Unraveling the Role of Podocyte Turnover in Glomerular Aging and Injury

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ABSTRACT

Podocyte loss is a major determinant of progressive CKD. Although recent studies showed that a subset of parietal epithelial cells can serve as podocyte progenitors, the role of podocyte turnover and regeneration in repair, aging, and nephron loss remains unclear. Here, we combined genetic fate mapping with highly efficient podocyte isolation protocols to precisely quantify podocyte turnover and regeneration. We demonstrate that parietal epithelial cells can give rise to fully differentiated visceral epithelial cells indistinguishable from resident podocytes and that limited podocyte renewal occurs in a diphtheria toxin model of acute podocyte ablation. In contrast, the compensatory programs initiated in response to nephron loss evoke glomerular hypertrophy, but not de novo podocyte generation. In addition, no turnover of podocytes could be detected in aging mice under physiologic conditions. In the absence of podocyte replacement, characteristic features of aging mouse kidneys included progressive accumulation of oxidized proteins, deposits of protein aggregates, loss of podocytes, and glomerulosclerosis. In summary, quantitative investigation of podocyte regeneration in vivo provides novel insights into the mechanism and capacity of podocyte turnover and regeneration in mice. Our data reveal that podocyte generation is mainly confined to glomerular development and may occur after acute glomerular injury, but it fails to regenerate podocytes in aging kidneys or in response to nephron loss.


Chronic loss of kidney function is of significant public health importance not only because of high prevalence but also because it is a major independent risk factor for cardiovascular morbidity and mortality. Aged kidneys display an increased susceptibility for progressive diseases, suggesting that overlapping molecular programs contribute to organ aging and disease progression. Recent data indicate that a decrease in the number of glomerular podocytes is an important predictor for kidney aging, and podocytes are considered the weak link in the progression of CKD. Animal models have shown that podocyte depletion of up to 20% can be tolerated before a scarring response takes place, but residual podocytes are unable to undergo cell division. Although it is widely believed that glomeruli have only a limited capacity to resolve lesions, cases of potential podocyte regeneration and disease reversal have been described. These recent observations have instigated hope of finding...
ways to stimulate kidney regeneration. So far, two potential podocyte progenitor niches, bone marrow cells and parietal epithelial cells, have been characterized. Bone marrow transplantation has an ameliorating effect in several animal models of glomerular disease, but the underlying mechanisms remain poorly understood. In humans, Y-chromosome–positive podocytes have been detected in kidneys transplanted from females to males. Located directly adjacent to podocytes, parietal epithelial cells express the stem cell markers CD24 and CD133 in human tissue and are capable of self-renewal, as well as differentiation into several cell types, including podocytes.

In this study, a novel flow cytometry–based quantitative method was devised to assess the regenerative capacity of podocytes during diphtheria toxin (DT)–mediated acute podocyte loss, models of chronic nephron loss, and kidney aging.

RESULTS

Parietal Epithelial Cells Are Populating the Glomerular Tuft during Kidney Development and Give Rise to Fully Differentiated Podocytes

Because recent literature suggests a contribution of parietal epithelial cells (PECs) for generating novel podocytes, we genetically labeled PECs with membrane-tagged enhanced green fluorescent protein (mG) in inducible hPODXL1.rTA; tetO.Cre;mT/mG mice exposed to doxycycline from embryonic day 8.5 to postnatal day 28 (Figure 1, A and B). Kidney sections from mice after postnatal kidney development showed mG-labeled podocytes, revealing their PEC origin. Of note, PEC-derived podocytes were already detectable in P1 kidneys (Figure 1C, Supplemental Figure 1, A–C). Strikingly, mG-labeled podocytes presented with foot processes indistinguishable from resident podocytes.

Figure 1. PECs are populating the glomerular tuft during kidney development and give rise to fully differentiated podocytes. (A) Schematic of inducible hPODXL1.rTA; tetO.Cre;mT/mG transgenic mice. (B) Labeling pattern in the glomerulus and possible ways of repopulation. (C) Expression of mG in PECs at P1 (arrow, labeled podocyte). Scale bar, 50 μm. (D) Podocyte foot process morphology in mG-labeled podocytes from hPODXL1.rTA; tetO.Cre;mT/mG and (E) hNPHS2.rTA; tetO.Cre;mT/mG mice. Scale bars, 10 μm overview, 1 μm inlays. EGFP, enhanced green fluorescent protein; PCA, core promoter of chicken β-actin.
podocytes genetically labeled in the hNPHS2.rtTA;tetO.Cre;mT/mG mouse model (Figure 1, D and E), revealing that PEC lineage-derived cells can give rise to fully differentiated podocytes.

Flow Cytometry–Based Quantification of Podocyte Turnover

Current quantitative techniques in glomerular lineage tracing rely heavily on evaluation of histologic sections, which have limitations regarding accuracy and are very time-consuming if performed on whole kidneys. We therefore used the mT/mG transgenic mouse model and a refined podocyte isolation technique to establish a flow cytometry–based approach for evaluation of podocyte plasticity (Figure 2A).20,21 With use of concomitant far red antipodocin staining of labeled podocytes, the mT/mG ratio could be reliably determined by flow cytometry (Figure 2, B and C). Evaluation of our technique showed a 99% overlap between genetically labeled podocytes and podocytes detected with the antibody, with a very low false-positive rate of about 1% (Figure 2, D–F).

Acute Podocyte Loss in a DT Model Leads to Limited Podocyte Turnover

We next tested whether podocyte regeneration can be stimulated in adult mice in response to acute podocyte loss. To establish a mouse model of acute podocyte loss, inducible DT receptor (iDTR) mice were crossed to hNPHS2.rtTA;tetO.Cre; mT/mG mice to specifically target podocyte ablation in a dose-dependent manner (Figure 3A).22 High doses (25–100 ng/g body weight) of DT resulted in massive albuminuria and loss of nearly all podocytes, whereas lower doses (2 and 5 ng/g body weight) resulted in partial cell ablation (Figure 3, B–D). A low dose (2 ng/g body weight) of DT was sufficient to cause a net

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**Figure 2.** Highly specific FACS analysis of podocyte populations. (A) Glomeruli were isolated by perfusion of the renal arteries with magnetic beads, followed by enzymatic digestion of the tissue and isolation of bead-filled glomeruli with a magnet. After digestion to single cells, podocytes were stained with a directly labeled antipodocin antibody. (B) mT/mG image of an hNPHS2.rtTA;TetO.Cre;mT/mG kidney previously induced with low efficiency. Scale bar, 10 μm. The same kidney was used for (C) analysis by flow cytometry. The podocyte population can be distinguished by antipodocin staining and can be split into mT- and mG-positive cells. (D) mT/mG image of an hNPHS2.Cre; mT/mG and (E) hNPHS2.rtTA;mT/mG glomerulus. Scale bar, 10 μm. (F) Validation of the flow cytometry–based method. Constitutively-labeled hNPHS2.Cre;mT/mG mice showed high specificity with very few false-positive cells, whereas no false-positive cells could be found in hNPHS2.rtTA;mT/mG mice. Error bars, SEM; n=3 per group. Scale bars, 10 μm. EGFP, enhanced green fluorescent protein.
loss of approximately 12% of all podocytes after 4 weeks without imposing any persistent gross damage to the tissue (Figure 3E, Supplemental Figure 1D). This injury led to a transient increase in albuminuria, which gradually decreased over 28 days (Figure 3F), similar to findings reported in rats.4 We then used the iDTR model to determine the influence of acute podocyte loss on podocyte regeneration. To induce podocyte loss, iDTR mice received DT, 2 ng/g body weight. Animals with a peak albumin-to-creatinine ratio >8 were considered responsive to DT, and their kidneys were analyzed by flow cytometry. Because podocyte ablation occurs only in mG-labeled podocytes (coexpressing DTR), but not in resident mT podocytes, we counted the total number of podocytes in glomerular sections and calculated the deviation from the control mean. When taking into account the ablated green podocytes, we could still detect a significant increase in the percentage of mT-labeled podocytes 4 weeks after administration of DT (19.53% control versus 26.86% iDTR; difference, 7.33 percentage points; P=0.03) (Figure 3, G and H), suggesting a distinct regenerative capacity of podocytes after acute podocyte loss.

Figure 3. Acute podocyte loss in a DT model leads to limited podocyte renewal. (A) Schematic of transgenic podocyte-specific iDTR mice. (B) Albuminuria is dose-dependent after DT injection. (C) mT/mG fluorescent images of iDTR animals treated with a high (50 ng/g body weight) or low (5 ng and 2 ng/g body weight) dose of DT. (D) Nephrin and active caspase-3 staining 10 days after injection of DT. Scale bar, 10 μm. (E) Number of podocytes per glomerular cross-section after injection of DT, 2 ng/g body weight; >30 glomeruli, ≥10 animals per group; error bars, SEM; **P<0.01. (F) Albumin-to-creatinine ratio after injection of DT, 2 ng/g body weight. Error bars, SEM; n.s., not significant; *P<0.05; **P<0.01; ***P<0.001. (G) Flow cytometric analysis 4 weeks after injection of DT, 2 ng/g body weight; n=10; error bars, SEM; *P<0.05. (H) Proportion of mT-positive cell increase in relation to ablated cells. EGFP, enhanced green fluorescent protein; PCA, core promoter of chicken β-actin.


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Analysis of Podocyte Regeneration in Models of Nephron Loss

The major compensatory mechanism for complete or partial nephron loss is hypertrophy of the remaining glomeruli, involving all glomerular cells including podocytes. To investigate whether this hypertrophic response also stimulates podocyte de novo generation from parietal epithelial cells, we used the unilateral nephrectomy (UNx) model (Figure 4, A–C). Validation of the model with morphometric and stereologic techniques confirmed a marked increase in kidney weight as well as mean glomerular volume, indicating that the remaining kidney underwent considerable adaptive hypertrophy in the 3 months following UNx, while podocyte number did not increase (Figure 4, D–F). After pulse-chase labeling of podocytes, we performed UNx in hNPHS2.rtTA;tetO.Cre;mt/mG mice and assessed the ratio of mt- and mG-positive podocytes in the explanted kidneys. Induction rates between left and right kidney were not different (Supplemental Figure 2, A and B). The remaining kidney was assessed after 12 weeks and compared with its baseline value. We could detect only a marginal shift toward mt-positive podocytes (2.6%), which was at the borderline of statistical significance (Figure 4G and Supplemental Figure 2, C and D). However, using labeled PECs (Figure 1A) or labeled bone marrow cells (Figure 5J) as potential sources of podocyte renewal did not indicate any podocyte de novo generation in response to UNx (Figure 4, H and I). In addition, no evidence for podocyte cell cycle entry could be detected on days 2 and 7 after UNx (Supplemental Figure 2F). Therefore, unilateral nephrectomy seems to result in compensatory glomerular hypertrophy, while recruitment of novel

Figure 4. Nephron loss is not associated with podocyte renewal from bone marrow or PEC compartment. (A) Schematic of transgenic hNPHS2.rtTA;tetO.Cre;mt/mG mice and labeling pattern. (B) Schematic of the UNx protocol. (C) Kidney weight 12 weeks after UNx compared with control animals; n=9; ***P<0.001. (D) Mean glomerular volume (*P<0.05) and (E) mean podocyte volume. n.s., not significant (P=0.06). (F) Number of podocytes per glomerulus. n.s., not significant (P=0.171). Error bars, SEM. (G) Flow cytometry analysis of labeled podocytes (n=9), (H) PECs (P=1, n=4) and (I) bone marrow cells (P=0.30, n=6) 12 weeks after nephrectomy compared with baseline values. Co, control; EGFP, enhanced green fluorescent protein; PCA, core promoter of chicken β-actin.
Figure 5. Podocytes are not renewed during aging. (A) Schematic of hNPHS2 rtTA;tetO.Cre;mT/mG mice. (B) Induction protocol. (C) Flow cytometry analysis at 4 weeks and 12 months. Error bars, SEM; n.s., not significant (P=0.55); n=12. mT/mG fluorescent images at (D) 4 weeks and (E) 12 months (arrow, mT-labeled podocyte). Scale bar, 10 μm. (F) Schematic of hPODXL rtTA;tetO.Cre;mT/mG mice. (G) Flow cytometry analysis at 4 weeks and 12 months. Error bars, SEM; n=6 per group; n.s., not significant (P=1). mT/mG fluorescent
podocytes to hypertrophic glomeruli does not substantially contribute to these adaptive mechanisms.

**Podocytes Are Not Renewed during Aging**

Aging is the most common reason for a decline in renal function, which is underlined by loss of podocytes and glomerulosclerosis. To address the role and capacity of podocyte turnover during aging, we performed pulse-chase experiments with inducible hNPHS2.rtTA;tetO.Cre;mT/mG mice, labeling the podocyte pool during development until P28 (Figure 5, A and B). After an aging period of 12 months, podocytes were analyzed by flow cytometry and compared with baseline values at 4 weeks. During this period no increase in mT-labeled podocytes could be detected, indicating a negligible podocyte turnover under physiologic conditions (Figure 5, C–E). To further substantiate the lack of podocyte turnover with age, we assessed potential podocyte replacement by PECs by pulse-chase labeling of hPODXL1.rtTA;tetO.Cre;mT/mG animals with doxycycline from E8.5 to P28 (Figure 5F). Quantitative analysis at 4 weeks revealed that approximately 1% of all podocytes was of PEC origin (Figure 5G). This percentage did not change when animals of the same cohort were assessed at 4, 8, and 12 months (Figure 5, H and I, Supplemental Figure 3, B and C). Therefore, despite intriguing reports about their regenerative potential, we could not detect any turnover of podocytes by genetically labeled PECs during normal aging. Because bone marrow–derived cells have been implicated in podocyte regeneration after injury, mG-positive bone marrow cells were transplanted into mT wild-type animals at 2 months of age after lethal irradiation, resulting in high chimerism and bone marrow engraftment (Figure 5, J and K, Supplemental Figure 4, B and C). Flow cytometric analysis 4 and 7 months after transplantation revealed that mG-positive podocytes remained <0.01% (Figure 3L). Cells that were positive for mG were detected in the renal interstitium and glomeruli (Figure 5, M and N, Supplemental Figure 4, D and E), but co-staining of GFP with the podocyte marker nephrin showed no overlap (Supplemental Figure 4F), supporting the conclusion that bone marrow–derived cells did not differentiate into podocytes during aging. In summary, these data indicate that podocyte turnover is absent during kidney aging.

**Kidney Aging Is Characterized by Podocyte Degeneration, Loss of Podocytes, and Glomerulosclerosis**

Because podocytes are not replaced during aging, we analyzed the degenerative changes of podocytes at increasing age of mice. Kidneys of aged mice exhibited a progressive accumulation of oxidized proteins and protein aggregates (Supplemental Figure 5, A and B). The number of podocytes decreased with age, while glomerulosclerosis, proteinuria, and serum creatinine increased, indicating that lack of podocyte turnover was associated with an age-related glomerulopathy (Supplemental Figure 5, C–G).

**DISCUSSION**

Podocytes have been identified as a key factor in kidney disease progression, and strategies are desperately sought to target podocytes for the prevention of ESRD. Yet many fundamental questions concerning podocyte lifespan, turnover, and regeneration remain to be elucidated. Using flow cytometry–based analysis of genetically labeled mouse podocytes, we were able to trace a large number of cells under physiologic and pathophysiologic conditions in an unbiased fashion. For this technique, the mT/mG reporter strain proved to be an excellent tool, providing a superb FACS quantifiable resolution of mT- and mG-labeled cells. Although absolute numbers varied depending on isolation efficacy, this approach allowed unbiased relative quantification of >100,000 podocytes per mouse kidney. This nearly eliminated the cumbersome and tedious counting of antibody–labeled podocytes in tissue sections, which, despite elaborated stereoscopic methods, remains to a certain degree subjective. With the high number of podocytes screened, it was also possible to detect small differences in turnover, which might be crucial to define the boundary between repair and progression toward glomerulosclerosis. In addition, this method should enable systematic screening for factors influencing turnover and repair. Together with further, recently optimized downstream applications, such as transcriptomic, proteomic, and epigenetic profiling, this could lead to the identification of “druggable” targets to enhance podocyte turnover or reduce further podocyte loss. Disadvantages of this method, however, are the loss of spatial resolution compared with microscopy-based approaches and the need for extensive mouse breedings to generate the desired mouse strains.

In addition to FACS analysis, this system could be used to resolve mG-labeled PEC-derived podocytes by immunofluorescence from adjacent mT-labeled podocytes at the level of cellular foot process formation. This documented for the first time that PECs can in fact form fully differentiated podocytes. The appearance of PEC-derived podocytes was exclusively observed during kidney development, indicating that PECs and podocytes, which originate from common progenitors, appear to be in a dynamic continuum only during kidney development.
Mechanistically, glomerular and hence podocyte response to injury can be divided into different subtypes: acute loss of podocytes, hypertrophy of podocytes, and chronic degeneration of podocytes. Acute podocyte loss may occur in certain types of glomerulonephritis, hypertrophy is found in response to any reduction of nephron mass and diabetes, and chronic degeneration appears to underlie podocyte aging. To assess the effects of acute podocyte loss on podocyte renewal, we chose the iDTR model, which had been extensively studied before.22,27 The advantage of this model is the dose-dependent specific ablation of podocytes without damaging other glomerular cell types. Our murine data correlated well with previously published data in rats showing that ablation of approximately 20% of podocytes leads to transient proteinuria.4 We speculated that the transient nature of proteinuria in this setting might be associated with a limited podocyte renewal. In fact, our results indicate that initially podocyte number was reduced by 19.3% while after 4 weeks with regressing proteinuria podocyte number only remained reduced by 12%; this means that 7.3% of podocytes appeared de novo after acute DT injury. Overall, this suggests a podocyte renewal of 38% of ablated cells (12% + 7.3% = 19.3%). However, our model does not allow identification of the source of this novel podocyte population because labeling of cells and expression of iDTR are linked. In addition, our quadruple transgenic approach could not differentiate between novel podocytes being incorporated on the glomerular tuft and possible “transitional” podocytes/PECs at the vascular glomerular stalk starting to express podocyte markers in response to acute podocyte injury. Subsequent studies with additional transgenic tools allowing for separate labeling of cells and expression of iDTR, respectively, will be required to determine whether these new podocytes are of PEC or any different origin.

Hypertrophic growth of the remaining kidney, nephrons, and glomeruli is probably the most commonly encountered renal repair mechanism and is highly clinically relevant. We therefore examined the UNx model in which we could not detect, at least in our experimental set-up, any podocyte turnover, a finding that confirms the work of others.24 These results confirm that glomerular and podocyte hypertrophy is the main adaptation mechanism, which is in line with previous reports.23,29 Although signaling pathways such as mammalian target of rapamycin that efficiently increase podocyte size have been identified,5,30,31 the lack of podocyte turnover in response to nephron loss makes these enlarged podocytes the obvious Achilles heel in progressive kidney diseases. Elucidating the cellular programs that control podocyte regeneration during glomerular development and acute podocyte loss might therefore provide a possible approach to stimulating cell differentiation into fully functional podocytes in response to nephron loss, thereby preventing progression of chronic kidney injury.

With the ever-increasing human lifespan in developed countries, age-related renal functional decline plays a more and more important role. To evaluate the effects of aging on glomeruli, chasing of pulse-labeled podocytes was done over the course of up to 12 months, representing half of the murine lifespan.32 During this time the ratio of mG- to mT-labeled podocytes did not change. Several studies report lack of podocyte proliferation under noninjury conditions using permanent labeling protocols.11,29 This in turn suggests that the podocyte pool in our study neither integrated any new cells nor generated new cells by cell division and hence essentially remained the same over the examined time period. This result corroborates the work of others and is in line with the nephron reserve hypothesis, which has recently gathered more and more scientific interest.28,33 It essentially postulates that we probably need only 25% of all our 2 million neonatally formed nephrons for a proper functioning kidney. But to sustain losses over our lifespan we have been endowed with a 300% surplus because we are not able to regenerate our highly complex metanephros. This is in contrast to lower vertebrates, such as zebrafish, which has only two rather simple pronephros but can regenerate them during its albeit much shorter life.34

In agreement with lack of age-dependent regeneration, aged podocytes exhibit a prominent degenerative aging phenotype with progressive accumulation of oxidized proteins and protein aggregates, podocyte loss, and ultimately glomerulosclerosis. These findings highlight the importance of cellular maintenance and stress adaptive mechanisms, such as autophagy, for long-term survival of podocytes.35

In summary, we developed a novel method to reliably and rapidly determine podocyte turnover. This approach revealed that podocyte regeneration is limited to glomerular development and podocyte loss caused by acute DT-induced cell death. It is conceivable that this regenerative capacity may occur in certain glomerular diseases or that such regeneration could potentially be stimulated to ameliorate glomerular disease. However, we can conclusively demonstrate that regenerative programs are absent in common forms of renal functional decline, including chronic nephron loss and kidney aging. Hypertrophy appears to be the main mechanism by which podocyte loss is compensated during aging and after reduction of nephron mass. However, despite the ability of podocytes to undergo significant hypertrophy, podocyte loss ultimately leads to renal failure. Uncovering the mechanisms that allow parietal epithelial cells to differentiate into podocytes may provide a novel approach to minimize the consequences of podocyte loss during aging and CKD.

CONCISE METHODS

Animals
All animal experiments were conducted according to the guidelines of the American Physiologic Society, as well as the German law for the welfare of animals, and were approved by local authorities (G-11/03, G-11/38, G-11/114). Mice were housed in a specific pathogen-free facility with free access to chow and water and a 12-hour day/night cycle. Breeding and genotyping was done according to standard procedures. hNPHS2:rtTA;tetO.Cre mice were a generous gift of
mice were a generous gift of Lawrence Holzman (Renal, Electrolyte, and Hypertension Division, University of Pennsylvania School of Medicine, Philadelphia, PA).

For the induction of mT deletion, mice received doxycycline hydrochloride (Sigma-Aldrich) via the drinking water (2 mg/ml with 5% sucrose) during pregnancy and nursing (embryonic deletion) up to P28. To increase induction efficiency, the mice received doxycycline (2 mg/ml) orally from P6 to P18 three times per week, starting with approximately 50 μl each time. DT (Sigma-Aldrich) was dissolved in PBS and injected intraperitoneally at 5 weeks.

For a complete discussion of the study methods, see the Supplemental Material.

Statistical Analyses

Statistical analyses were performed with GraphPad Prism. The data were analyzed with the D’Agostino-Pearson test for normality distribution. An unpaired two-tailed t test was used for Figures 3, E–G, 4D, and 5C. A paired two-tailed t test was used for Figure 4G. A two-tailed Wilcoxon matched pairs test was used for Figure 4, H and I; a two-tailed Mann–Whitney U test was used for Figure 5, G and I; and a one-tailed Mann–Whitney U test was used for Figure 4, E and F.

ACKNOWLEDGMENTS

The work in the laboratory of T.B.H. has been generously supported by the German Research Foundation DFG (E.G. and T.B.H.), by the Excellence Initiative of the German Federal and State Governments (BIOSS II–EXC 294 to T.B.H. and R.Z.; GSC–4 Spermann Graduate School to N.W., T.B.H., N.S., and R.Z.), by the Else Kröner Fresenius Excellence Initiative of the German Federal and State Governments and the German Research Foundation DFG (F.G. and T.B.H.), by the German Heart Foundation (BMBF Gerontosys II-NephAge (T.B.H.)).

DISCLOSURES

None.

REFERENCES


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2013050452/-/DCSupplemental.
Supplementary Material

Complete Methods

UNx

Mice were anesthetized with ketamine/xylazine and body temperature was maintained with a warmed operation table (Föhr Medical Instruments, Germany), infrared light during recovery and warmed solutions. After deep anaesthesia a left sided flank incision was made. The vasculature of the left kidney was ligated with Mersilene 3-0 (Ethicon, Germany) and the kidney removed. The wound was closed with Vicryl 5-0 (Ethicon, Germany). After regaining consciousness adequate pain relief was given. The explanted kidney was perfused with magnetic beads and used for podocyte isolation.

Bone marrow transplantation

Recipient mT/mG wild type mice were lethally irradiated with 900 cGy in two doses given 5 h apart. Bone marrow cells were isolated from femura and tibiae of donor mG mice and injected intravenously (5x10^6 cells in 100 µl sterile PBS). After irradiation, mice were maintained on antibiotic water containing Enrofloxacin (10 mg/l, Bayer, Germany) for 4 weeks. For the determination of chimerism, peripheral blood cells were stained with Alexa Fluor 647 anti-mouse CD45 (clone 30-F11, BioLegend) and the ratio of mT/mG positive cells was determined via flow cytometry (CyAn ADP, Beckman Coulter).

Genotyping

Tail biopsies were incubated at 95°C in an alkaline lysis reagent (25 mM NaOH, 0,2 mM EDTA), neutralized with 40 mM TrisHCl and subsequently used for PCR with the following primers:

\textbf{NPHS2.rTA} forward CGC ACT TCA GTT ACT TCA GGT CC TC and reverse GCT TAT GCC TGA TGT TGA TGC; \textbf{pPEC.rTA} forward AAT CGA GAT GCT GGA CAG GCA TCA TAC CCA and reverse GGC ATA GAA TCG GTG GTA GGT GTC TCT CTT; \textbf{TetOCre}
forward GCA TAA CCA GTG AAA CAG CAT TGC TG and reverse GGA CAT GTT CAG
GGA TCG CCA GGC G; \textit{mT/mG} forward (common) CTC TGC TGC CTC CTG GCT TCT
and reverse (mutant) TCA ATG GGC GGG GGT CGT T and (wild type) CGA GGC GGA
TCA CAA GCA ATA; \textit{iDTR} forward (common) AAA GTC GCT CTG AGT TGT TAT and
reverse (mutant) CAT CAA GGA AAC CCT GGA CTA CTG and (wild type) GGA GCG GGA
GAA ATG GAT ATG.

\textbf{Urinary and serum measurements}

Urinary albumin and creatinine were measured with a Microflural Microalbumin Test kit
(Progen) and an enzymatic Creatinine PAP kit (Labortechnik) following the manufacturer’s
instructions. Albumin-to-creatinine ratio is given as mg albumin/mg creatinine.

\textbf{Immunofluorescence staining of kidney sections}

\textit{mT/mG} mouse kidneys were perfused and incubated with 4 % PFA in PB at 4 °C for 3 hours.
Kidneys were immersed in 15 % sucrose in PBS for 2 h and 30% sucrose overnight, then
frozen in OCT compound and sectioned at 6 µm (Leica Kryostat). The sections were
counterstained with Hoechst 33342 (Invitrogen). For paraffin embedding, the kidneys were
incubated in 4 % PFA for 24 hours and then dehydrated in ethanol. PAS staining was done
with 3 µm sections. Antigen retrieval was performed on 6 µm sections with citrate buffer (pH
6.0) for 30 min. Images were taken with a Zeiss fluorescence microscope with Apotome
mode or a Zeiss laser scan microscope equipped with a 63x water immersion objective. To
determine podocyte cell number WT1-positive cells were counted in >30 glomerular sections
per mouse and the mean value of each animal used for statistical analysis.

\textbf{Immunohistochemical staining of kidney sections}

Kidneys were fixed in 4% paraformaldehyde in PBS, embedded in paraffin and sectioned at
4 µm (Leica Microtome). After deparaffinization, rehydration and antigen retrieval in 10mM
sodium citrate buffer, sections were blocked with 2% BSA in PBS and incubated overnight
with primary antibodies as indicated, followed by peroxidase blocking in 3% H$_2$O$_2$ for 10
minutes and incubation with HRP conjugated secondary antibodies (Dako) for 1 hour. DAB (Dako) was applied for 6 minutes. Sections were counterstained with hematoxylin.

**Antibodies**

The following antibodies were used: rabbit anti-active caspase 3 (AF835, R&D), mouse anti-PCNA (M08979, Dako), guinea pig anti-nephrin (GP-N2, Progen), rabbit anti-WT1 (ab15249, Abcam), mouse anti-desmin (M0760, Dako), rabbit anti-podocin (P0372, Sigma), rabbit anti-GFP (MBL-598, Biozol), rabbit anti-nitrotyrosine (AB5411, Millipore) and guinea pig anti-p62 (GP62-C, Progen). Fluorophore-conjugated secondary antibodies and nuclear staining reagent (To-Pro-3 T3605, Hoechst 33342) were obtained from LifeTechnologies.

**Isolation of podocytes**

Glomeruli were isolated by Dynabead perfusion as recently described in detail (19, 26). After subsequent digestion to single cells, the cells were fixed in 4% PFA, permeabilized with 0.5% Triton and stained with anti-podocin antibody (P0372, Sigma) labeled with Alexa Fluor 647 (Zenon labeling kit, Invitrogen).

**Flow cytometry**

Isolated and stained glomerular cells were resuspended in FACS buffer (0.5% BSA, 5 mM EDTA in PBS) and measured with FACS Calibur (BD).

**Histological analyses**

Quantitative stereological analyses of kidney sections were performed as described previously (1). Briefly, the mean glomerular volume (mean $v(Glom)$) was determined from the mean glomerular profile area (mean $A(Glom)$) of ~ 100 systematically sampled glomerular profiles per animal (2). The physical dissector principle was applied for counting podocytes (Q−) as described, using semithin sections (3, 4). The numerical density of podocytes in glomeruli ($NV(P/Glom)$) was calculated as the quotient of the sum of Q− divided by the dissector volume. The number of podocytes per glomerulus ($N(P,Glom)$) was calculated
multiplying $NV(P/Glom)$ and mean $\nu(Glom)$. Six glomerular profiles were evaluated (corresponding to 352–500 reference points). All results were corrected for embedding shrinkage (1).
References


**Supplementary Figure 1.** PEC induction and diphtheria toxin mediated targeted podocyte ablation

(A) Embryonically induced hPODXL.rtTA;tetO.Cre;mT/mG kidney at day 1 after birth (P1) Scale bar, 100 µm. (B and C) 63x magnification of P1 kidney shows expression of mG in parietal epithelial cells around mature glomeruli (arrow: mG-positive podocyte). Scale bar, 10 µm. (D) Four weeks after 2 ng diphtheria toxin injection PAS, nephrin and PCNA staining is indistinguishable from control litter mates, whereas WT1 staining shows reduced numbers of podocytes. Scale bar, 100 µm PAS, 10 µm IF.
Supplementary Figure 2:

(A) Schematic of comparison of induction levels of the two kidneys of the same mouse. (B) Comparison of induction levels between right and left kidney at the same time point shows no significant directional differences (p=0.9082). (C) Schematic of UNx in hNPHS2.rtTA;tetO.Cre;mT/mG mice. (D) Flow cytometry analysis of mG-labeled podocytes 12 weeks after nephrectomy compared to baseline values (p=0.0387). (E) The animals did not develop proteinuria 3 months after nephrectomy (p=0.3207). Error bars, SEM. n.s., not significant. (F) mean podocyte volume. n.s., not significant, p=0.057. (G) PCNA/WT1 staining of kidney sections 2 days or 7 days after unilateral nephrectomy shows activation of PCNA in parietal cells after 7 days (arrows), but no colocalization with WT1 at either time point. Scale bar, 10 µm.

S2. Unilateral nephrectomy

(A) Schematic of comparison of induction levels of the two kidneys of the same mouse. (B) Comparison of induction levels between right and left kidney at the same time point shows no significant directional differences (p=0.9082). (C) Schematic of UNx in hNPHS2.rtTA;tetO.Cre;mT/mG mice. (D) Flow cytometry analysis of mG-labeled podocytes 12 weeks after nephrectomy compared to baseline values (p=0.0387). (E) The animals did not develop proteinuria 3 months after nephrectomy (p=0.3207). Error bars, SEM. n.s., not significant. (F) mean podocyte volume. n.s., not significant, p=0.057. (G) PCNA/WT1 staining of kidney sections 2 days or 7 days after unilateral nephrectomy shows activation of PCNA in parietal cells after 7 days (arrows), but no colocalization with WT1 at either time point. Scale bar, 10 µm.
S3. Physiological podocyte turnover during aging: podocytes and PECs

(A) Representative PAS stainings of the renal cortex of hNPHS2.rtTA;tetO.Cre;mT/mG mice at 4 weeks and 12 months. Scale bar, 100 µm (B) Flow cytometry analysis of hPODXL.rtTA;tetO.Cre;mT/mG mice showed no increase of mG-positive podocytes at 4 months, 8 months and 12 months compared to 4 weeks. Error bars, SEM. n.s., not significant (4w vs. 12mo p = 0.6804). n≥6 per group. (C) mT/mG fluorescent images of 4 months and 8 months points showed labeled parietal cells and rare mG podocytes (arrows). Scale bar, 10 µm.
Supplementary Figure 4:

A. GFP desmin GFP nephrin

B. 4mo 7mo

BMT

flow cytometry

4mo 7mo

Chimerism

% CD45+ PBMCs

% GFP+

% Tomato+

G

bone marrow

liver

kidney

lung

spleen

stomach

D

D' D''

E

7mo

E' E''

F

4mo 7mo

GFP nephrin

GFP desmin

Supplementary Figure 4:
S4. Physiological podocyte turnover during aging: bone marrow cells

(A) Irradiated mT wild type mice received mG-labeled donor bone marrow. The kidneys were analyzed 4 months and 7 month after irradiation. (B) After 4 weeks > 89 % of CD45-positive peripheral blood mononuclear cells were mG-labeled. Error bar, SEM. (C) Tissue sections 11 days after bone marrow transplantation (BMT) showed mG-positive bone marrow and single cells in the liver, kidney, lung, spleen and stomach lining. Scale bar, 50 µm. After (D) 4 and (E) 7 months mG-positive cells have increased in the kidney (scale bar 100 µm), but not in the glomeruli (D’, D”, E’ and E”, scale bar 10 µm). (F) Staining of mG-positive cells in the kidney 4 months or 7 months after bone marrow transplantation did not reveal colocalization with podocyte marker nephrin or mesangial marker desmin. Scale bar, 10 µm. Arrows, mG-labeled glomerular cells.
S5. Accumulation of oxidized proteins and deposition of p62 aggregates in podocytes are hallmarks of kidney aging.

(A) Accumulation of the oxidative stress marker nitrotyrosine in glomeruli of aging mice. Scale bars, 20 µm. (B) Immunofluorescence staining of mouse kidney sections for p62 and the podocyte marker protein podocin reveals accumulation of p62 positive protein aggregates (arrows) in podocytes of aging mice. Scale bars, 5 µm. (C) Significantly increased albuminuria in 22 month old C57BL/6 wild type mice compared to 4 month old C57BL/6 wild type mice (n>9, *** p=0.0003). (D) Significantly increased serum creatinine in 22 month old C57BL/6 wild type mice compared to 4 month old C57BL/6 wild type mice (n>12, * p=0.020). (E) The number of podocytes per glomerular cross section at 4 and 22 months (** p=0.0052). Error bars, SEM. n≥3, respectively. (F) Glomerulosclerosis score at 4 and 22 months (*** p<0.0001). Error bars, SEM. n≥3, respectively. (G) PAS stainings at 4 and 22 months. Scale bars, 20 µm.