>flox/flox</sup> (genotype: Tsc2<sup>flox/wt</sup>, Nphs2-Cre<sup>+/−</sup>) and Nphs2-Cre<sup>−/−</sup> littermates were used as controls.
During the study, animals were housed in a temperature-controlled room (22˚C) with a 12-h light/dark cycle with free access to diet and water. A standard laboratory diet (Labo H Standard, Nosan Corporation, Yokohama, Japan) was administered ad libitum from weaning. All animal care and procedures were performed in accordance with Animal Research Reporting In Vivo Experiments guidelines [18]. All research staff handling with animals was trained in accordance with the recommendations of the Institutional Animal Care and Use Committee of National Center for Global Heal and Medicine.

Survival time
For survival analysis, at least ten Tsc2Δpodocyte mice were followed open end for max. The survival time of Nphs2-Cre (n = 9), Tsc2flax/flax (n = 10), and Tsc2Δpodocyte (n = 32) mice was checked until 16 weeks of age and evaluated using the Kaplan-Meier method. For rapamycin treatment, Nphs2-Cre (n = 17), Tsc2flax/flax (n = 16), and Tsc2Δpodocyte (n = 8) mice were intraperitoneally injected with rapamycin (LC Laboratories, Woburn, MA) at 2 mg kg⁻¹ of body weight every other day from 4 to 11 weeks of age. Saline injection for control was performed similarly (Nphs2-Cre [n = 7], Tsc2flax/flax [n = 9], and Tsc2Δpodocyte [n = 26]). All mice were monitored every 2 weeks beginning at 3 weeks of age. As described later, Tsc2Δpodocyte mice had significantly shorter survival than control mice without humane intervention due to renal dysfunction. When mice exhibited reduced locomotor activity and hypothermia, blood urea nitrogen (BUN) was measured using an Arkray Spotchem D (Arkray, Kyoto, Japan). In case of BUN over 50 mg/dL, as a specific endpoint criterion, the affected mice were euthanized immediately. There were no mice that were euthanized before reaching the experimental endpoint. The numbers of mice that died without humane intervention and euthanized after reaching the experimental endpoint were summarized in S1 Table.

Serum and urine analysis
At 3, 5 and 7 weeks of age, mice were individually placed in metabolic cages (Shinano Manufacturing, Tokyo, Japan) with free access to diet and water, and urine was collected for 16 h. Urinary albumin and creatinine levels were measured on a Hitachi 7180 analyser (Hitachi Inc., Tokyo Japan), and the albumin-to-creatinine ratio (ACR) was calculated. Body weight and fasting plasma glucose levels were measured, and blood samples were obtained as described previously [19]. Serum creatinine (SCr), total protein, albumin, uric acid, BUN, HDL-cholesterol (HDL-C), total cholesterol (TC), triglyceride, Na, K and Cl were measured using an Arkray Spotchem D (Arkray, Kyoto, Japan).

Histological assessments
At defined experimental time points, mice were deeply anesthetized with sevoflurane (Maruishi Pharmaceutical Co., Ltd, Osaka, Japan). The sacrificed mice were perfused with 0.9% NaCl solution and then both kidneys were excised. Kidneys were fixed in 10% phosphate-buffered formalin, embedded in paraffin and deparaffinized in xylene; then 2-μm sections were stained with periodic acid-Schiff (PAS) and Masson’s trichrome. Glomerulosclerotic injury was graded based on the severity of glomerular damage, essentially as reported previously [20]. A glomerulosclerotic index was then calculated using the formula: Glomerulosclerotic index = (1 × n₁) + (2 × n₂) + (3 × n₃) + (4 × n₄) / (n₀ + n₁ + n₂ + n₃ + n₄), where nₙ is the number of glomeruli at each grade of glomerulosclerosis. At least 50 glomerular sections were randomly assessed in each mouse (n = 3/genotype), and this analysis was performed with the observer masked to the treatment groups. For an evaluation of glomerular size, glomerular diameters were assessed in 20 glomerular sections that were randomly selected from each mouse (n = 3/
genotype), measured by using ImageJ processing software version 1.50i [21], and the averages of the glomerular diameters per glomerular section were calculated. For immunofluorescence studies, 4-μm frozen sections of OCT-embedded frozen kidneys were fixed in ice-cold acetone, blocked with 3% bovine serum albumin and incubated with primary antibodies—rabbit anti-Wilms tumor 1 (WT1) (1:50, sc-192, Santa Cruz Biotechnology, Dallas, TX), anti-synaptopodin (1:50, sc-50459, Santa Cruz Biotechnology) and anti-podocin (1:100, P0372, Sigma-Aldrich, St. Louis, MO) polyclonal antibodies and developed using FITC-conjugated swine anti-rabbit immunoglobulins polyclonal antibody (1:20, F020502, Dako; Agilent Technologies, Santa Clara, CA). Cell nuclei were counterstained with Hoechst 33342 and mounted with Fluoromount. The numbers of double-positive cells (WT1 and Hoechst 33342) were counted in more than 20 glomerular sections that were randomly selected from each mouse (n = 6–8/genotype) and the averages of the double-positive cells per glomerular section were calculated.

**Transmission electron microscopic analysis**

Kidney samples were fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.4), postfixed with 1% osmium tetroxide, dehydrated, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined with a transmission electron microscope (H-7100, Hitachi Ltd., Tokyo, Japan). Glomerular basement membrane thickness was assessed in 22–25 fields in the glomeruli, which were randomly selected from each mouse, and was measured by using ImageJ processing software version 1.50i [21].

**Isolation of glomeruli and culture of primary podocytes**

Glomeruli of Tsc2<sup>Δpodocyte</sup> and control mice were isolated by magnetic bead isolation [22]. Isolated glomeruli were cultured on type I collagen-coated multiwell plate dishes (AGC Techno Glass Co. Ltd., Shizuoka, Japan) in RPMI 1640 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% fetal bovine serum (GE Healthcare, Chicago, IL) supplemented with 100 U ml<sup>−1</sup> penicillin and 100 μg ml<sup>−1</sup> streptomycin (Thermo Fisher Scientific, Inc., Waltham, MA) in a 37˚C humidified incubator with 5% CO<sub>2</sub>. Explant primary podocytes were used for subsequent analyses. The podocytes isolated from these mice were stained with WT1, a podocyte marker, and the ratio of the number of WT1-positive cells to the number of the explant cells was 96.3 ± 2.1%. For an LC3B assay, the cultured podocytes were treated with or without 10 μM chloroquine for 24 h before analysis.

**RNA extraction and quantitative real-time PCR**

Total RNA was isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany), and cDNA was synthesized using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Fast SYBR Green and TaqMan Fast Advantage (Thermo Fisher Scientific) were used for real-time PCR analysis and the expression levels of each mRNA were quantified using the standard curve method and normalized relative to the levels of expression of β-actin or GAPDH mRNA in the same sample.

**Microarray analysis**

Total RNA isolated from the podocytes of Tsc2<sup>Δpodocyte</sup> and Tsc2<sup>flcx/flcx</sup> mice was subjected to microarray analysis. RNA quality and integrity were determined using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany). All samples were analysed with Agilent SurePrint G3 Mouse GE 8x60K microarray (Agilent Technologies). Sample labelling, microarray hybridization and washing were performed according
to the manufacturer’s instructions using the One-Color Microarray-Based Gene Expression Analysis Protocol. Data extraction was performed using Feature Extraction Software, and the Feature Extraction Software-derived output data files were further analysed using GeneSpring software (version 14.8, Agilent Technologies). Differentially expressed mRNAs were selected on the basis of a fold-change $\geq 1.5$ at $P < 0.05$ between the \(Tsc2^{\text{podocyte}}\) mice and control samples by the Benjamini-Hochberg procedure. To facilitate gene microarray data analysis, Ingenuity Pathway Analysis software (Qiagen, Redwood City, CA) was used for in silico genomics network analysis to search for possible biological processes, pathways and networks.

**Immunoblot analysis**

Cultured podocytes were lysed in RIPA buffer containing phosphatase inhibitor (Nacalai Tesque, Kyoto, Japan). The samples were resolved by 7.5% or 10–20% SDS-PAGE and transferred to Immobilon-P Transfer Membranes (Merck Millipore, Billerica, MA). The membranes were incubated with the antibodies indicated below and washed and incubated with secondary antibodies. Blots were visualized using an enhanced chemiluminescence system (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare). Images were captured with a ChemiDoc XRS+ system and processed using ImageLab software (Bio-Rad Laboratories, Hercules, CA). Antibodies used in this study were as follows: rabbit anti-tuberin (1:1000, sc-893, Santa Cruz Biotechnology), anti-WT1 (1:500, sc-192, Santa Cruz Biotechnology), anti-p62 (1:5000, P0067, Sigma-Aldrich), anti-LC3B (1:5000, L7543, Sigma-Aldrich), anti-phosphorylated unc-51-like kinase 1 (ULK1) (Ser757) (1:1000, #6888, Cell Signaling Technology, Beverly, MA), anti-phosphorylated p70S6K (Thr389) (1:1000, #9205, Cell Signaling Technology), anti-phosphorylated eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) (Ser65) (1:1000, #9451, Cell Signaling Technology) polyclonal antibodies and mouse anti-β-tubulin monoclonal antibody (1:1000, 05–661, Upstate Biotechnology, Lake Placid, NY).

**Quantitative analysis of autophagic activity in vivo**

GFP-LC3 transgenic mice provided by N. Mizushima (The University of Tokyo, Tokyo, Japan) were used to analyse autophagic activity in vivo [23]. \(Tsc2^{\text{podocyte}}\) and control mice were subsequently crossed with GFP-LC3 transgenic mice. The numbers of GFP-LC3 puncta in podocytes were observed with an LSM880 confocal microscope (Zeiss, Oberkochen, Germany), counted in 20 glomeruli randomly selected from each mouse (\(n = 3\)/genotype) and quantified.

**Human kidney biopsy specimens**

Clinically indicated renal biopsies were performed in obese patients (body mass index $> 25$ kg m$^{-2}$) with CKD at Chiba-Higashi National Hospital. Three human kidney biopsy specimens from patients diagnosed with FSGS perihilar variant were stained with rabbit anti-phospho-S6 ribosomal protein (pS6) monoclonal antibody (Ser235/236, 1:400, #4858, Cell Signaling Technology) and counterstained with haematoxylin. pS6-positive glomeruli were counted, and the percentage of pS6-positive glomeruli in 10–26 glomeruli was calculated for each patient. Human kidney biopsy specimens obtained from three patients with abnormal results on urinalysis but with no glomerular abnormality on kidney biopsy were used as normal controls.

**Statistics**

Kaplan-Meier analysis was conducted using IBM SPSS software version 20. Data are expressed as means $\pm$ s.d. for normally distributed variables and median (interquartile range) for non-
normally distributed variables. Differences between the two groups for normally distributed variables were tested using Student’s two-sided t-test, and nonparametric data were analysed using the Mann–Whitney U-test. Differences among more than three groups were analysed using parametric (one-way analysis of variance) or nonparametric (Kruskal–Wallis test) statistical methods. All calculations were performed with Microsoft Excel 2016 or IBM SPSS software version 20. P < 0.05 was considered significant.

**Study approval**

All animal protocols and experiments were approved by the Institutional Animal Care and Use Committee of National Center for Global Heal and Medicine (no. 18068). Human renal biopsies were performed at Chiba-Higashi National Hospital after written informed consent was received from participants prior to inclusion in the study. The protocol concerning the use of biopsy samples was approved by the ethics committee of Chiba-Higashi National Hospital (no. 27–14).

**Results**

*Tsc2 deletion in podocytes causes death due to renal failure*

We generated *Tsc2*Δ*podocyte* mice by crossing homozygous floxed *Tsc2* mice (*Tsc2*fl/fl) with *Cre*-recombinase transgenic mice that had *Cre* gene under the control of a murine *Podocin* (*Nphs2*) promoter (*Nphs2-Cre*). *Tsc2*Δ*podocyte* mice were born at the expected Mendelian ratio and divided into three types according to genotype: *Nphs2-Cre*, *Tsc2*fl/fl and *Tsc2*Δ*podocyte* (Fig 1A). To verify the depletion of *Tsc2* in podocytes, we examined mRNA from primary cultured podocytes. The *Tsc2* mRNA level showed an 80% ± 0.42% reduction in *Tsc2*Δ*podocyte* mice compared with control mice (Fig 1B), and TSC2 protein was barely detected in *Tsc2*Δ*podocyte* mice. (Fig 1C). We also examined the tissue distribution of *Tsc2* mRNA, including the renal cortex; however, *Tsc2* mRNA levels did not differ in the tissues examined between *Tsc2*Δ*podocyte* mice and control mice (Fig 1D).

*Tsc2*Δ*podocyte* mice were normoglycemic and nonobese, and initially appeared normal. However, Kaplan-Meier analysis indicated that *Tsc2*Δ*podocyte* mice had significantly shorter survival (*P* < 0.01) than control mice (Fig 2A). A dramatic loss of animals was detected in *Tsc2*Δ*podocyte* mice after 4 weeks of age, and all of the *Tsc2*Δ*podocyte* mice examined died by 10 weeks of age. SDS-PAGE analysis revealed that *Tsc2*Δ*podocyte* mice started to develop albuminuria at approximately 3 weeks of age (Fig 2B). The urinary ACR also remained significantly higher in *Tsc2*Δ*podocyte* mice than in control mice up to 7 weeks of age (Fig 2C). The levels of serum albumin and total protein in *Tsc2*Δ*podocyte* mice began to decrease at 5 weeks of age. On the other hand, the levels of BUN and Scr began to increase in *Tsc2*Δ*podocyte* mice (S2 Table). We found increased levels of K, TC, triglyceride and HDL-C in *Tsc2*Δ*podocyte* mice at 7 weeks of age (S2 Table), and also observed massive ascites in *Tsc2*Δ*podocyte* mice. There were few sex differences in the biochemical parameters examined (S2 Table). These findings indicate that specific deletion of *Tsc2* in podocytes led to death from renal dysfunction.

We further generated *Tsc2*Δ*podocyte* mice of the C57BL/6 strain and found that these mice showed kidney dysfunction such as proteinuria and hypoalbuminemia and increased levels of TC and HDL-C (S1A–S1D Fig) at 24 weeks of age. However, the levels of BUN and Scr in *Tsc2*Δ*podocyte* mice were comparable to those of the control mice (BUN in *Tsc2*fl/fl: 43.2 ± 8.8 mg/dL, BUN in *Tsc2*Δ*podocyte*: 47 ± 9.6 mg/dL; Scr in *Tsc2*fl/fl: 0.32 ± 0.14 mg/dL, Scr in *Tsc2*Δ*podocyte*: 0.14 ± 0.06 mg/dL). Kaplan-Meier analysis also revealed that these mice showed the same trend as the ICR strain (S1E Fig).
Tsc2Δpodocyte showed an increased glomerular size and FSGS

The kidney weight of Tsc2Δpodocyte mice was comparable with that of control mice at 4 weeks of age (Tsc2Δpodocyte: 0.38 ± 0.08 g, n = 16; Tsc2flox/flox: 0.33 ± 0.12 g, n = 30; P = 0.87), whereas significant differences were observed at 8 weeks of age (Tsc2Δpodocyte: 0.44 ± 0.10 g, n = 5; Tsc2flox/flox: 0.64 ± 0.08 g, n = 9; P < 0.01). Morphologically, podocytes of Tsc2Δpodocyte mice underwent hypertrophy, and the podocyte foot process was lost at 4 weeks of age (Fig 3A and 3B). However, the thickness of the glomerular basement membrane was almost comparable in Tsc2Δpodocyte mice and controls at 4 weeks of age (Tsc2Δpodocyte: 0.17 ± 0.05 μm; Tsc2flox/flox):

https://doi.org/10.1371/journal.pone.0229397.g001
Fig 2. mTORC1 activation in podocytes causes proteinuria and increased mortality. (A) Kaplan-Meier survival plots for Tsc2Δpodocyte and control mice. A significant increase in mortality was found in Tsc2Δpodocyte compared with control mice. Nphs2-Cre (black line), n = 9; Tsc2flax/flax (blue line), n = 10; Tsc2Δpodocyte (red line), n = 32. (B) At 3 weeks, Tsc2Δpodocyte became albuminuric. SDS-PAGE gel shows a microliter of urine was loaded for each lane. (C) Seven-week follow-up of Tsc2Δpodocyte for proteinuria (n = 10 per group). Results are expressed as means ± s.d. White bar, Nphs2-Cre; dotted bar, Tsc2flax/flax; black bar, Tsc2Δpodocyte. Results are expressed as means ± s.d. *P < 0.05 compared with age-matched controls.

https://doi.org/10.1371/journal.pone.0229397.g002
Fig 3. mTORC1 activation in podocytes resulted in progressive glomerulosclerosis. (A) Tsc2\textsuperscript{Δpodocyte} developed progressive glomerulosclerosis between 4 and 8 weeks of age. Renal tissues from Tsc2\textsuperscript{Δpodocyte} and control mice at 4, 6, and 8 weeks of age were stained with periodic acid-Schiff (PAS). Representative glomeruli from Tsc2\textsuperscript{Δpodocyte} and control mice are shown. Various degrees of glomerulosclerosis, partial glomerulosclerosis and protein casts in tubules (asterisks), and glomerulosclerosis with synchia formation (arrowhead) are shown. Scale bar: 50 μm. (B) Transmission electron microscopy (TEM) shows partial flattening and disorganization of podocyte foot processes (arrows). Scale bar: 5 μm. (B, bottom) Glomerular basement membrane thickness in Tsc2\textsuperscript{Δpodocyte} at 4 weeks of age was comparable with that in age-matched control mice (within arrowheads). Scale bar: 1 μm.

https://doi.org/10.1371/journal.pone.0229397.g003
0.17 ± 0.04 μm; \( P = 0.98 \)). The glomerulosclerosis index was higher in \( Tsc2^{Δpodocyte} \) mice than in control mice at 4 weeks of age and increased with age (S2A Fig). At 6 weeks of age, \( Tsc2^{Δpodocyte} \) mice showed an increased glomerular size, FSGS and proteinaceous casts (Fig 3A and S2B Fig). Interestingly, crescent formation was found in some glomeruli of \( Tsc2^{Δpodocyte} \) mice at 8 weeks of age; most of the crescents were fibrotic (Fig 3A and S2C Fig).

We next examined podocyte distribution in glomeruli using the podocyte markers podocin and synaptopodin. At 4 weeks of age, there were no obvious differences in the expression patterns of podocin and synaptopodin in the glomeruli of \( Tsc2^{Δpodocyte} \) and control mice. However, their expressions were lost in some glomeruli of \( Tsc2^{Δpodocyte} \) mice at 6 weeks of age (Fig 4A). We then determined the average number of podocytes per glomerulus by counting WT1-positive podocytes in \( Tsc2^{Δpodocyte} \) mice from 4 to 8 weeks of age. The number of podocytes in \( Tsc2^{Δpodocyte} \) mice at 6 to 8 weeks of age, but not at 4 weeks of age, was significantly decreased compared with control mice (Fig 4B). We further assessed the correlation between the number of WT1-positive podocytes and biochemical parameters and found that the number of WT1-positive podocytes in \( Tsc2^{Δpodocyte} \) mice was negatively correlated with the urinary ACR, BUN, TC and HDL-C and positively associated with serum albumin (S3 Fig). Furthermore, WT1-positive podocytes began to be excreted in the urine of \( Tsc2^{Δpodocyte} \) mice at 5 weeks of age (Fig 4C).

**Tsc2 deficiency reduces autophagic activity in podocytes**

To explore the molecular mechanism of podocyte dysfunction in \( Tsc2^{Δpodocyte} \) mice, we conducted microarray analysis using the total RNA of primary podocytes isolated from \( Tsc2^{Δpodocyte} \) and control \( Tsc2^{flox/flox} \) mice. We found that 858 genes were differentially expressed between these groups (fold-change difference \( ≥ 1.5 \), \( P < 0.05 \), S4A Fig). IPA analysis of the differentially expressed genes showed significant enrichment for pathways involved in glycolysis I, gluconeogenesis I, NRF2 (nuclear factor, erythroid derived 2, like 2)-mediated oxidative stress response, glutathione-mediated detoxification, SPINK1 general cancer pathway, and MIF regulation of innate immunity (S4B Fig). In addition, the network analysis in IPA mapped the significant genes to network in mTOR signalling activating pathway, in which an inhibition of autophagy regulation is predicted (S4C Fig). Taken together, we hypothesized that \( Nrf2 \) may be activated in the podocytes of \( Tsc2^{Δpodocyte} \) mice. \( Nrf2 \) is a transcription factor that translocates to the nucleus in response to oxidative stress to activate the transcription of various detoxifying enzymes [24]. Moreover, the \( Nrf2/Keap1 \) ubiquitination and degradation system is associated with the phosphorylation of p62, which is an autophagy-related molecule that is also modulated by mTORC1 activity [25]. Accordingly, we speculate that mTORC1 inhibits autophagic degradation and increase the intracellular level of p62, leading to noncanonical activation of \( Nrf2 \) in the podocytes of \( Tsc2^{Δpodocyte} \) mice. The level of p62 was substantially increased in the podocytes of \( Tsc2^{Δpodocyte} \) mice compared with control mice (Fig 5A). Decreased formation of LC3 type II, an autophagy-related protein, was also observed in the podocytes of \( Tsc2^{Δpodocyte} \) mice, concomitant with the increased phosphorylation of ULK1 at Ser757 and 4E-BP1 at Ser65 (Fig 5A). In addition, \( FIP200 \) and \( ATG101 \) genes, which are involved in the initiation of autophagy, were significantly decreased in the podocytes of \( Tsc2^{Δpodocyte} \) mice compared with control mice (Fig 5B). Finally, we crossed \( Tsc2^{Δpodocyte} \) mice with GFP-LC3 transgenic mice (GFP-LC3 Tg) to evaluate autophagic activity in vivo. The number of GFP-LC3 puncta was significantly decreased in \( Tsc2^{Δpodocyte} \) mice at 4 weeks of age compared with age-matched control mice (Fig 5C).
Fig 4. Podocytes in Tsc2Δpodocyte mice were excreted in urine from glomeruli with progression of renal functional decline. (A) Immunofluorescence staining of podocin and synaptopodin showed decreased signal intensity in Tsc2Δpodocyte mice (arrows). (B, top) The number of podocytes was decreased in the glomeruli of Tsc2Δpodocyte mice. Representative images of WT1 positive (red) and Hoechst 33342-positive (blue) podocytes are shown. (B, bottom) The graph shows the average number of podocytes per glomerulus in each group. Each dot represents the mean number of WT1-positive cells in about 20 glomeruli from the indicated mouse, and horizontal lines represent the mean number of
Rapamycin treatment extends survival in \( Tsc2^{Δpodocyte} \) mice

Next, we evaluated the effects of rapamycin in \( Tsc2^{Δpodocyte} \) mice. Rapamycin, an inhibitor of mTORC1, was administered via intraperitoneal injection. Rapamycin impaired proteinuria and extended the survival of \( Tsc2^{Δpodocyte} \) mice, although \( Tsc2^{Δpodocyte} \) mice exhibited renal dysfunction and died by 10 weeks after birth without rapamycin treatment, as revealed above (Fig 6A and 6B). Albuminuria vanished 1 week after rapamycin treatment (Fig 6C). \( Tsc2^{Δpodocyte} \) mice exhibited significantly higher levels of BUN and SCr. However, rapamycin treatment decreased these levels to those comparable to the control (S3 Table). Morphologically, rapamycin treatment restored podocyte hypertrophy, foot process effacement, FSGS and proteinaceous casts (Fig 6D). We next conducted microarray analysis using the total RNA of primary podocytes isolated from rapamycin-treated \( Tsc2^{Δpodocyte} \) mice. We found expression of 858 genes were significantly differed in \( Tsc2^{Δpodocyte} \) mice compared with control, and also found that expression of 810 genes out of the 858 genes was normalized in rapamycin-treated \( Tsc2^{Δpodocyte} \) mice (S5A Fig). Furthermore, the levels of intracellular p62 and LC3 type II in podocytes isolated from rapamycin-treated \( Tsc2^{Δpodocyte} \) mice were comparable to those from control mice (S5B Fig). There were no apparent differences in the number of GFP-LC3 puncta between these groups, implying that \( in \; vivo \) autophagic activity was also restored in the podocytes of rapamycin-treated \( Tsc2^{Δpodocyte} \) mice (Fig 6D and S5C Fig).

mTORC1 is activated in podocytes in patients with CKD

To explore whether mTORC1 activity is associated with obesity-related renal functional decline, we examined mTORC1 activation in vivo. First, we examined mTORC1 activation in kidney of \( db/db \) mice, used as an obese model of genetic diabetes, at 24 weeks of age. The \( db/db \) mice showed a significantly higher urinary ACR compared with age-matched \( db/m \) mice (\( db/db \): 574 ± 175 mg g \(^{-1}\) creatinine; \( db/m \): 22 ± 3 mg g \(^{-1}\) creatinine; \( n = 5 \) group; \( P < 0.01 \)) and featured glomerulosclerosis at 24 weeks of age (Fig 7A). We found that phosphorylation of p70 S6 kinase, a direct phosphorylation target of mTORC1, was enhanced in primary cultured podocytes isolated from \( db/db \) mice (S5A Fig). Furthermore, the levels of intracellular p62 and LC3 type II in podocytes isolated from rapamycin-treated \( Tsc2^{Δpodocyte} \) mice were comparable to those from control mice (S5B Fig). There were no apparent differences in the number of GFP-LC3 puncta between these groups, implying that \( in \; vivo \) autophagic activity was also restored in the podocytes of rapamycin-treated \( Tsc2^{Δpodocyte} \) mice (Fig 6D and S5C Fig).

Discussion

This study revealed that podocyte-specific deletion of \( Tsc2 \) contributes to severe podocyte injury, leading to massive proteinuria, end-stage renal dysfunction and increased mortality. \( Tsc2^{Δpodocyte} \) mice were normoglycemic and nonobese but showed an increased glomerular size, glomerulosclerosis, proteinaceous casts, crescent formation and increased tubulointerstitial fibrotic lesions, with a pattern that was similar to that of FSGS in humans. Recent work reported that podocyte-specific \( Tsc1 \) knockout mice, which lack the TSC1-TSC2 heterodimer
Fig 5. mTORC1 hyperactivation led to a decreased autophagic activity in the podocytes. (A) Primary cultured podocytes were isolated from Tsc2<sup>Δpodocyte</sup> and wild-type controls, followed by western blot analyses of TSC2, phospho-4EBP1, LC3B type II, p62, phospho-ULK1, and β-tubulin. Arrows indicate specific bands corresponding to each indicated protein. (B) FIP200 and ATG101 mRNA expressions were decreased in the primary cultured podocytes from Tsc2<sup>Δpodocyte</sup>. (C) Representative fluorescence images of glomeruli of Nphs2-Cre<sup>−</sup> (top, left), Tsc2<sup>flox/flox</sup>- (top, right) and Tsc2<sup>Δpodocyte</sup> GFP-LC3 transgenic mice (bottom, left) at 4 weeks of age. The white box indicates the location of the magnified figure. Scale bar: 50 μm. (bottom, right) Quantitative analysis of autophagic activity in vivo. Graph bars show the number of GFP-LC3 dots per glomerulus from the indicated mice (n = 3 per group). The number of GFP-LC3 dots per glomerulus was counted in 20 independent visual fields from the indicated mice. Results are expressed as means ± s.d. *P < 0.05 compared with age-matched controls.
complex, exhibit structural abnormalities such as FSGS with occasional crescent formation and podocyte vacuolation [26]. Additionally, podocyte-specific Tsc1 knockout mice show features of diabetic nephropathy and mTORC1 hyperactivation is present in podocytes of patients with diabetic nephropathy [27, 28]. In addition to the above findings, we found an increase in the number of mitochondria in the podocytes of Tsc2 Δ podocyte mice. Similar findings were reported in cardiac-specific Tsc2-deficient mice, which showed structural abnormalities of mitochondria, although the mitochondrial function was maintained [29].

To explore the underlying mechanisms of the renal functional decline in Tsc2 Δ podocyte mice, we performed microarray analysis, finding that Tsc2 deletion in podocytes may modulate the Nrf2-mediated oxidative stress response pathway (S4B Fig). We also revealed an increased abundance of p62 and a decreased abundance of LC3B type II in the podocytes of Tsc2 Δ podocyte mice by suppressing autophagic activity through the mTOR-ULK1 pathway (Fig 5). We further revealed that inhibition of mTORC1 activity in the podocytes of Tsc2 Δ podocyte mice by rapamycin injection attenuated the podocyte dysfunction, including the impaired autophagic activity and structural abnormalities, preventing the massive proteinuria, end-stage renal dysfunction and increased mortality seen in controls. Considering these findings, we conclude that mTORC1 hyperactivation in podocytes could impair the autophagy and cause cytoplasmic accumulation of p62, leading to Nrf2 activation via dissociation of the Nrf2/Keap1 complex [25]. Autophagy is a conserved mechanism of intracellular degradation that maintains homeostasis and cell integrity and its dysregulation has been suggested to cause a variety of disease processes [30]. Podocytes exhibit an unusually high level of constitutive autophagy, and a recent report showed that podocyte-specific deletion of the Atg5 gene which is known as one of the autophagy conjugation systems led to podocyte injury such as proteinuria, foot process effacement, vacuolation and progressive development of glomerulosclerosis, which are similar to the structural abnormalities observed in Tsc2 Δ podocyte mice [31, 32]. However, podocyte-specific Atg5-deficient mice did not exhibit end-stage renal dysfunction and increased mortality, which is inconsistent with the characteristics of Tsc2 Δ podocyte mice. Zhou et al. [33] reported that mTORC1 exerts a dual inhibitory effect on autophagy, blocking autophagy not only at the initiation stage via suppression of the ULK1 complex, but also at the degradation stage via inhibition of lysosomal function. One possible explanation for the severe characteristics of Tsc2 Δ podocyte mice may be a dual suppressive effect of mTORC1 on autophagy, leading to severe podocytopathy. However, functional investigations are required.

Obesity leads to CKD. Moreover, obese patients show proteinuria and some patients have nephrotic-range proteinuria and progressive loss of renal function [34]. The pathologic features of obese patients with CKD include glomerulomegaly and FSGS [35, 36], and these features were observed in the renal biopsy specimens analysed in the present study (Fig 7C). To further investigate the involvement of mTORC1 in obesity-related kidney dysfunction, we observed mTORC1 activity in renal biopsy specimens from obese patients with CKD. As shown in Fig 7C, obese patients with FSGS exhibited an increase in mTORC1 activity in podocytes and the parietal cells of Bowman’s capsule, in contrast to nonobese patients. An increased mTORC1 activity has been reported in cellular crescents from patients with crescentic glomerular diseases [26]. An increased mTORC1 activity in podocytes and the parietal cells of Bowman’s capsule may be related to crescent and scar formation in CKD, but the underlying mechanisms remain to be resolved. We also found increased mTORC1 activity in tubulointerstitial regions of obese patients with CKD (Fig 7C). Recently, van der Heijden et al. [37] reported that high-fat diet-challenged mice exhibited upregulation of pro-inflammatory genes and infiltrating macrophages in the tubulointerstitium. High-fat diet-induced obesity may cause the infiltration of macrophages into tubulointerstitial regions accompanied by the activation of mTORC1,
Fig 6. Rapamycin prevented death from renal dysfunction in Tsc2Δpodocyte. (A) Tsc2Δpodocyte mice treated with rapamycin had improved survival compared to vehicle-treated Tsc2Δpodocyte mice. From 4 to 11 weeks of age, rapamycin was injected intraperitoneally (2 mg/kg body weight) twice a day (bold vertical line). Nphs2-Cre with rapamycin treatment (black line), n = 17; Tsc2Δpodocyte with rapamycin treatment (blue line), n = 16; Tsc2Δpodocyte with rapamycin treatment (red line), n = 8; vehicle-treated Nphs2-Cre (dashed black line), n = 7; vehicle-treated Tsc2Δpodocyte (dashed blue line), n = 9; vehicle-treated Tsc2Δpodocyte (dashed red line), n = 26. (B) Eight-week follow-up of rapamycin-treated Tsc2Δpodocyte for proteinuria (n = 6 per group).
leading to chronic low-grade inflammation and renal functional decline. Nonetheless, further experimental investigations are required.

The major limitation of the current study is the lack of information on the pathogenesis of mTORC1 activation in podocytes from obese patients with CKD. mTORC1 is an important factor in protein synthesis that is activated by amino acids. Recent reports showed that increased levels of branched-chain amino acids (BCAA) were associated with T2DM and obesity [38]. Furthermore, Giesbertz et al. [39] reported increased levels of BCAA and α-ketoisocaproic acid, the transamination product of leucine, in plasma of db/db mice and that adipose tissues contribute most to the changes in plasma BCAA. Obese mice show a decreased protein level and activity of the mitochondrial BCAA transferase and the rate-limiting branched-chain keto acid dehydrogenase complex [40]. Therefore, disturbed expression of genes related to the metabolism of amino acids in adipose tissue may significantly contribute to the metabolism of BCAA, leading to the activation of mTORC1 in podocytes. Disturbed expression of cytokines and growth factors could be another causative factor for obesity-related kidney dysfunction. Inflammatory cytokines are modulated in the glomeruli of obesity-related glomerulopathy [41]. Lee et al. [42] also reported that IκB kinase β, a downstream kinase in the tumor necrosis factor α-signalling pathway, phosphorylates TSC1, resulting in the activation of mTORC1. However, it is uncertain whether the levels of tumor necrosis factor α or other cytokines were increased in the obese patients with CKD examined in this study because of the sample limitations. In addition, it is difficult to dissect out the individual contributions of obesity and T2DM to renal functional decline. Indeed, mTORC1 target genes and mTOR mRNA itself were reported to be induced in glomeruli from patients with diabetic nephropathy [28]. We further analysed the levels of Tsc1 and Tsc2 mRNA in diabetic nephropathy using the Nephroseq database (https://www.nephroseq.org) and found that Tsc2 mRNA was also significantly decreased in both glomeruli (Glom) and the tubulointerstitial (TubInt) from patients with diabetic nephropathy (Glom in healthy living donor: 0.92 ± 0.31; Glom in diabetic nephropathy: 0.71 ± 0.46 [P = 0.002]; TubInt in healthy living donor: 0.13 ± 0.24; TubInt in diabetic nephropathy: 0.02 ± 0.27 [P = 0.02]). However, in this study, we revealed Tsc2Δpodocyte mice were normoglycemic and nonobese but showed a similar histological pattern of FSGS in obese patients with CKD, which has not been reported in the analyses of podocyte-specific Tsc1 knockout mice [26, 27]. Moreover, we have also found that mTORC1 is activated in podocytes of nondiabetic obese patients with CKD, so an evaluation of the involvement of the Tsc2 gene in nondiabetic obese patients with CKD might provide valuable clues for understanding the pathogenesis of obesity-related renal diseases.

In conclusion, mTORC1 hyperactivation in podocytes leads to severe renal dysfunction caused by the induction of oxidative stress and impairment of autophagic activity in podocytes. mTORC1 may play important roles in maintaining podocyte functions, and inhibition of mTORC1 activity in podocytes could be a key therapy for obesity-related kidney dysfunction.
Fig 7. mTORC1 is activated in podocytes in patients with CKD. (A) Representative photomicrographs of periodic acid-Schiff (PAS) staining in the kidney of db/db and db/m mice. Db/db mice exhibited glomerulosclerosis at 24 weeks of age. Scale bar: 50 μm. (B) Western blot analysis of p70 S6 kinase (p70S6K) phosphorylation using primary cultured podocytes isolated from db/db and db/m mice at 24 weeks of age. β-tubulin served as the internal control. (C) mTORC1 is activated in glomeruli of obese patients with CKD. Human kidney biopsy specimens from normal controls and obese patients with CKD were immunostained with anti-phospho-S6 ribosomal protein (pS6) antibody and counterstained with hematoxylin. pS6 protein was detected in podocytes (arrowheads), parietal cells lining Bowman’s capsule (arrow), and tubulointerstitial regions (asterisk) of obese patients with CKD. Representative low-magnification (top) and high-magnification (bottom) images are shown.

https://doi.org/10.1371/journal.pone.0229397.g007
Supporting information

S1 Fig. Analysis of podocyte-specific Tsc2 knockout mice (Tsc2\textsuperscript{Δpodocyte}) in C57BL/6 strain. (A-D) Twenty-four-week follow-up of Tsc2\textsuperscript{Δpodocyte} (solid line) and homozygous floxed Tsc2 mice (Tsc2\textsuperscript{flx/flx}, dotted line) for urine albumin-to-creatinine ratio (A), serum albumin (B), total cholesterol (C) and HDL-cholesterol (D) (n = 3–5/group). ACR, albumin-to-creatinine ratio; ALB, albumin; TC, total cholesterol; HDL-c, HDL-cholesterol; dotted bars, Tsc2\textsuperscript{flx/flx}; black bars, Tsc2\textsuperscript{Δpodocyte}. (E) Kaplan-Meier survival plots for Tsc2\textsuperscript{Δpodocyte} (solid line) and control Tsc2\textsuperscript{flx/flx} mice (dashed line). The results were expressed as mean ± SD. *P < 0.05 to the age-matched control. (PDF)

S2 Fig. Tsc2\textsuperscript{Δpodocyte} showed an increased glomerular size and focal segmental glomerulosclerosis. (A) Morphometric analysis using PAS-stained renal tissues. Fifty glomeruli were randomly selected from the indicated mice (n = 3/group) and their glomerulosclerosis indices assessed. The bar graph shows the glomerulosclerosis index in Nphs2-Cre mice (white bars), Tsc2\textsuperscript{flx/flx} mice (dotted bars) and Tsc2\textsuperscript{Δpodocyte} mice (black bars). The results are expressed as the mean ± s.d. *P < 0.05 versus the age-matched control. (B) Tsc2\textsuperscript{Δpodocyte} mice show increased glomerular size. Renal sections from Tsc2\textsuperscript{Δpodocyte} and control mice at 6 weeks of age were stained with periodic acid-Schiff. Twenty glomeruli were randomly selected in each mouse (n = 3/genotype), and glomerular diameters were measured by using ImageJ. The results are expressed as the mean ± s.d. *P < 0.05 versus the age-matched control. (C) Renal sections from Tsc2\textsuperscript{Δpodocyte} and control mice at 8 weeks of age were stained with Masson’s trichrome. Scale bar: 100 μm. (PDF)

S3 Fig. Correlation between biochemical parameters and the numbers of WT1-positive podocytes per glomerulus. The numbers of podocytes were counted in each glomerulus of 3, 5 and 7 weeks of age Tsc2\textsuperscript{Δpodocyte} and control mice. The X-axis shows (A) the urine albumin-to-creatinine ratio (ACR), (B) blood urea nitrogen (BUN), (C) total cholesterol (TC), (D) HDL-cholesterol (HDL-c) and (E) serum albumin (ALB). △, Nphs2-Cre; ○, Tsc2\textsuperscript{flx/flx}; ◯, Tsc2\textsuperscript{Δpodocyte} (n = 6/group). (PDF)

S4 Fig. Microarray analysis of primary podocytes isolated from Tsc2\textsuperscript{Δpodocyte} and control Tsc2\textsuperscript{flx/flx} mice. (A) Volcano plot showing top differentially expressed genes among Tsc2\textsuperscript{Δpodocyte} and Tsc2\textsuperscript{flx/flx} mice. (B) The significantly expressed genes between Tsc2\textsuperscript{Δpodocyte} and Tsc2\textsuperscript{flx/flx} mice were inputted to IPA for pathway enrichment analysis. Of these genes, IPA analysis further identified 625 genes, 388 of which were significantly increased and 237 of which were significantly decreased in Tsc2\textsuperscript{Δpodocyte} mice. The figure shows some of the top pathways identified by IPA (–log[P-value], >1.3; z-score, >2.0; threshold value, 0.05). P-values here are from right-tailed Fisher’s exact test. (C) Network analysis on differentially expressed genes between Tsc2\textsuperscript{Δpodocyte} and Tsc2\textsuperscript{flx/flx} mice mapped to networks involved in the mTOR signaling activating pathway. (PDF)

S5 Fig. Differential expression analysis in podocytes isolated from Tsc2\textsuperscript{Δpodocyte} and rapamycin-treated Tsc2\textsuperscript{Δpodocyte} mice (Rapa-Tsc2\textsuperscript{Δpodocyte}). (A) Expression of 858 genes were significantly different in Tsc2\textsuperscript{Δpodocyte}, and 810 out of 858 genes were normalized in rapamycin-treated Tsc2\textsuperscript{Δpodocyte} mice. Rapamycin-treatment also caused disturbed expression of 76 genes in Rapa-Tsc2\textsuperscript{Δpodocyte} mice, although those levels were similar both in Tsc2\textsuperscript{Δpodocyte} and Tsc2\textsuperscript{flx/flx}.
mTORC1 hyperactivation in podocytes promotes focal segmental glomerulosclerosis

floxed mice. (B) Primary cultured podocytes were isolated from Tsc2APodocyte mice 1 week after rapamycin treatment, followed by western blot analyses of LC3B type II, p62 and phospho-ULK1 (Ser757). The arrow indicates the band corresponding to LC3B type II. β-tubulin served as the internal control. (C) The graph bars show the number of GFP-LC3 puncta in each glomerulus from Tsc2APodocyte-GFP-LC3 transgenic mice. The results are expressed as the mean ± s.d. N.S., not statistically significant.

S1 Raw images. Uncropped original images of gels and blots presented in the figures of this study.

S1 Table. Overview of the number of mice used in survival analyses. There were no mice that were euthanized before reaching the experimental endpoint. The numbers of mice that died without humane intervention and euthanized after reaching the experimental endpoint were also summarized.

S2 Table. Characteristics of Nphs2-Cre, Tsc2floxed/floxed and Tsc2APodocyte mice. Data are expressed as mean ± SD (n = 10). Analysis of variance was used between groups; and multiple testing corrections were performed using the Tukey’s method. ACR, urine albumin to creatinin; BUN, blood urea nitrogen; Cre, creatinine; TP, total protein; ALB, alubumin; TC, total cholesterol; TG, triglyceride; HDL-c, high density lipoprotein-cholesterol. aP < 0.05 vs. Nphs2-Cre, bP < 0.05 vs. Tsc2APodocyte.

S3 Table. Characteristics of rapamycin-treated Nphs2-Cre, Tsc2floxed/floxed and Tsc2APodocyte mice. Data are expressed as mean ± SD (n = 5). Analysis of variance was used between groups; and multiple testing corrections were performed using the Tukey’s method. There were no significant differences in the biochemical parameters among rapamycin-treated Nphs2-Cre, Tsc2floxed/floxed and Tsc2APodocyte mice. Abbreviations are as in S2 Table.

S4 Table. Clinical characteristics of normal control subjects and patients diagnosed with FSGS perihilar variant.

Acknowledgments
We thank Nami Hosaka, Eri Takahashi (Department of Diabetic Complications, Diabetes Research Center, Research Institute, NCGM), Takao Naganuma (Department of Molecular Metabolic Regulation, Diabetes Research Center, Research Institute, NCGM) and Kazue Takeda (Department of Allergy and Clinical Immunology, NCCHD) for their technical help.

Author Contributions
Conceptualization: Hiroyuki Unoki-Kubota, Yasuo Terauchi, Masaomi Nangaku, Masato Kasuga, Yasushi Kaburagi.

Data curation: Wakiko Iwata, Hiroyuki Unoki-Kubota.
Formal analysis: Wakiko Iwata, Hiroyuki Unoki-Kubota, Arisa Igarashi.

Funding acquisition: Hiroyuki Unoki-Kubota, Yasushi Kaburagi.

Investigation: Wakiko Iwata, Hiroyuki Unoki-Kubota, Hideki Kato, Akira Shimizu, Toshiyuki Imasawa.

Methodology: Wakiko Iwata, Hiroyuki Unoki-Kubota, Hideki Kato, Michihiro Matsumoto, Tetsuo Noda, Masaomi Nangaku.

Project administration: Wakiko Iwata, Hiroyuki Unoki-Kubota, Masato Kasuga, Yasushi Kaburagi.

Resources: Hiroyuki Unoki-Kubota, Michihiro Matsumoto, Tetsuo Noda, Masato Kasuga, Yasushi Kaburagi.

Supervision: Masato Kasuga.

Visualization: Wakiko Iwata, Hiroyuki Unoki-Kubota, Hideki Kato, Akira Shimizu, Toshiyuki Imasawa, Arisa Igarashi.

Writing – original draft: Wakiko Iwata, Hiroyuki Unoki-Kubota, Hideki Kato, Toshiyuki Imasawa, Kenji Matsumoto.

Writing – review & editing: Hiroyuki Unoki-Kubota, Yasuo Terauchi, Masaomi Nangaku, Masato Kasuga, Yasushi Kaburagi.

References

1. Finucane MM, Stevens GA, Cowan MJ, Danaei G, Lin JK, Paciorek CJ, et al. National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9.1 million participants. Lancet. 2011; 377(9765):557–567. https://doi.org/10.1016/S0140-6736(10)62037-5 PMID: 21295846

2. Malik VS, Willett WC, Hu FB. Global obesity: trends, risk factors and policy implications. Nat Rev Endocrinol. 2012; 9(1):13–27. https://doi.org/10.1038/nrendo.2012.199 PMID: 23165161

3. Wang Y, Chen X, Song Y, Caballero B, Cheskin LJ. Association between obesity and kidney disease: a systematic review and meta-analysis. Kidney Int. 2008; 73(1):19–33. https://doi.org/10.1038/sj.ki.5002586 PMID: 17928825

4. Eckardt KU, Coresh J, Devuyst O, Johnson RJ, Köttgen A, Levey AS, et al. Evolving importance of kidney disease: from subspecialty to global health burden. Lancet. 2013.; 382(9887):158–169. https://doi.org/10.1016/S0140-6736(13)60439-0 PMID: 23727165

5. Maric-Bilkant C. Obesity and diabetic kidney disease. Med Clin North Am. 2013; 97(1):59–74. https://doi.org/10.1016/j.mcna.2012.10.010 PMID: 23290730

6. Cornu M, Albert V, Hall MN. mTOR in aging, metabolism, and cancer. Curr Opin Genet Dev. 2013; 23(1):53–62. https://doi.org/10.1016/j.gde.2012.12.005 PMID: 23317516

7. Huang J, Dibble CC, Matsuzaki M, Manning BD. The TSC1-TSC2 complex is required for proper activation of mTOR complex 2. Mol Cell Biol. 2008; 28(12):4104–4115. https://doi.org/10.1128/MCB.00289-08 PMID: 18411301

8. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. Cell. 2012; 13; 149(2):274–293. https://doi.org/10.1016/j.cell.2012.03.017 PMID: 22500797

9. Khamzina L, Veilleux A, Bergeron S, Marette A. Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: possible involvement in obesity-linked insulin resistance. Endocrinology. 2005; 146(3):1473–1481. https://doi.org/10.1210/en.2004-0921 PMID: 15604215

10. Catalán V, Gómez-Ambrosi J, Rodríguez A, Ramírez B, Andrade P, Rotellar F, et al. Expression of S6K1 in human visceral adipose tissue is upregulated in obesity and related to insulin resistance and inflammation. Acta Diabetol. 2015; 52(2):257–266. https://doi.org/10.1007/s00392-014-0632-9 PMID: 25118997

11. Morris BJ, Carnes BA, Chen R, Donlon TA, He Q, Grove JS, et al. Genetic variation in the raptor gene is associated with overweight but not hypertension in American men of Japanese ancestry. Am J Hypertens. 2015; 28(4):508–517. https://doi.org/10.1093/ajh/hpu188 PMID: 25249372
12. Lee PL, Tang Y, Li Huawei, Guertin DA. Raptor/mTORC1 loss in adipocytes causes progressive lipodystrophy and fatty liver disease. Mol Metab. 2016; 11; 5(6):422–432. https://doi.org/10.1016/j.molmet.2016.04.001 PMID: 27257602

13. Liu M, Bai J, He S, Villarreal R, Hu D, Zhang C, et al. Grb10 promotes lipolysis and thermogenesis by phosphorylation-dependent feedback inhibition of mTORC1. Cell Metab. 2014; 3; 19(6):967–980. https://doi.org/10.1016/j.cmet.2014.03.018 PMID: 24746805

14. Xiang X, Lan H, Tang H, Yuan F, Xu Y, Zhao J, et al. Tubercous sclerosis complex 1-mechanistic target of rapamyacin complex 1 signaling determines brown-to-white adipocyte phenotypic switch. Diabetes. 2015; 64(2):519–528. https://doi.org/10.2337/db14-0427 PMID: 25213336

15. Tran CM, Mukherjee S, Ye L, Frederick DW, Kissig M, Davis JG, et al. Rapamycin blocks induction of the thermogenic program in white adipose tissue. Diabetes. 2016; 65(4):927–941. https://doi.org/10.2337/db15-0502 PMID: 26858361

16. Belteki G, Haigh J, Kabacs N, Haigh K, Sison K, Costantini F, et al. Conditional and inducible transgene expression in mice through the combinatorial use of Cre-mediated recombination and tetracycline induction. Nucleic Acids Res. 2005; 33(5):e51. https://doi.org/10.1093/nar/gni051 PMID: 15784609

17. Shigeyama Y, Kobayashi T, Kido Y, Hashimoto N, Asahara S, Matsuda T, et al. Biphase response of pancreatic beta-cell mass to ablation of tuberous sclerosis complex 2 in mice. Mol Cell Biol. 2008; 28(9):2971–2979. https://doi.org/10.1128/MCB.01695-07 PMID: 18316403

18. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 2010; 8: e1000412. https://doi.org/10.1016/j.molcel.2013.08.003 PMID: 24011591

19. Ichimura Y, Waguri S, Sou YS, Kageyama S, Hasegawa J, Ishimura R, et al. Phosphorylation of p62 regulates autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. Mol Biol Cell. 2004; 15(3):1101–1111. https://doi.org/10.1091/mbc.E03-09-0704 PMID: 14699058

20. Maric C, Sandberg K, Hinojosa-Laborde C. Glomerulosclerosis and tubulointerstitial fibrosis are attenuated with 17-estradiol in the aging dahl salt sensitive rat. J Am Soc Nephrol. 2004; 15(6):1546–1556. https://doi.org/10.1097/01asn.0000128219.65330.ea PMID: 15153505

21. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012; 9(7):671–675. https://doi.org/10.1038/nmeth.2088 PMID: 22930834

22. Takemoto M, Asker N, Gerhardt H, Lundkvist A, Johansson BR, Saito Y, et al. A new method for large scale isolation of kidney glomeruli from mice. Am J Pathol. 2002; 161(3):799–805. https://doi.org/10.1016/S0002-9440(10)64239-3 PMID: 12213707

23. Mizushima N, Yamamoto A, Matsui M, Yoshimori T, Ohsumi Y. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. Mol Biol Cell. 2004; 15(3):1101–1111. https://doi.org/10.1091/mbc.E03-09-0704 PMID: 14699058

24. Nguyen T, Nici P, Pickett CB. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. J Biol Chem. 2009; 284(20):13291–13295. https://doi.org/10.1074/jbc.R900010200 PMID: 19182219

25. Ichimura Y, Waguri S, Sou YS, Kageyama S, Hasegawa J, Ishimura R, et al. Phosphorylation of p62 activates the Keap1-Nrf2 pathway during selective autophagy. Mol Cell. 2013; 51(5):618–631. https://doi.org/10.1016/j.molcell.2013.08.003 PMID: 24011591

26. Mao J, Zeng Z, Xu Z, Li J, Jiang L, Fang Y, et al. Mammalian target of rapamycin complex 1 activation in podocytes promotes cellular crescent formation. Am J Physiol Renal Physiol. 2014; 307(9):F1023–1032. https://doi.org/10.1152/ajprenal.00018.2014 PMID: 24990893

27. Inoki K, Mori H, Wang J, Suzuki T, Hong S, Yoshida S, et al. mTORC1 activation in podocytes regulates the thermogenic program in white adipose tissue. Diabetes. 2016; 65(4):927–941. https://doi.org/10.1128/MCB.01695-07 PMID: 24746805

28. Gödel M, Hartleben B, Herbach N, Liu S, Zschiedrich S, Lu S, et al. Role of mTOR in podocyte function and diabetic nephropathy in humans and mice. J Clin Invest. 2011; 121(6):2197–2209. https://doi.org/10.1128/JCVI.01177-10 PMID: 21606597

29. Taneike M, Nishida K, Omiya S, Zarrinpashneh E, Misaka T, Kitazume-Taneike R, et al. mTOR hyperactivation by ablation of tuberous sclerosis complex 2 in the mouse heart induces cardiac dysfunction with the increased number of small mitochondria mediated through the down-regulation of autophagy. PLoS One. 2016; 11(3):e0152628. https://doi.org/10.1371/journal.pone.0152628 PMID: 27023784

30. Kim J, Klionsky DJ. Autophagy, cytoplasm-to-vacuole targeting pathway, and pexophagy in yeast and mammalian cells. Annu Rev Biochem. 2000; 69:303–342. https://doi.org/10.1146/annurev.biochem.69.1.303 PMID: 10966461
31. Hartleben B, Gödel M, Meyer-Schwesinger C, Liu S, Ulrich T, Köbler S, et al. Autophagy influences glomerular disease susceptibility and maintains podocyte homeostasis in aging mice. J Clin Invest. 2010; 120(4):1084–1096. https://doi.org/10.1172/JCI39492 PMID: 20200449

32. Tagawa A, Yasuda M, Kume S, Yamahara K, Nakazawa J, Chin-Kanasaki M, et al. Impaired podocyte autophagy exacerbates proteinuria in diabetic nephropathy. Diabetes. 2016; 65(3):755–767. https://doi.org/10.2337/db15-0473 PMID: 26384385

33. Zhou J, Tan SH, Nicolas V, Bauvy C, Yang ND, Zhang J, et al. Activation of lysosomal function in the course of autophagy via mTORC1 suppression and autophagosome-lysosome fusion. Cell Res. 2013; 23(4):508–523. https://doi.org/10.1038/cr.2013.11 PMID: 23337583

34. Praga M, Hernández E, Morales E, Campos AP, Valero MA, Martínez MA, et al. Clinical features and long-term outcome of obesity-associated focal segmental glomerulosclerosis. Nephrol Dial Transplant. 2001; 16(9):1790–1798. https://doi.org/10.1093/ndt/16.9.1790 PMID: 11522860

35. Kambham N, Markowitz GS, Valeri AM, Lin J, D'Agati VD. Obesity-related glomerulopathy: an emerging epidemic. Kidney Int. 2001; 59(4):1498–1509. https://doi.org/10.1046/j.1523-1755.2001.0990041498.x PMID: 11260414

36. Praga M, Morales E. Obesity, proteinuria and progression of renal failure. Curr Opin Nephrol Hypertens. 2006; 15(5):481–486. https://doi.org/10.1097/01.mnh.0000242172.06459.7c PMID: 16914959

37. Van der Heijden RA, Bijzet J, Meijers WC, Yakala GK, Kleemann R, Nguyen T.Q, et al. Obesity-induced chronic inflammation in high fat diet challenged C57BL/6J mice is associated with acceleration of age-dependent renal amyloidosis. Sci Rep. 2015; 5:16474. https://doi.org/10.1038/srep16474 PMID: 26653579

38. Lynch CJ, Adams SH. Branched-chain amino acids in metabolic signalling and insulin resistance. Nat Rev Endocrinol. 2014; 10(12):723–736. https://doi.org/10.1038/nrendo.2014.171 PMID: 25287287

39. Giesbertz P, Padberg I, Rein D, Ecker J, Höfte AS, Spanier B, et al. Metabolite profiling in plasma and tissues of ob/ob and db/db mice identifies novel markers of obesity and type 2 diabetes. Diabetologia. 2015; 58(9):2133–2143. https://doi.org/10.1007/s00125-015-3656-y PMID: 26058503

40. She P, Van Horn C, Reid T, Hutson SM, Cooney RN, Lynch CJ. Obesity-related elevations in plasma leucine are associated with alterations in enzymes involved in branched-chain amino acid metabolism. Am J Physiol Endocrinol Metab. 2007; 293(6):E1552–E1563. https://doi.org/10.1152/ajpendo.00134.2007 PMID: 17925455

41. Wu Y, Liu Z, Xiang Z, Zeng C, Chen Z, Ma X, et al. Obesity-related glomerulopathy: insights from gene expression profiles of the glomeruli derived from renal biopsy samples. Endocrinology. 2006; 147(1):44–50. https://doi.org/10.1210/en.2005-0641 PMID: 16210374

42. Lee DF, Kuo HP, Chen CT, Hsu JM, Chou CK, Wei Y, et al. IKK beta suppression of TSC1 links inflammation and tumor angiogenesis via the mTOR pathway. Cell. 2007; 10; 130(3):440–455. https://doi.org/10.1016/j.cell.2007.05.058 PMID: 17693255