Partial podocyte replenishment in experimental FSGS derives from nonpodocyte sources

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Kaverina NV, Eng DG, Schneider RRS, Pippin JW, Shankland SJ. Partial podocyte replenishment in experimental FSGS derives from nonpodocyte sources. Am J Physiol Renal Physiol 310: F1397–F1413, 2016. First published April 13, 2016; doi:10.1152/ajprenal.00369.2015.—The current studies used genetic fate mapping to prove that adult podocytes can be partially replenished following depletion. Inducible NPHS2-rtTA/tetO-Cre/RS-ZsGreen-R reporter mice were generated to permanently label podocytes with the ZsGreen reporter. Experimental focal segmental glomerulosclerosis (FSGS) was induced with a cytotoxic podocyte antibody. On FSGS day 7, immunostaining for the podocyte markers p57, synaptopodin, and podocin were markedly decreased by 44%, and this was accompanied by a decrease in ZsGreen fluorescence. The nuclear stain DAPI was absent in segments of reduced ZsGreen and podocyte marker staining, which is consistent with podocyte depletion. Staining for p57, synaptopodin, podocin, and DAPI increased at FSGS day 28 and was augmented by the ACE inhibitor enalapril, which is consistent with a partial replenishment of podocytes. In contrast, ZsGreen fluorescence did not return and remained significantly low at podocytes. In contrast, ZsGreen fluorescence did not return and inhibiting enalapril, which is consistent with a partial replenishment of necrosis, and a depletion in number (28, 43). A large body of such as FSGS, podocyte injury leads to apoptosis, detachment, and membranous nephropathy (43). In these diseases, a common response to injury by podocytes is that they change ease, and the primary cells injured in several glomerular diseases such as focal segmental glomerulosclerosis (FSGS), minimal change disease, and membranous nephropathy (43). In these diseases, a common response to injury by podocytes is that they change shape (called effacement) because their actin cytoskeleton has been activated. In progressive forms of glomerular diseases such as FSGS, podocyte injury leads to apoptosis, detachment, necrosis, and a depletion in number (28, 43). A large body of literature shows that when the total podocyte number decreases below a certain threshold in glomerular disease, glomerulosclerosis ensues (6, 25, 51). Moreover, the magnitude of glomerulosclerosis correlates directly with the extent of podocyte depletion (51).

One might expect that, in response to depletion, podocytes would simply proliferate to restore and preserve their number. In contrast to the majority of other kidney cell types that readily proliferate to replace cells, adult podocytes are unable to proliferate. This is largely due to changes in cell cycle proteins that limit DNA synthesis and mitosis (12, 30, 52, 56). However, using cell-specific markers to identify and quantify podocytes, it has recently been recognized that under certain circumstances podocyte numbers will increase following depletion despite the absence of podocyte proliferation (55). In this context, administration of angiotensin I-converting enzyme (ACE) inhibitors (22, 29, 43, 56), angiotensin receptor blockers (20a), corticosteroids (54), retinoids (55), and an improved diabetic milieu all increase the density of podocyte markers following an initial phase of depletion (31). These changes in immunostaining, interpreted as partial or complete podocyte replenishment, are typically accompanied by improved kidney function, reduced proteinuria, and lower glomerulosclerosis, stressing the importance of podocyte replenishment.

Despite these informative reports of podocyte replenishment, there is currently limited genetic proof that adult podocyte regeneration derives from nonpodocyte sources. Accordingly, we employed mouse molecular genetic lineage tracing to prove that, in experimental glomerular disease typified by podocyte depletion, the partial increase in podocytes induced by ACE inhibition is not due to their proliferation, but rather it derives from nonpodocyte sources. We also examined the contribution of podocytes to cells lining the Bowman’s capsule that coexpressed both parietal epithelial cell (PEC) and podocyte proteins.

METHODS

Reporter Mice Used for Study

Inducible podocyte reporter mice. To study podocyte regeneration following induction of experimental FSGS, inducible NPHS2-rtTA/tetO-cre/RS-ZsGreen-R mice were generated. These mice are bred using the Tet-On system that allows temporally specific and permanent reporter labeling of podocytes upon doxycycline administration. The podocin-tetracycline-controlled transactivator protein (rtTA) mice, generously provided by Dr. Jeffrey Kopp (National Institutes of Health, Bethesda, MD), and previously described by Shigehara et al. (45), were crossed with commercially available tetO-cre mice (B6.Cg-Tg(tetO-cre)1Jaw/J) and RS-ZsGreen-R (B6.Cg-Gt(Rosa)26Sortm6(CAG-ZsGreen1)HzEl/J).

ADULT PODOCYTES ARE TERMINALLY differentiated, highly specialized glomerular epithelial cells that form the outer layer of the glomerular filtration barrier (4). Podocytes are the primary cells injured in several glomerular diseases such as focal segmental glomerulosclerosis (FSGS), minimal change disease, and membranous nephropathy (43). In these diseases, a common response to injury by podocytes is that they change shape (called effacement) because their actin cytoskeleton has been activated. In progressive forms of glomerular diseases such as FSGS, podocyte injury leads to apoptosis, detachment, necrosis, and a depletion in number (28, 43). A large body of
mice from The Jackson Laboratory (Bar Harbor, ME). Triple transgenic mice were identified by genotyping for the presence of each transgene.

To specifically and permanently label podocytes within a defined temporal window, NPHS2-rtTA/tetO-cre/RS-ZsGreen-R mice were administered doxycycline hydrochloride via feed at 625 mg/kg starting at weaning (3 wk of age) for 28 days ad libitum (Harlan TD.01306) (Fig. 1). Chow was changed weekly. To ensure specific labeling of podocytes within this specific temporal window and to preclude the possibility of spontaneous expression of the podocyte reporter, a minimum 1-wk wash out period for doxycycline was performed before experiments were initiated. A survival kidney biopsy was performed to confirm podocyte labeling. Mice were housed in the animal care facility of the University of Washington under specific pathogen-free conditions with ad libitum access to food and water. Animal protocols were approved by the University of Washington Institutional Animal Care and Use Committee.

**Podocyte confetti reporter mice.** To study the potential ability of podocytes to regenerate following the induction of experimental FSGS, adult (8–10 wk old) NPHS2Cre/Confetti TG/WT (podocin-cre/confetti) reporter mice, generously provided by Dr. Katalin Susztak (University of Pennsylvania, Philadelphia, PA) and described by Tao et al. (47), were utilized. In brief, podocin-cre/confetti mice are a stochastic multicolor reporter line that contain the Brainbow-2.1 cassette in the Rosa26 locus. Following podocin cre-mediated excision of the loxP-flanked neomycin resistance cassette, there is stochastic activation of one of four fluorescent colors in podocytes driven by the CAGG promoter. These colors are cytoplasmic-targeted red fluorescent protein (cRFP), membrane-targeted cyan fluorescent protein (mCFP), nuclear-targeted green fluorescent protein (mCFP), and membrane-targeted cyan fluorescent protein (mCFP), nuclear-targeted green fluorescent protein (mCFP), and nuclear-targeted green fluorescent protein (mCFP), nuclear-targeted green fluorescent protein (mCFP), and nuclear-targeted green fluorescent protein (mCFP).
tein (nGFP), or cytoplasmic-targeted yellow florescent protein (cYFP).

Experimental FSGS with Abrupt Podocyte Depletion

Experimental FSGS was induced in NPHS2-Cre/RS-ZsGreen-R and NPHS2Cre/R26R-ConfettiTG/WT mice with a cytotoxic sheep antipodocyte antibody as previously described (32, 54, 55). This antibody induces abrupt podocyte depletion accompanied by glomerulosclerosis (32, 54, 55). Adult mice were given two doses of sheep antiglomerular antibody at 12 mg IgG/20 g body wt via ip injection 24 hours apart.

On day 3, NPHS2-Cre/RS-ZsGreen-R mice were randomized into two treatment groups: 1) group 1 received drinking water (the vehicle for enalapril); 2) group 2 received the ACE inhibitor enalapril (75 mg/ml) refreshed weekly. Mice were killed on either day 7 or day 28. On day 7, NPHS2Cre/R26R-ConfettiTG/WT mice were randomized and killed on either day 7 or day 28 of disease. Four animals without disease that did not receive the cytotoxic sheep antiglomerular antibody were killed and used as baseline controls.

At death, mice were perfused with 10 ml of ice-cold PBS to remove excess red blood cells. Kidneys were split in half, and one half was fixed overnight at 4°C in 10% neutral buffered formalin (Globe Scientific, Paramus, NJ), rinsed in 70% ethanol, processed, and embedded in paraffin. The other half was fixed for 45 min in 4% paraformaldehyde solution (PFA) in PBS (Aldrich, Burlington, MA) and processed as described above. Antigen retrieval was performed with the avidin/biotin blocking kit (Vector Laboratories). After antigen retrieval, sections were incubated overnight at 4°C with the appropriate primary antibodies: mouse antibody to nephrin ( Fitzgerald Industries, Concord, MA), mouse antibody to synaptopodin (Fitzgerald Industries), rabbit antibodies to podocin (Abcam, Cambridge, MA), and anti-p57 (Santa Cruz Biotechnology). The appropriate biotinylated secondary antibody (Vector Laboratories) was applied, followed by streptavidin and Alexa Fluor 594 conjugate (Life Technologies–Molecular Probes, Grand Island, NY). All immunofluorescence samples were mounted using Vectashield medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Antibodies were not required to detect confetti reporter.

BrdU/FDU Labeling of Mice to Assess Proliferation

To quantitate cell proliferation, 10 ul/g body wt of commercially available 5-bromo-2-deoxyuridine (BrdU) and 5-fluoro-2'-deoxyuridine (FUD; Amersham Cell Proliferation Labeling Reagent; GE Healthcare Life Sciences, Little Chalfont, UK) was administered via the tail vein. To visualize the multicolor confetti reporter, 4-μm cryosections were washed with PBS (pH 7.4) to remove OCT and then blocked with Background Buster (Accurate Chemical & Scientific, Westbury, NY) for 30 min to minimize nonspecific protein interactions. Endogenous biotin activity was quenched with the avidin/biotin blocking kit (Vector Laboratories). Sections were then incubated overnight at 4°C with the appropriate primary antibodies: guinea pig antibody to nephrin (Fitzgerald Industries, Concord, MA), mouse antibody to synaptopodin (Fitzgerald Industries), rabbit antibodies to podocin (Abcam, Cambridge, MA) and anti-p57 (Santa Cruz Biotechnology). The appropriate biotinylated secondary antibody (Vector Laboratories) was applied, followed by streptavidin and Alexa Fluor 594 conjugate (Life Technologies–Molecular Probes, Grand Island, NY). All immunofluorescence samples were mounted using Vectashield medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Antibodies were not required to detect confetti reporter.

Identifying PECs in Inducible Podocyte Reporter Mice

To determine whether a subset of reporter-labeled podocytes migrated away from the glomerular tuft and began to express PEC proteins de novo, we performed staining for the following markers: claudin-1, PAX8, and SSEA6. For two-dimensional (2D) analysis, 4-μm cryosections were washed with PBS (pH 7.4) to remove OCT and then blocked with an avidin/biotin kit and Background Buster (as described above). Tissue sections were incubated overnight at 4°C with rabbit antibodies to PAX8 (10336-1-AP; Protein Tech Group; Rosemount, IL) and to SSEA6 (a gift from the Irwin Gelman laboratory) (21). Biotinylated anti-rabbit secondary antibody (Vector Laboratories) was applied, followed by streptavidin and Alexa Fluor 594 conjugate (Life Technologies–Molecular Probes). Positive double staining was defined as a yellow-colored cell, which results from overlap between the red (claudin-1, PAX8, SSEA6) and green (ZsGreen) signals, respectively.

For 3D image analysis, 10-μm cryosections were stained with rabbit antibody to claudin-1 (Abcam). Biotinylated anti-rabbit secondary antibody (Vector Laboratories) was applied followed by streptavidin and Alexa Fluor 594 conjugate (Life Technologies–Molecular Probes) as described above.

Triplet Staining for Reporter, Podocytes, and BrdU-Labeled Cells

To detect any proliferating in experimental FSGS, BrdU immunostaining was performed. Paraffin-embedded tissue was processed as described above and incubated at 4°C overnight with an anti-BrdU antibody (GE Healthcare Life Sciences). The podocytes were identified by immunostaining with podocin (Abcam). Living Colors ZsGreen Monoclonal Antibody (Clontech Laboratories, Mountain View, CA) was used to detect the ZsGreen reporter. For podocin-cre/confetti mice, an FITC 488-conjugated anti-GFP antibody, which binds to all immunostaining occurred via precipitation of diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO). Counterstaining was performed with PAS by washing slices in fresh 0.5% periodic acid (Sigma-Aldrich) for 8 min, double-distilled H2O for 5 min, and incubating sections for 10 min at room temperature with Schiff’s reagent (Sigma-Aldrich). Sections were washed twice for 5 min in fresh 0.5% sodium metabisulfate (Sigma-Aldrich) and washed for 5–10 min under running tap water. Slides were dehydrated in ethanol and mounted with Histomount. To measure podocyte density, we used the correction factor method previously reported by Venkatareddy et al. (49).

Assessment of Glomerular Injury and Podocyte Density

Immunostaining for p57 was performed in both reporter mouse strains with periodic acid-Schiff (PAS) counterstaining to quantify podocyte number and assess glomerulosclerosis. In brief, paraffin sections were processed as described above, with antigen retrieval in 1 mM EDTA, pH 8.0. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide and sections were incubated overnight at 4°C with a primary rabbit anti-p57 (1:800; Santa Cruz Biotechnology, Santa Cruz, CA) followed by rabbit-on-rat HRP-polymer (Biocare Medical, Concord, CA). Visualization of
four of the confetti variants (Rockland Immunochemicals), was used to detect all four confetti reporters at the 488-nm wavelength channel (GFP). As a negative control, all staining was performed without primary antibodies.

Either biotinylated anti-rabbit IgG or anti-mouse IgG followed by streptavidin conjugated with Alexa Fluor 488, Alexa Fluor 594, or Alexa Fluor 647 were applied to detect the primary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA).

**Microscopy**

Two-dimensional images were examined using a Leica TCS SPE II laser scanning confocal microscope and an EVOS FL Cell Imaging System using ×400 and ×600 magnification. Z-series stacks were collected on a Leica TCS SPE II laser scanning confocal microscope at 0.5-μm intervals. Imaris 7.3.1 (Bitplane Scientific Solutions, Badenerstrasse, Switzerland) and Volocity (Improvision; PerkinElmer, Waltham, MA) were used to generate 3D reconstructions.

**Statistical Analysis**

Groups were compared using a two-tailed unpaired Student’s t-test with minimum significance set at $P < 0.05$.

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**RESULTS**

**Podocyte Density Is Higher in NPHS2-rtTA/tetO-cre/RS-ZsGreen-R Mice Given Enalapril**

We began by trying to prove that podocyte replacement occurs in this FSGS model of mice with abrupt podocyte depletion induced by a cytotoxic antibody, and that replenishment derives from nonpodocyte origins. Accordingly, we generated an inducible podocyte reporter mouse (NPHS2-rtTA/tetO-Cre/RS-ZsGreen-R) to specifically address this question. Podocyte density was first determined by the correction factor method previously reported by Venkatareddy et al. (49) using p57 staining. Using that method, podocyte density was 48.5% lower on day 7 of FSGS compared with baseline (129.16 ± 17.13 vs. 250.66 ± 9.95 × 10⁶ podocytes/um³ glomerular tuft volume, $P < 0.001$ vs. baseline) (Fig. 2, A, B, and E). Similar to what we recently reported in other mouse strains (7, 54), podocyte density was 30.0% higher at day 28 compared with day 7 (129.16 ± 17.13 vs. 184.33 ± 8.07 × 10⁶ podocytes/um³ glomerular tuft volume, $P < 0.05$ vs. day 7), but did not reach

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**Fig. 2. Podocyte depletion in inducible podocin-reporter mice (NPHS2-rtTA/tetO-cre/RS-ZsGreen-R). Sections were imaged and analyzed using p57 immunostaining followed by podocyte density measurement (49). A–D: representative p57 and periodic acid Schiff (PAS) staining. A: p57 staining (brown, nuclear) is detected in baseline glomeruli. B: at day 7 of FSGS, p57 staining was markedly reduced in glomeruli (marked by dashed circles), consistent with podocyte depletion. Proteinaceous material was noted in some tubules (marked by P). C and D: at FSGS day 28, p57 staining was increased, the majority of glomeruli had a near normal podocyte number, but not all have recovered (marked by circles). E: podocyte density decreased abruptly by FSGS day 7 but was significantly higher in FSGS mice given water only at day 28 (FSGS+water). FSGS and fed enalapril (FSGS+enalapril) showed even greater improvement with an even higher density of podocytes than the FSGS mice that drank water only.
baseline density ($P < 0.05$ vs. baseline). When enalapril was administrated beginning on day 3 of FSGS until death at day 28, podocyte density was 11% higher than in mice that received drinking water alone ($184.33 \pm 8.066$ vs. $205.9 \pm 18.82 \times 10^6$ podocytes/um$^2$ glomerular tuft volume, $P < 0.05$ vs. day 28 + water). These data show that, following an abrupt decline in podocyte density at day 7 in NPHS2-rtTA/tetO-Cre/RS-ZsGreen-R mice, podocyte density was significantly higher by day 28, with a 45.5% replenishment of lost podocytes, and was augmented by enalapril that resulted in a 63.1% replenishment of lost podocytes (Fig. 2, C–E). Replenishment of lost podocytes was calculated from the increase in the average number of podocytes between day 7 and day 28, divided by the average number lost between baseline and day 7 of FSGS.

Partial Repletion in Podocyte Number Derives From a Nonpodocyte Origin

Permanent labeling of podocytes with ZsGreen fluorescence was induced by administering doxycycline (Fig. 1A). Following a wash out period of at least 1 wk, all mice underwent a survival biopsy prior to disease induction to ensure and evaluate reporting in each individual mouse. The data from that kidney biopsy was considered the baseline. Following a surgical healing phase of at least 1 wk, FSGS was induced as described in the methods section (Fig. 1A). As is desired in an inducible reporter mouse, ZsGreen labeling of podocytes was not detected in triple transgenic mice fed standard diet (without doxycycline), nor in double-transgenic mice without the NPHS2-rtTA transgene-fed doxycycline chow (Fig. 1, B and D).

In all mice that were given doxycycline, ZsGreen was detected in a characteristic podocyte distribution when viewed on frozen tissue samples via confocal microscopy (Fig. 1C). Note that ZsGreen detection on frozen tissue samples did not require use of an antibody because the induced and transiently expressed fluorescence was easily detected.

A group of mice were randomly killed at day 7 of FSGS. ZsGreen fluorescence was markedly reduced in segments of a subset of individual glomeruli in focal areas (Fig. 3C). DAPI costaining was also performed to make sure that the segmental decrease in ZsGreen was not simply attributable to injured podocytes “losing” or “leaking” the ZsGreen reporter, but rather that it was due to depleted cells. As shown in Figure 3C, reduced/absent ZsGreen fluorescence on day 7 of FSGS colocalized with segments of absent DAPI nuclear staining, which is consistent with reduced cell number. This is in keeping with the decrease in podocyte density described earlier (Fig. 2). At FSGS day 28, DAPI staining was higher than at day 7, similar to baseline (Fig. 3E). However, ZsGreen fluorescence remained low/absent in many glomerular segments at day 28 despite an increase in DAPI staining (Fig. 3E). Similarly, in FSGS day 28 mice that were given enalapril, DAPI staining was markedly increased compared with what was observed at day 7, but ZsGreen also remained low/absent (Fig. 3G). Compared with baseline, quantitation showed that ZsGreen fluorescence was decreased by 45, 39.5, and 42.1% in mice at day 7, at day 28 in mice that received water, and at day 28 in mice that received enalapril, respectively. Taken together, these results show that there was a marked and sustained decrease in podocyte labeling in FSGS mice. The decrease was not simply due to a leaky reporter because cell number measured by DAPI staining decreased.

We used p57 as a podocyte marker (Fig. 3) as reported previously (7, 34, 36, 46, 54, 56). The podocyte reporter ZsGreen costained with p57, and the results are shown in Figure 3, B, D, F, and H. As expected in mice at baseline, ZsGreen and p57 staining was abundant and colocalized in most cells (Fig. 3B). On day 7 of FSGS, both ZsGreen and p57 decreased together (Fig. 3D). By day 28 of FSGS in mice that were given water, staining for p57 was higher than it was at day 7, but ZsGreen fluorescence remained markedly low (Fig. 3F). Similarly, by day 28 of FSGS in enalapril-treated animals (Fig. 3H), ZsGreen fluorescence remained low, similar to levels at day 7, but there was an even higher number of p57$^+$ cells than in the animals at day 28 of FSGS fed water only.

Taken together, these results show that both ZsGreen-labeled podocytes and the podocyte marker p57 decreased on day 7 of FSGS, consistent with abrupt podocyte depletion, confirmed by reduced DAPI staining. However, at day 28, there was partial repletion of podocytes judged by p57 and DAPI staining. However, this was not accompanied by increased ZsGreen staining. This is consistent with the partial replenishment of podocytes from one or more nonpodocyte sources.

Podocyte Replenishment Derives From Nonpodocyte Sources in Inducible NPHS2-rtTA/tetO-Cre/RS-ZsGreen-R Podocyte Reporter Mice

To further prove that partial replenishment of podocytes in an inducible podocyte reporter with experimental FSGS derives from nonpodocyte sources, double-staining was performed for the permanent podocyte reporter (ZsGreen) and the podocyte proteins podocin (Fig. 4) and synaptopodin (Fig. 5). As expected at baseline (before disease induction), ZsGreen fluorescence (green) (Figs. 4A and 5A) overlapped with podocin (red) (Fig. 4B) and synaptopodin (red) (Fig. 5B), which creates a yellow color (Figs. 4C and 5C). Figure 4, D–F and Figure 5, D–F show that by day 7 of FSGS, the intensity of ZsGreen, podocin, and synaptopodin were all reduced, consistent with podocyte depletion. However, by day 28 of FSGS in the water only group, and even more so in the water-plus-enalapril group, increased staining for podocin and synaptopodin were readily detected (Fig. 4, H and K, and Fig. 5, H and K). In contrast, ZsGreen fluorescence was not increased, but was rather similar to the reduced levels of expression seen at day 7 (Fig. 4, G and J, and Fig. 5, G and J).

The higher expression of three podocyte proteins at day 28 together with increased DAPI staining are consistent with replacement of podocytes. However, because ZsGreen was markedly reduced following disease induction and did not increase thereafter, the source of new podocytes was not native ZsGreen labeled podocytes, but rather from a nonpodocyte source.

Podocyte Regeneration Was Not Due to Podocyte Proliferation

Pulsed doses of BrdU were used to assess proliferation in NPHS2-rtTA/tetO-Cre/RS-ZsGreen-R mice. As expected, BrdU staining was not detected in glomeruli at baseline (Fig. 6A). This was not a false negative because BrdU was

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detected in occasional tubular epithelial cells. BrdU staining was not detected in the glomerular tuft at day 7 of FSGS, but was occasionally observed on the Bowman’s capsule (Fig. 6B). Likewise, on day 28 of FSGS, in mice that were treated with water and enalapril, BrdU staining was not detected in the glomerular tuft (Fig. 6, C and D), similar to what we reported previously in other mouse strains in this model (7, 12). These results are consistent with the absence of proliferation in cells contributing to the partial replenishment of podocytes in FSGS.

Fig. 3. Podocyte replenishment from nonpodocyte sources in inducible NPHS2-rtTA/tetO-cre/RS-ZsGreen-R podocyte reporter mice. A and B: at baseline (after reporter induction with doxycycline; before disease induction). A: administration of doxycycline permanently labels podocytes with the ZsGreen reporter, which is detected without the use of an antibody and green fluorescence in a typical podocyte distribution. B: representative photograph showing podocyte staining for p57 (red, nuclear, dashed arrow) and podocytes expressing both ZsGreen and p57 (yellow, solid arrows). C and D: at day 7 (d7) of FSGS. C: following administration of a cytotoxic antiglomerular antibody, there is a segmental decrease in ZsGreen reporter fluorescence at day 7 of FSGS (example is demarcated by the white line), which overlaps with a corresponding absence of cell nuclei (DAPI staining), coincides with a decrease in p57 staining (D). E and F: at day 28 (d28) of FSGS. E: there were more cell nuclei at day 28 of FSGS, but ZsGreen fluorescence remained low in segments where DAPI was detected. Glomerulus is demarcated by the white dashed circle. F: despite substantial increases in staining for p57, there was little if any increase in fluorescence for the ZsGreen reporter. G and H: enalapril-treated FSGS mice. Enalapril was added to the drinking water on day 3 of FSGS and continued until death at day 28. G: although there were more nuclei and p57+ cells at day 28 of FSGS+enalapril, ZsGreen fluorescence remained low in glomeruli.
Fig. 4. Podocin staining in NPHS2-rtTA/tetO-cre/RS-ZsGreen-R podocyte reporter mice. Confocal images of ZsGreen reporter (green, left), podocin staining (red, middle), and overlap of expression when the images are merged (yellow, right). A–C: at baseline. ZsGreen reporter (A) colocalized with podocin (B) in characteristic podocyte distribution and creates a merged yellow color (C, solid arrows). Dashed arrows indicate podocin-stained podocytes that are ZsGreen negative. D–F: at day 7 of FSGS. ZsGreen fluorescence (D) was markedly reduced at day 7 of FSGS, consistent with a decrease in podocin staining (E). F: colocalization of two colors (ZsGreen and podocin) (solid arrows). G–I: at day 28 of FSGS. ZsGreen fluorescence (G) was not increased compared with day 7 of FSGS. Podocin staining (H) was readily detected in podocytes. Expression and staining overlaps to create a yellow color (I, solid arrows). Dashed arrows indicate podocin-stained podocytes that are ZsGreen negative. J–L: Enalapril-treated mice at day 28 of FSGS. ZsGreen reporter (J) remained low. Podocin staining (K) was readily detected in podocytes. Expression and staining overlaps to create a yellow color (L, solid arrows). Dashed arrows indicate podocin-stained podocytes that are ZsGreen negative. These results show that podocytes do not regenerate themselves, but rather they derive from a nonpodocyte source.
Podocyte Regeneration in NPHS2^{Cre}/R26R-Confetti^{TG/WT} Mice with Experimental FSGS

Next, to further confirm podocyte regeneration in this model following podocyte depletion, we utilized NPHS2^{Cre}/R26R-Confetti^{TG/WT} mice, which are podocyte-specific confetti reporter mice (47). The average number of podocytes per glomerular cross-section, as measured by p57 staining, was significantly lower at day 7 (5.6 ± 0.3 vs. 9.9 ± 0.1, \( P < 0.0001 \) vs. baseline). However, at day 28 of FSGS, podocyte number was significantly higher than it was at day 7 (7.6 ± 1.3 vs. 5.6...
Fig. 6. Podocyte replenishment was not due to podocyte proliferation in NPHS2-rtTA/tetO-Cre/RS-ZsGreen- R mice. A: representative confocal image showing that podocytes (podocin/ZsGreen” cells) and BRDU do not overlap. BRDU is detected in occasional tubular epithelial cells but was not present in glomeruli at baseline (blue, solid arrow). B: BRDU staining increased at day 7 of FSGS (dashed arrows) but is not detected in podocytes on the glomerular tuft. C and D: on day 28 of FSGS in both the water (C) and enalapril-treated mice (D), BRDU staining does not colocalize with podocin” or ZsGreen” cells. However, it should be noted that there were occasional BRDU” cells along the Bowman’s capsule (see Fig. 4D, dashed arrow) and migration of ZsGreen” podocytes to the Bowman’s capsule (see Fig. 4D, arrowhead).

Regeneration of Podocytes in NPHS2Cre/R26R-ConfettiTG/WT Mice Coexpress Podocyte Proteins

To ensure that the fluorescence for each of the multicolored reporter-labeled podocytes (no antibody needed) indeed coexpressed podocyte proteins in NPHS2Cre/R26R-ConfettiTG/WT mice, cryosections were stained with antibodies to podocin (Fig. 8, A–C), synaptopodin (Fig. 8, D–F), and nephrin (Fig. 8, G–I).

Podocin and reporters. All four reporters (green, red, blue, yellow) overlapped with podocin staining (magenta) in baseline mice prior to FSGS (Fig. 8A). When a reporter and podocin overlap, they create a white color. By day 7 of FSGS, individual podocyte reporters, as well as podocin staining, decreased in segments of glomeruli (Fig. 8B). However, by day 28, the majority of glomeruli once again contained multiple reporter-labeled cells, which colocalized with podocin staining (Fig. 8C).

Fig. 5. Synaptopodin staining in NPHS2-rtTA/tetO-cre/RS-ZsGreen-R podocyte reporter mice. Confocal images of ZsGreen reporter (green, left), synaptopodin staining (red, middle), and overlap of expression when the images are merged (yellow, right). A–C: at baseline, ZsGreen reporter (A) colocalized with synaptopodin (B) in a characteristic podocyte distribution and creates a merged yellow color (C, solid arrows). D–F: at day 7 of FSGS, ZsGreen fluorescence (D) was markedly reduced at day 7 of FSGS, consistent with a decrease in synaptopodin staining (E). F: colocalization of two colors (ZsGreen and synaptopodin) (solid arrows). Dashed arrows indicate synaptopodin-stained podocytes that are ZsGreen negative. G–I: at day 28 of FSGS, ZsGreen fluorescence (G) was not increased compared with that on day 7 of FSGS. Synaptopodin staining (H) was readily detected in podocytes. Expression and staining overlaps to create a yellow color (I, solid arrows). Dashed arrows indicate synaptopodin-stained podocytes that are ZsGreen negative. These results show that podocytes do not regenerate themselves, but rather they derive from a nonpodocyte source.
Synaptopodin and reporters. All four reporters (green, red, blue, yellow) overlapped with synaptopodin staining (magenta) in baseline mice prior to FSGS (Fig. 8D). By day 7 of FSGS, individual podocyte reporters, as well as synaptopodin staining, decreased in segments of glomeruli (Fig. 8E). However, by day 28, the majority of glomeruli once again contained multiple reporter-labeled cells, which colocalized with synaptopodin staining (Fig. 8F).

Nephrin and reporters. All four reporters (green, red, blue, yellow) overlapped with nephrin staining (magenta) in baseline mice prior to FSGS (Fig. 8G). By day 7 of FSGS, individual podocyte reporters, as well as nephrin staining, decrease in segments of glomeruli (Fig. 8H). However, by day 28, the majority of glomeruli once again contained multiple reporter-labeled cells, and these colocalized with nephrin staining (Fig. 8I).

These data show that the four podocyte reporters and three podocyte proteins decrease by day 7 in this model of FSGS, consistent with podocyte depletion, similar to what we have reported with several other mouse strains (32, 34, 54, 55). However, by day 28 of disease, the podocyte number was significantly increased. The appearance of all four reporters in recovered glomeruli and the de novo expression of podocin, nephrin, and synaptopodin in re-
porter-labeled cells are consistent with podocyte regeneration.

Augmented Podocyte Labeling in NPHS2Cre/R26R-ConfettiTgWt Mice Is Not Due to Podocyte Proliferation

Because podocytes are permanently labeled, the increase in the four reporters could have potentially been due to proliferation of podocytes. To rule out proliferation of these remaining podocytes, sections were stained for BrdU and visualized with all four reporter colors converted to green. BrdU was not detected in reporter-labeled podocytes at baseline, nor at day 7 or day 28 (Fig. 9). This was not a false negative because BrdU staining in tubules and occasional PECs in disease served as internal positive controls (Fig. 9, A–C). Taken together, this model of FSGS was characterized by an abrupt depletion of podocytes, followed by partial replacement. Because podocyte proliferation of remaining podocytes was not detected, these data are consistent with podocyte replacement from one or more nonpodocyte sources.

Labeled Podocytes Are Detected on the Bowman’s Capsule in Inducible Podocyte Reporter Mice With FSGS

Several groups of researchers have reported that podocytes migrate to the Bowman’s capsule in experimental disease (14, 26, 40). In the current study, following doxycycline induction of permanent podocyte labeling (with ZsGreen) in NPHS2-rtTA/tetO-Cre/RS-ZsGreen-R mice, ZsGreen was limited to
podocytes (Fig. 1C). No ZsGreen was detected in cells lining the Bowman’s capsule in mice at baseline.

In inducible podocyte reporter mice with FSGS, occasional ZsGreen-labeled cells were detected along Bowman’s capsule on days 7 and 28 (Fig. 10). Quantification of all glomeruli on the kidney section showed that at day 7 of FSGS, 0.743% of glomeruli had ZsGreen cells lining Bowman’s capsule (P < 0.001 vs. baseline), and that at day 28, 0.6% of glomeruli have ZsGreen cells lining Bowman’s capsule (P < 0.01 vs. day 7). In FSGS mice given enalapril, 4.7 ± 0.743% of glomeruli had ZsGreen cells lining Bowman’s capsule at day 28 (P < 0.05 vs. FSGS + water) (data not shown).

To determine whether labeled podocytes that migrated to the Bowman’s capsule coexpressed proteins that are considered markers of PECs, costaining was performed with ZsGreen, and shown). capsules at 0.743% of glomeruli had ZsGreen cells lining Bowman’s capsule.

DISCUSSION

When glomerular disease-induced podocyte injury leads to apoptosis, detachment, and other forms of loss, the inability of podocytes to self-replicate leads to an overall decrease in their number (8, 42). A decrease in adult podocyte number is a critical determinant for the onset and magnitude of glomerulosclerosis (3, 23, 35). We and others have reported that adult terminally differentiated podocytes cannot proliferate because of cell cycle inhibition (53) or mitotic catastrophe (19), and adult podocytes cannot self-renew following abrupt or chronic depletion in glomerular diseases, nor can they replicate in the aging kidney (32, 50). Yet several lines of evidence show that following a decrease in number, podocyte density can improve under certain conditions (24). For example, ACE inhibition, (56) angiotensin-receptor blockers (48), corticosteroids, (54) retinoids (55), and an improved diabetic milieu (57) can enhance podocyte number when they are administered following podocyte depletion under experimental conditions. The accompanying partial or full replenishment occurs in the absence of podocyte proliferation. Several groups of researchers, including ours, have data supportive of the notion that podocytes can be partially or fully replenished from nonpodocyte sources (2, 34). In the current studies we used labeling and cell lineage tracing in podocyte transgenic reporter mice to show that in an experimental model of abrupt podocyte depletion, podocytes themselves cannot self-renew, but that their number can be partially replenished in the absence of proliferation.

To directly address podocyte replenishment using transgenic labeling, we began by generating an inducible podocyte reporter mouse (NPHE2-rtTA/tetO-Cre/RS-ZsGreen-R). The strength of this process is that the reverse tetracycline-controlled transactivator (rtTA) translocates to the nucleus and recognizes the tetO sequences of the tetO-Cre transgene only when mice are given doxycycline. Cre-recombinase then excises the loxP-flanked STOP cassettes so that podocytes are specifically and permanently labeled by the ZsGreen reporter, only during a defined temporal window. Thus following the doxycycline wash out period, any cell labeling (ZsGreen) had only during a defined temporal window. Thus following the doxycycline wash out period, any cell labeling (ZsGreen) had
injured glomeruli at day 7, measured by segmental decreases in p57 staining. This was accompanied by a decrease in ZsGreen fluorescence. The latter did not require an antibody for detection. To ensure that the decrease in p57 and ZsGreen fluorescence was not simply due to de-differentiation of podocytes or to loss/leakage of the ZsGreen reporter, DAPI staining was performed, which labels all cell nuclei. The results showed that in segmental areas of reduced ZsGreen and p57 staining, DAPI staining was not detected. As expected on day 7 in this model of podocyte depletion, staining for the podocyte markers podocin and synaptopodin were also markedly reduced. Taken together, these data prove that a decrease in podocytes occurred.

We have previously reported that in this FSGS model, following abrupt podocyte depletion, there is some partial replenishment of podocytes at day 28 (7). This was based on...
the quantitation of podocyte markers. Moreover, when corticosteroids (54–56), retinoids (21), and ACE inhibitors (55) are given to FSGS mice of this model following depletion of podocytes, podocyte replenishment, as judged by measuring podocyte markers, is further augmented. In the current study, the results were similar in that staining for p57, podocin, and synaptopodin was greater on day 28 than it was on day 7, and it was augmented by enalapril. Noteworthy is that DAPI staining also confirmed a higher number of glomerular cells at day 28. A major finding in the current studies was that, despite increased staining for p57, podocin, and synaptopodin was greater on day 28 than it was on day 7, and it was augmented by enalapril. Noteworthy is that DAPI staining also confirmed a higher number of glomerular cells at day 28. A major finding in the current studies was that, despite increased staining for p57, podocin, and synaptopodin, and DAPI, ZsGreen fluorescence did not increase at day 28. Indeed, quantitation of ZsGreen showed that there was not an increase from day 7 to day 28 in disease in FSGS mice given water or enalapril. These data are consistent with the notion that, in an inducible podocyte reporter mouse following abrupt podocyte depletion, partial replenishment of podocytes did occur and was augmented by enalapril, but that the source of new podocytes was derived from nonpodocyte sources.

BrdU was administered frequently over the course of disease to measure cumulative DNA synthesis. BrdU staining was not detected in the glomerular tuft in FSGS mice. This was not a false negative because, as expected, BrdU staining was detected in tubular cells and used as an internal positive control. Moreover, as expected, proliferation of podocytes measured by BrdU staining was absent, as we have previously reported (7).

To determine whether podocyte replacement in this FSGS model was monoclonal or polyclonal in origin, we used NPHS2^Cre/R26R-Confetti^TG/WT mice, previously described by Tao et al. (47). This breed enables simultaneous examination of up to four different reporters for podocytes, all driven by the podocin promoter (14, 47). As expected in mice at baseline and before disease induction, individual podocytes were randomly labeled with GFP, YFP, CFP, and RFP in similar distributions. Similar to reports by Tao et al. (47) describing the doxorubicin model of FSGS in the reporter mouse, our results showed a decrease in all four podocyte reporters in a cytotoxic antibody model of experimental FSGS. By day 28 of disease, when podocyte density was higher, all four reporters were again detected. Moreover, by day 28, all four reporters colocalized with the podocyte proteins podocin, synaptopodin, and nephrin. BrdU staining was not detected in labeled podocytes. These data strongly support podocyte regeneration from a nonpodocyte source that is polyclonal in origin, because regeneration from the native podocytes would have been BRDU positive.

We recognize that a potential weakness is that podocyte labeling in the NPHS2^Cre/R26R-Confetti^TG/WT mice used in these studies was not driven by an inducible promoter, and therefore, the podocin-driven cre-recombination was always on (37). However, we reasoned that the results are robust because the DAPI data supported depletion of podocytes and subsequent replenishment of podocytes. Thus the changes in podocyte labeling could not be attributed simply to podocyte injury causing loss of reporter labeling. The nonpodocyte source or sources appear to activate known podocyte markers of expres-

Fig. 11. Permanently labeled podocytes coexpress the PEC protein PAX8 in FSGS. A: at baseline. Podocytes labeled with the ZsGreen reporter (green, dashed arrows) were restricted to the glomerular tuft; staining for PAX8 (red, solid arrow) was restricted to PECs along Bowman’s capsule. B: at day 7 of FSGS. There were segmental decreases in ZsGreen reporter (green), PAX8^+^ cells were greater in number and formed cellular crescents along Bowman’s capsule (solid arrow). C: at day 28 of FSGS in mice fed water. ZsGreen fluorescence labeling podocytes remains reduced overall, with a subset having migrated to Bowman’s capsule and colocalized with the PEC protein PAX8 (yellow, dashed arrow). This is consistent with podocytes migrating to the Bowman’s capsule, where they coexpress a PEC protein. D: at day 28 of FSGS in mice given enalapril. ZsGreen labeled podocytes (green) have migrated to the Bowman’s capsule and coexpress PAX8 (yellow, dashed arrow).
sion as part of the replenishment/regeneration process, and given the multireporter characteristics of the confetti reporter utilized, more than one precursor cell per glomerulus appears to be involved (at least from the stage of activation of podocin). The fact that both yellow or green and red or blue cells are observed in any one glomerulus after injury support this assertion, even if “flipping” is occurring. If an inducible model incorporating the confetti reporter prior to injury were to be used, we would suggest that we would simply observe fewer cells of multiple colors after injury. Regarding bicoloration we and others observed, it is unclear at present how we should interpret Nphs2 expression in podocytes. Neither Tao et al. (47) nor Hackl et al. (14) mention observing bicolored cells in podocytes when they used podocin-driven constitutive Cre, although Tao et al. refer to the ability to detect bicolored cells when using a genetic background homozygous for confetti.

The intent of the current studies was to prove that following abrupt depletion of podocytes at day 7 of FSGS by 48.5% of normal, podocytes can be replenished, albeit partially, from nonpodocyte sources. Although we did not determine the source or sources of podocyte replacement, two sources need to be considered: glomerular PECs (7, 14–16, 18, 50), and cells of renin lineage (1, 20a, 32–34). Both are increased following ACE inhibition (2, 20a). Using fate-mapping techniques, Appel et al. (2) and Wanner et al. (50) showed that in adolescent reporter mice, a subset of PECs gives rise to podocytes. Seminal studies by Romagnani and colleagues have convincingly shown that a subpopulation of PECs serve as adult podocyte progenitors (18, 38, 39). One of the earliest clues of a role for PECs was the identification of a small number of cells lining Bowman’s capsule, in human kidneys, that coexpress both a podocyte and a PEC protein (5). Such cells, called different names by different authors (44), have since been shown to exist in rats (10, 11, 13) and mice (2, 13). The PEC has been considered the cell of origin on the basis of its location. Smeets and Moeller confirmed significant recruitment of labeled PECs into glomerular tuft in juvenile and adult mice (2, 20). We and others have shown that the number of cells lining Bowman’s capsule that coexpress both a podocyte and a PEC protein increase in states of podocyte depletion (7) and in aging (36), and that their quantity is augmented by ACE inhibition (56), corticosteroids (54), and retinoids (55). The location suggests that these cells were of PEC origin, and follow-up studies in an inducible PEC reporter mouse confirmed this origin in many cells that coexpress PEC and podocyte proteins.

More recently, several groups have shown that podocytes themselves do migrate to Bowman’s capsule following podocyte depletion (9, 14, 26, 27, 40, 41). Hackl et al. (14) used an inducible reporter mouse; however, no researchers have reported whether migrating podocytes begin to coexpress PEC proteins. Accordingly, we used the inducible podocyte reporter...
mice in the current study to determine whether this phenomenon exists. As expected, at baseline before disease in mice that received doxycycline to induce permanent podocyte labeling, ZsGreen fluorescence was restricted to podocytes in the glomerular tuft, and was not detected along Bowman’s capsule. However, in mice with FSGS at days 7 and 28, a subset of glomeruli contained ZsGreen-labeled cells lining Bowman’s capsule. Moreover, 60% of podocytes that migrated to Bowman’s capsule began to coexpress three PEC proteins (claudin-1, Pax8, SSeCKS). These results show that in this model of FSGS, the sources of cells lining Bowman’s capsule that coexpress both a PEC and podocyte protein derive from both PEC and podocyte origins. The biological significance of these findings is not yet known. Using immunostaining techniques, Kuppe et al. (17) recently reported that podocytes adhere to Bowman’s capsule. Our studies using a genetic method support this notion, and together, one can speculate that synchial Kuppe et al. (17) recently reported that podocytes adhere to Bowman’s capsule. Moreover, 60% of podocytes that migrated to Bowman’s capsule contained ZsGreen-labeled cells lining Bowman’s capsule. Furthermore, mice were unable to endogenously regenerate podocytes during the repair of podocyte-specific injury.  

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


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**PODOCYTE REPLENISHMENT**

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