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Glomerular parietal epithelial cells contribute to adult podocyte regeneration in experimental focal segmental glomerulosclerosis

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Abstract

Since adult podocytes cannot adequately proliferate following depletion in disease states there has been interest in the potential role of progenitors in podocyte repair and regeneration. To determine if parietal epithelial cells (PECs) can serve as adult podocyte progenitors following disease-induced podocyte depletion, PECs were permanently labeled in adult PECrtTA/LC1/R26 reporter mice. In normal mice, labeled PECs were confined to Bowman's capsule, while in disease (cytotoxic sheep anti-podocyte antibody), labeled PECs were found in the glomerular tuft in progressively higher numbers by days 7, 14 and 28. Early in disease, the majority of PECs in the tuft co-expressed CD44. By day 28, when podocyte numbers were significantly higher and disease severity was significantly lower, the majority of labeled PECs co-expressed podocyte proteins but not CD44. Neither labeled PECs on the tuft, nor podocytes stained for the proliferation marker BrdU. The *de novo* expression of phospho-ERK colocalized to CD44 expressing PECs, but not to PECs expressing podocyte markers. Thus, in a mouse model of focal segmental glomerulosclerosis typified by abrupt podocyte depletion followed by regeneration, PECs undergo two phenotypic changes once they migrate to the glomerular tuft. Initially these cells are predominantly activated CD44 expressing cells coinciding with glomerulosclerosis, and later they predominantly exhibit a podocyte phenotype which is likely reparative.

Keywords

progenitor; glomerulosclerosis; CD44; ERK; glomerulus; podocin; nephrin; synaptopodin

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Introduction

Classic focal segmental glomerulosclerosis (FSGS), the leading cause of nephrotic syndrome in the US, is considered a disorder of adult terminally differentiated glomerular visceral epithelial cells, also called podocytes.[1] Classic FSGS is characterized by a depletion in podocytes,[1] and when podocyte number is depleted below a critical threshold, glomerulosclerosis ensues.[2, 3] In contrast to other kidney cells, adult podocytes are unable to adequately proliferate, and thus cannot replenish their numbers following depletion in disease.[4] Thus, several groups are attempting to identify possible progenitors that might replace podocytes.

Several lines of evidence support that neighboring glomerular parietal epithelial cells (PECs) are attractive candidates to serve as podocyte progenitors.[5-10] In reporter mice, a subset of adolescent podocytes do arise from PECs.[11-13] In adults, a subset of cells lining Bowman's capsule co-express both a podocyte and a PEC protein in normal rats, mice and humans, raising suspicion that PECs serve a podocyte progenitor role.[10, 14, 15] Furthermore, their number increases when diseased animals are given ACE-inhibitors[16], corticosteroids[17] and retinoids[18] and when diabetic mice have their metabolic milieu improved.[19] A subset of human PECs co-express progenitor markers.[20] When isolated and injected into mice with experimental FSGS, they engraft within the glomerular tuft, and begin to express markers, and acquire ultrastructural features of podocytes.[21] Recent studies showed that PECs adopt a podocyte phenotype when miR-193a levels are lowered.[22]

However, several studies have dampened enthusiasm for the notion that PECs serve as adult podocyte progenitors. Using reporter mice, Berger et al,[13] Sakamoto et al,[23] Hackl et al,[24] and Miyazaki et al [25] showed that in disease, adult podocytes move from the tuft to Bowman's capsule, and begin to co-express PEC proteins, and may even become PECs. Cell fate mapping studies of PECs have failed to show a progenitor niche in models of aging,[12, 13] and glomerular hypertrophy following partial nephrectomy.[13] Studies also showed that the expression of podocyte specific proteins in PECs might simply reflect alterations in protein degradation.[26]

Taken together, the controversial nature for the role for PECs as potential adult podocyte progenitors may depend on model systems used, species studied and other factors. The purpose of the current study was to determine if a subset of adults PECs that migrate to the glomerular tuft acquire features of podocytes, following an abrupt depletion in podocyte number that leads to glomerulosclerosis. Accordingly, an experimental model of FSGS characterized by an abrupt and substantial decline in podocyte number[16-18, 27, 28] followed by a phase of regeneration was induced in reporter mice where PECs were fate mapped following the induction of permanent labeling of adult PECs.

Results

Expression of β-Gal Reporter was induced in PECs in reporter mice—To determine if glomerular parietal epithelial cells (PECs) serve as possible adult podocyte progenitors in this FSGS model of abrupt and marked podocyte depletion, studies were

performed in PEC-Reverse Tetracycline-Transactivator (rtTA)/LC1/Rosa26 reporter (R26R) (PEC-reporter) mice. As reported previously,[11, 29] doxycycline induced the expression of β-gal in PECs, measured by both enzymatic X-gal staining, and by immunohistochemistry for β-gal (Figure S1). In normal mice prior to disease induction (i.e. at baseline), both the enzymatic reaction and fluorescent staining for β-gal was restricted to cells along Bowman's capsule within the glomerulus (Figure S1). Approximately 70% of PECs reported positive at baseline, similar to previously reported. [11, 29] As expected, mice that did not receive doxycycline administration, or mice without all the transgenes did not have labeled PECs (data not shown).

Glomerulosclerosis and albuminuria in PEC reporter mice—Experimental FSGS with a significant decrease in podocytes accompanied by glomerulosclerosis was successfully induced in PEC-reporter mice. Immunostaining for sheep IgG was performed to confirm deposition of the podocyte cytotoxic antibody, and examples are shown in Figure S2. Despite antibody binding to all glomeruli, and as expected with focal glomerular diseases, an average of 22±5% of glomeruli had evidence of glomerulosclerosis at d7 ($P < 0.00001$ vs. baseline)(Figure 1). Glomerulosclerosis increased to involve 33±8% of all glomeruli by d14 ($P<0.00001$ vs baseline). However by d28, the number of glomeruli with evidence of disease was lower on average than d14, but was not significant due to the large variation between animals at this time point (24±7%; $P = 0.06$ vs d14). Subsequent analysis included all glomeruli, and was not limited to the subset with sclerosis.

Within individual glomeruli, disease severity, assessed by scoring 55±5 gloms in each animal on a scale of (0-4),[27] was progressively higher (worse disease) at d7 (0.38±0.12 vs. 0.03±0.03, $p<0.0001$vs. baseline) and d14 (1.00±0.30, $p<0.001$ vs. d7)(Figure 1). Importantly, the sclerosis score was significantly lower by d28 (0.55±0.12, $P<0.01$ vs. d7 and d14). Albuminuria was assessed in individual mice at all time points by measuring the urinary albumin-to-creatinine ratio. The albumin to creatinine ratio increased significantly at day 7 (335.3±55.51 vs. 0.42±0.16 at baseline, $P<0.001$). By day 14, albuminuria was reduced significantly (159.5±40, $P<0.01$ vs d7). There were no significant changes at day 21 (121.8±15.93) and day 28 (143.7±23.39). Taken together, proteinuria and glomerulosclerosis both increase early in disease, with significant improvements by day 28.

Podocytes regenerate in PEC reporter mice with FSGS independent of proliferation—Although there was no significant differences in glomerular tuft area at any time point in disease compared to baseline, unbiased stereology was used to measure the average number of podocytes per mm^2 of glomerular tuft, by counting the number of p57 positive cells/glomerular cross section/tuft cross-sectional area, and the results are shown in Figure 1. Podocyte number was significantly lower at d7 (2159±448 vs 3355±398, $p<0.001$ vs. baseline, a 36% decrease) and at d14 (1712±263 vs 3355±398, $P<0.0001$ vs baseline, a 49% decrease). The decrease in the average number of podocytes between d14 and d7 was not significant (1712±263 vs 2159±448, a 21% decrease; $P>0.05$ vs. d7, $P<0.0001$ vs. baseline) (Figure 1). However the number of podocytes was 44% higher at d28 (2461±452 vs 1712 ±263, $P<0.01$ vs. d14).

Because of the potential concern that the decrease in p57 staining at d7 and 14 might simply reflect podocyte injury or dedifferentiation, and not actual loss of podocytes, co-staining for the nuclear marker DAPI with both podocin and p57 was performed. As shown in supplemental Figure S3, in areas of segmental reduction and/or absence of staining for podocin and p57, DAPI staining was also absent. This is consistent with actual depletion of podocytes. If injured podocytes had simply dedifferentiated, these areas would have still contained nuclei. These data show that (i) the decrease in podocyte number and podocyte markers was indeed due to loss of podocyte and not simply a manifestation podocyte injury or dedifferentiation; (ii) the higher number of podocytes observed at day 28 compared to earlier time points was due to regeneration of podocytes and not simply repair or redifferentiation. In order to determine if the higher number of podocytes at d28 was due proliferation of podocytes, BrdU staining was performed (Supplementary Figure 4). BrdU was rarely detected in the glomerular tuft at any time point, suggesting that another source was responsible for the 44% increase in podocyte number at d28 (see below).

Taken together, this data was consistent with podocyte regeneration and the improvement in glomerulosclerosis, was consistent with repair.

PECs migrated onto the glomerular tuft in FSGS—In order to determine if PECs migrated onto the glomerular tuft following podocyte depletion, and then to determine the characteristics of the migrating PEC subpopulations in this model of FSGS, LacZ (the reporter for PECs) was detected by enzymatic reaction for X-gal, and immunohistochemistry for β-gal (Figure 2). Figure 2A shows that the average percent of glomeruli with β-gal positive cells in the tuft was 2.7±2.3% at baseline (per 100 counted). The number of glomeruli with β-gal positive cells in the tuft increased progressively at d7 (12.7±7.2%, $P<0.01$ vs. baseline), d14 (16±3.2%, $P<0.05$ vs. d7), and at d28 (26.75±5.4%, $P<0.01$ vs. d14). In mice with FSGS, 5-10% of glomeruli with labeled PECs in the tuft, β-gal staining was decreased along Bowman's capsule. One explanation for this is PEC injury in this model due to proteinuria.[44] Another is that labeled PECs that migrated to the tuft are replaced by other progenitors.[27]

We next used unbiased stereology to further analyze those glomeruli with β-gal positive cells in the tuft. The average number of β-gal positive cells within the tuft per mm^2 cross-sectional area was 477±187 at baseline, 865±392 at d7 ($P=0.05$ vs. baseline), 614±269 at d14 (P not significant vs. d7), and 1528 ±241 at d28 ($P<0.0001$ vs. baseline; $P<0.001$ vs. d14; $p<0.01$ vs d7) (Figure 2B). Figure 2C-N shows examples of low and high power pictures of X-gal and PAS staining, and β-gal staining at baseline and at the different disease time points.

These data show that in a PEC reporter mouse where labeling was induced by doxycycline prior to disease, the percentage of glomeruli with labeled PECs in the tuft increased progressively during disease, and that within these glomeruli, the average number of β-gal positive cells that moved from Bowman's capsule on to the glomerular tuft also increased significantly.

A subset of PECs on the glomerular tuft co-expressed podocyte markers— Having determined that PECs moved onto the glomerular tuft following podocyte depletion in FSGS, double immunohistochemistry was performed for the PEC reporter β-gal, and the podocyte markers p57, podocin or synaptopodin in order to determine if a subset of these PECs co-expressed podocyte proteins. As shown in Figures 3 & 4, a subset β-gal cells on the glomerular tuft co-expressed p57, podocin and synaptopodin. The number of β-gal$^+$ p57$^+$ cells within the glomerular tuft was higher at d7 (432±332 vs. 184±238, P not significant vs. baseline), d14 (421±209, P not significant vs. D7) and d 28 (1128±171 P<0.001 vs. d14) (Figure 3A). Figure 3B-I shows examples of co-staining for β-gal and p57. The number of β-gal$^+$ podocin$^+$ cells within the glomerular tuft was not changed at d7 (268±184 vs. 352±308, P not significant vs. baseline). However, the number of β-gal$^+$ podocin$^+$ cells was higher at d14 (753±189, P <0.01 vs. D7) and d28 (1089±206, P<0.05 vs. D14) (Figure 3J). Figure 3K-R shows examples of co-staining for β-gal and podocin. A subset of labeled PECs on the tuft also co-expressed synaptopodin (β-gal$^+$ synaptopodin$^+$) (Figure 4).

This data shows that a significant number of β-gal$^+$ cells (i.e. permanently labeled PECs) that migrated on to the glomerular tuft during disease co-expressed three different podocyte proteins (p57, podocin, synaptopodin). Their number increased during the course of disease, and coincided with a higher number of podocytes a day 28 compared to earlier disease time points.

PECs on the glomerular tuft did not stain for BrdU—Double immunohistochemistry was performed for BRDU and β-gal in order to determine two outcomes: did β-gal labeled PECs proliferate within the tuft or along Bowman's capsule? As previously observed,[16, 17] following the assessment of 100 glomeruli per animal, proliferation was barely detected within the glomerular tuft in this model of FSGS. Quantification of the number of β-gal$^+$BRDU$^+$ cells within the glomerular tuft showed values near zero, which did not change significantly throughout the time course (Supplemental Figure 4). This was not a false negative, because as expected, occasional tubular epithelial cells express BrdU. However, a number of β-gal$^+$BRDU$^+$ cells were noted lining Bowman's capsule, with an average number per glom at d7 (0.64±0.18), d14 (0.46±0.13), and 28 (0.35±0.12) being significantly (P<0.001) higher than at baseline (0.04±0.03). These data show that the number of PECs migrating to the tuft was not “matched” proliferation of β-Gal positive labeled PECs. Finally, proliferating β-Gal positive PECs do not co-express podocyte markers. These data show that podocyte regeneration was accompanied by proliferation of PEC's that were restricted to Bowman's capsule, but not proliferation of native podocytes, or labeled PECs once they migrated to the tuft, under the experimental conditions used in these experiments.

A subset of PECs on the glomerular tuft co-express the activation marker CD44—Smeets et al have shown that CD44 is a marker of activated PECs in experimental and human FSGS.[29-32] As expected, CD44 staining was barely detected in normal glomeruli (Figure 5). However, there was an increase in the number of β-gal cells along Bowman's capsule (i.e. PECs) that co-expressed CD44 in disease. Cells co-staining for CD44 and β-gal on the tuft increased significantly at d7 (3.7±2% vs 0.6±0.4% area per glomerulus with positive signal, p<0.001 vs. baseline), and at d14 (5.8±2.4%; P not significant vs. d7) of

disease. However the number decreased significantly at d28 compared to d14 (3.8±2.6% vs 5.8±2.4%, $p<0.05$ vs. D14).

PECs did not co-express CD44 and a podocyte protein—Because CD44 has been considered a stem/progenitor marker in certain renal [33] and non-renal cells,[34] triple staining was used to determine if a subset of PECs that differentiated into podocytes also co-expressed CD44. No triple staining was detected for β-gal/CD44/synaptopodin at any time point studied (data not shown). These data suggest that CD44 is likely not a progenitor marker for PECs, but rather indicates activation and a pro-fibrotic response as previously reported by Smeets and Moeller. [29, 30, 35, 36]

MAP-Kinase signaling increases in activated PECs—We have previously demonstrated the de novo expression of phospho-ERK in cells lining Bowman's capsule in experimental FSGS.[16, 17, 37] In the current study we sought to determine which subset of PECs co-expressed phospho-ERK in disease. Figure 6 shows three results. First, similar to previous reports,[16, 17, 37] phospho-ERK is not detected in normal mice, but increases in PECs during disease, identified by the detection of β-gal$^+$ phospho-ERK$^+$ cells lining Bowman's capsule. Second, triple staining showed that phospho-ERK was co-expressed in a subset of CD44$^+$ staining cells (i.e. phospho-ERK$^+$β-gal$^+$CD44$^+$ cells) restricted to Bowman's capsule. Third, phospho-ERK was not detected in the subset of PECs co-expressing a podocyte protein (i.e. phospho-ERK$^+$β-gal$^+$synaptopodin$^+$ cells). Taken together, phospho-ERK co-localized to the subset of activated PECs that express CD44 along Bowman's capsule, and in a subset of activated PECs that migrated to the glomerular tuft.

Discussion

A large body of literature shows that following injury in glomerular disease, a decrease in podocyte number below a certain threshold, leads to glomerular scarring.[2] Experimental and human studies have shown that podocytes can regenerate.[16-19, 38-40] However, because adult podocytes do not proliferate, any regeneration is dependent on podocyte progenitors.[6] The neighboring glomerular parietal epithelial cells (PECs) have been of particular interest as a potential source of podocyte progenitors.[7, 41] This paradigm is perhaps better established in human studies than in experimental studies. Using an inducible and permanent genetic labeling approach to fate map PECs, the current study shows that following a marked abrupt depletion of podocytes, a subpopulation of PECs migrate from Bowman's capsule to the glomerular tuft, where they acquire several classical characteristics of podocytes.

We next investigated the potential role of PECs as podocyte progenitors, by inducing the aforementioned model of FSGS in PEC-Reverse Tetracycline-Transactivator (rtTA)/LC1/Rosa26 reporter (R26R) reporter mice. Following the administration of a cytotoxic anti-podocyte antibody, podocyte number decreased by 29% from baseline by d7, with a nadir of depletion at d14 by 46% from baseline. Similar to other reports,[2, 3] this marked reduction in podocyte number was initially accompanied by the development of glomerulosclerosis, and albuminuria. The earlier phase of loss was followed by a phase of regeneration, where podocyte number was significantly higher at d28 of disease with an increase of 51% from

d14, which put the average podocyte loss at only 18% less than baseline, a significant recovery. This was temporally accompanied by improved glomerulosclerosis and albuminuria.

The strength of this inducible fate mapping approach is that PECs are only permanently labeled in the temporal window following the administration of doxycyline. Upon withdrawal of doxycycline, no further cells can be labeled, and we allowed a washout period before inducing disease. Our results show that in normal reporter mice given doxycycline, labeled PECs within glomeruli were essentially restricted to Bowman's capsule as previously reported.[11] A very rare labeled PEC was detected in glomerular tufts (less than 2.7±2.3% of glomeruli). However, following the depletion of podocytes in FSGS, labeled PECs were readily detected in glomerular tufts in approximately one quarter of all glomeruli. In addition, the number of glomerular tufts containing labeled PECs increased temporally during the course of disease. Moreover, within individual glomeruli, the number of labeled PECs within the tuft increased over the course of disease. These findings lead to us ask what was the nature of the subset of PECs that migrated from Bowman's capsule to the glomerular tuft during the course of FSGS.

As expected in normal reporter mice following induction with doxycycline, labeled PECs along Bowman's capsule did not co-express any of the three podocyte markers. A first major finding was that in FSGS, a subset of labeled PECs (β-gal positive) located in a glomerular tuft location co-expressed three podocyte markers - p57, podocin or nephrin. Their number increased progressively during the course of disease, and was highest at d28 when podocyte number was at its maximal of the times examined following the onset of FSGS. By d28, when podocyte number was significantly higher than the earlier time points and was about 80% of normal, glomeruli with labeled PECs co-expressing podocyte proteins were often times indistinguishable from normal, and scarring was barely detected within these individual glomeruli.

A BrdU pulse chase was given at the onset of disease to determine if podocyte regeneration was due to proliferation. Our data showed that BrdU staining was rarely detected in native podocytes, nor in labeled PECs on the tuft that co-expressed podocyte proteins. This is consistent with the absence of podocyte proliferation as an immediate response of disease induction. Together, these data show that in this model of FSGS, a subset of cells of PEC origin that migrated to the glomerular tuft differentiated into podocytes (defined by the de novo expression of three podocyte markers). Because of the inducible nature of this cell fate mapping approach, it is highly unlikely that podocytes co-expressing β-gal staining was due to de novo (re) expression of β-gal. Moreover, disease was only induced following a washout period (i.e. no more doxycycline was administered), and therefore, any cells detected on the tuft (or elsewhere) had to arise from the originally labeled cells.

The findings in the current study that PECs might serve as adult podocyte progenitors are consistent with reports in human systems.[21] However, although PECs do give rise to a subset of podocytes during adolescence,[11] this is the first report to show that adult PECs can serve as adult podocyte progenitors in PEC reporter mice in disease. How then does one explain the absence of this paradigm in these reporter mice previously? The most likely

interpretation is that the mouse models studied to date were not characterized by a marked depletion in podocyte number. Second, the models studied were not associated with podocyte regeneration.

Recent studies have shown that PECs do participate in FSGS.[1, 7] PECs are activated in FSGS, defined as the de novo expression of CD44, where they contribute to glomerulosclerosis by producing extracellular matrix proteins considered of Bowman's capsule origin.[30, 32, 42] CD44 is a marker of activated PECs in certain glomerular diseases, because this subset of PECs is likely responsible for the accumulation of basement membrane proteins leading to focal glomerular scarring. A second finding in the current studies was that a subset of PECs begin to express CD44 *de novo* in this model of FSGS. Temporally, CD44 expression was initially limited to PECs along Bowman's capsule. As more PECs migrated to the tuft, the number of β-gal$^+$CD44$^+$ cells in the tuft also increased, which was maximal at d14 of disease. This coincided with glomerulosclerosis. The overall number of β-gal$^+$CD44$^+$ cells in the tuft then decreased during the period of regeneration, while the number of β-gal positive cells expressing podocyte proteins increased. These data suggest that activation is the predominant initial response by PECs following marked podocyte depletion in podocyte number, similar to that reported by Hakroush and colleagues.[43]

Because CD44 has been used as a progenitor marker during kidney development,[33] we asked if this subset of PECs gave rise to podocytes. Triple staining showed labeled PECs that co-expressed CD44 did not triple stain for a podocyte protein. This was consistent with the notion that in disease, cells of PEC origin that have migrated to the glomerular tuft can undergo one of two fates, with opposing biological impacts: a podocyte progenitor fate, or a CD44 fate. These fates seem independent from one another. Based on several studies,[30, 32, 42] PECs co-expressing CD44 are likely to be injurious, leading to scarring and synechial attachments, whereas PECs that co-express podocyte proteins are likely reparative as this regeneration contributes to a higher number of podocytes, which is accompanied by lower scarring.

Finally, we have previously reported that the activated form of ERK (phospho-ERK) is *de novo* expressed in PECs in proteinuric podocyte diseases such as FSGS.[17, 44] We next asked if the expression of phospho-ERK might help explain, and possibly determine, the fate of PECs in this model. A third finding in this study was that the *de novo* expression of phospho-ERK in disease was restricted to the subset of PECs lining Bowman's capsule that co-expressed CD44 and not to the subset that took on a podocyte phenotype. Further studies are needed to confirm this proposed biological role that might distinguish the ultimate fate of PECs in response to a primary podocyte injury. Furthermore, it would be of interest to determine the fate of these cells if phospho-ERK was inhibited pharmacologically or genetically.

In several glomeruli in which PECs migrated to the glomerular tuft, the number of cells along Bowman's capsule that expressed the PEC reporter was reduced, and/or the intensity of either X-gal or β-gal staining was reduced in those glomeruli. The finding could be due to several explanations. First, doxycycline was used to temporally induce permanent labeling

in a subpopulation of PECs during the window of administration of the reagent. It was then withdrawn for at least one week to "clear out", before the experiments were performed, and therefore, no additional PECs can be labeled during disease. If a PEC therefore moves from their original location along Bowman's capsule to the tuft, one might expect that the overall number of originally labeled PECs will indeed be reduced along Bowman's capsule when these cells leave. Second, we have stated that PECs do proliferate, but we did not state that this response was sufficient to maintain those PECs that migrated to the tuft. Third, a small fraction of PECs that derived from the proliferation of labeled PECs would indeed express β-Gal, but because not all PECs are labeled following doxycycline pulsing, we cannot expect that all cells derived from PEC proliferation would be labeled.

In summary, we have shown that following primary podocyte injury leading to abrupt depletion, podocytes can regenerate, independent of their proliferation. We postulate that PECs respond secondarily to podocyte injury, by initially favoring an activated phenotype characterized by CD44 expression that coincides with the activation of ERK, and the development of glomerulosclerosis (Figure 7). As these events diminish, a subset of PECs that migrate to the glomerular tuft become podocyte progenitors, coinciding with podocyte regeneration, and a decrease in glomerular scarring.

Methods

Animals

PEC Reporter Mice—In order to study the fate of PECs following the induction of experimental FSGS, inducible PEC-Reverse Tetracycline-Transactivator (rtTA)/LC1/Rosa26 reporter (R26R) mice (called PEC-reporter mice in this manuscript) on a BTBR background were genotyped and used as an inducible PEC reporter as previously reported.[13, 32, 45] PECs, and their progeny, permanently express β-galactosidase (β-gal) only following doxycycline administration. Thus to specifically and permanently genetically label PECs within a defined window of time, 23 transgenic animals aged 10-12 weeks were administered doxycycline hydrochloride via feed at 625mg/kg, for 14 days ad libitum (HarlanTD.01306). Chow was changed weekly. The serum half-life of doxycycline is less than one day in humans, and is cleared very quickly in mice.[46] However, a one week wash out period for doxycycline was performed, to ensure labeling only within the specific temporal window prior to disease precluding the possibility of spontaneous expression of the PEC reporter within the tuft. Mice were housed in the animal care facility of the University of Washington under specific pathogen-free conditions with ad libitum food and water. Animal protocols were approved by the University of Washington Institutional Animal Care and Use Committee (2968-04).

Experimental FSGS—Experimental focal segmental glomerulosclerosis (FSGS) was induced in PEC-reporter mice with a cytotoxic sheep anti-podocyte antibody, as previously described[17, 1827, 47] This cytotoxic antibody induces abrupt podocyte depletion, accompanied by glomerulosclerosis.[17, 18, 28]

FSGS was induced in 17 PEC reporter mice with 2 doses of sheep anti-glomerular antibody at 10mg/20g body weight via IP injection, 24 hours apart. Mice were randomly sacrificed on

d7 (n=6), d14 (n=5), and d28 (n=6). Normal baselines (n=6) were either sacrificed one week after doxycycline treatment, or 14 days after administration of control Sheep IgG in PBS.

At sacrifice, mice were perfused with 10ml 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, USA), rinsed in 70% Ethanol, processed and embedded in paraffin. The other half was fixed for 45 minutes in 4% Paraformaldehyde Solution (PFA) in PBS (Affymetrix, Santa Clara, California, USA), washed in 30% sucrose overnight at 4°C, patted dry, rinsed briefly in OCT, embedded in OCT, and frozen in a dry ice 100% Ethanol bath. Sections were then cut from the paraffin or OCT blocks at 4-10μm thickness.

Sheep anti-podocyte antibody binding—To verify the binding of injected sheep anti-glomerular antibody to podocytes within the glomerulus, immunofluorescence staining for sheep IgG was performed. Rabbit anti-sheep IgG H&L (FITC) polyclonal antibody (Abcam, Cambridge, MA) was utilized on cryosections as previously described.[27]

BrdU labeling of mice to assess proliferation—BrdU was administered to quantitate cell proliferation. All animals received 3 consecutive daily IP injections, 24 hours apart, of 10ul per gram body weight 5-bromo-2-deoxyuridine and 5-fluoro-2′-deoxyuridine (BrdU-Amersham Cell Proliferation Labeling Reagent) (GE Healthcare Life Sciences, Little Chalfont, UK). Following the induction of permanent labeling of PECs in PEC-reporter mice with doxycycline, and a one week washout period, baseline mice were given 3 consecutive daily doses of BrdU and sacrificed 48 hours after the last injection. Mice given FSGS were given 3 consecutive doses of BrdU starting the day after the last dose of the antibody or control sheep IgG and randomly sacrificed at d7, 14 or 28.

Immunostaining—All immunostains described below were performed and analyzed on each mouse enrolled in the study (i.e. at baseline and in every time point in disease). Indirect immunoperoxidase and immunofluorescence staining were performed on 4μm tissue sections from mouse renal biopsies fixed in formalin and embedded in paraffin as described previously,[15, 48] with minor modifications. In brief, paraffin was removed using Histoclear (National Diagnostics, Atlanta, GA, USA) and sections were rehydrated in a graded series of ethanol. Except where noted otherwise, antigen retrieval was performed by boiling in 10mM citric acid buffer pH 6.0. Nonspecific protein binding was blocked using Background Buster (Accurate Chemical & Scientific, Westbury, NY, USA) or Bloxall Blocking Solution (Vector Laboratories, Burlingame, CA, USA) for 20 minutes at room temperature. Endogenous biotin activity was suppressed with an Avidin/Biotin Blocking Kit by Vector Laboratories. Antibodies were diluted in 1% IgG free BSA in PBS. Initial primary antibodies were incubated overnight at 4°C. In the case of double and triple immunostaining, subsequent primary antibodies were incubated either overnight at 4°C or for 3 hours at room temperature. Secondary antibodies and streptavidin conjugates were incubated for one hour at room temperature. All immunofluorescence samples were mounted using Vectashield mounting medium with DAPI (Vector Labs, Burlingame, CA, USA).

Assessment of Glomerulosclerosis, Albuminuria and Podocyte Depletion— Immunostaining was performed for p57 with Periodic Acid Schiff (PAS) counterstaining to measure podocyte number and assess glomerulosclerosis, as previously reported.[16, 17, 19, 27, 28] 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 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, USA) followed by rabbit on rodent HRP-polymer (Biocare Medical, Concord, CA, USA). Visualization of immunostaining was by precipitation of diaminobenzidine (DAB; Sigma-Aldrich , St. Louis, Missouri, USA). Counterstaining was performed with Periodic Acid Schiff (PAS) by washing slides in fresh 0.5% periodic acid (Sigma-Aldrich, St Louis, MI, USA) for 8 minutes, washed for 5 minutes in ddH2O, sections were incubated for 10 minutes at room temperature with Schiffs Reagent (Sigma-Aldrich, St Louis, MI, USA), washed 2× for 5 minutes in fresh 0.5% sodium metabisulfate (Sigma-Aldrich, St Louis, MI, USA), and washed for 5-10 minutes under running tap water. Slides were dehydrated in ethanol and mounted with Histomount. An average of 50±10 glomeruli in each animal was graded as previously reported.[16, 17, 19, 27, 28]

Urinary mouse albumin was measured by radial immunodiffusion assay (RID) as previously described.[49] Briefly, rabbit anti-mouse albumin antibody (Accurate Chemical, Westbury, NY) and rabbit serum (Pel-Freez, Rogers, AR) were incorporated into a thin layer of 1.5% type I, low EEO agarose gel (Sigma-Aldrich) in 0.5 M veronal buffer. A small volume of urine was placed in a well cut into the agarose gel. As the urine diffused from the well, mouse albumin reacted with the antibody in the agar and formed a halo of precipitation around the well. Measurement of the halo diameter was related directly to the albumin concentration in the urine. This was compared to a calibration curve prepared from known concentrations of purified fraction V mouse albumin standards (MP Biomedicals, Irvine, CA). Creatinine was measured in the urine via a colorimetric assay (Cayman Chemical, Ann Arbor, MI) and an albumin to creatinine ratio was calculated.

Identifying labeled PECs in reporter mice—Two methods were used to identify PECs that were permanently labeled with beta-galactosidase (β-gal): (i) enzymatic reaction, (ii) immunostaining for β-gal. Enzymatic β-gal staining was performed on frozen tissues sectioned at 4-10μm thick, adhered to superfrost slides and allowed to dry for 10 minutes at room temperature. Slides were rehydrated in PBS 2× for 5 minutes and tissue circled with a PAP pen. Freshly made and preheated X-gal solution (5mM K3(CN)6, 5mM K4(CN)6, 2mM MgCl2, 1mg/ml X-gal, in 1×PBS pH7.4) was placed onto each section for 1-2 hours at 37°C. Slides were rinsed 1× in PBS, washed 2× for 4 minutes in PBS, washed 1× in ddH20. Counterstaining was performed with Periodic Acid Schiff (PAS) Immunohistochemical staining was performed with a chicken polyclonal primary antibody to beta-galactosidase (1:2000, Abcam, Cambridge, MA, USA), followed by a biotinylated goat anti-chicken secondary antibody (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). An AlexaFluor594 conjugated streptavidin (1:100, Life Technologies, Eugene, OR, USA) was used for detection. Negative controls included tissues without β-gal and β-gal reporter positive tissue with no primary antibody applied.

Identifying Podocytes in PEC reporter mice—Podocytes were identified by immunostaining for the following markers: p57, podocin and synaptopodin. The following primary antibodies were used respectively: rabbit polyclonal anti-p57 (1:1500, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal anti-NPHS2 (1:400, abcam, Cambridge, MA, USA) and mouse monoclonal anti-synaptopodin (1:4, Fitzgerald Industries International, Acton, MA, USA). Either donkey anti-rabbit IgG or donkey anti-mouse IgG conjugated with AlexaFluor488 (green signal) were used to detect the primary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). To determine if a subset of cells of PEC origin that migrated to the glomerular tuft begin to de novo express podocyte proteins, double staining was performed for β-gal and p57, and β-gal and podocin. Tissue was first stained for β-gal as described above, using an Alexa594 conjugated secondary for a red signal under fluorescence. Following this, tissue was re-blocked using Background Buster (Accurate Chemical & Scientific, Westbury, NY, USA), and then incubated with p57, podocin or synaptopodin primary antibodies overnight at 4°C.

Quantification of β-gal and podocyte markers—Unbiased stereology was used for quantitation, by measuring the glomerular tuft area, used as a denominator for appropriate measurements. To measure tuft area, glomerular cross-sections were analyzed on an EVOS FL Cell Imaging System using 20× magnification. Tuft contours were manually traced, excluding the urinary space. Tuft area was measured for 50 consecutive glomeruli for each animal. All quantified cell counts were normalized against that particular animal's average glomerular tuft cross-sectional area. An average of 95 ± 23 glomeruli were quantified per animal. Glomeruli with poor β-gal staining were excluded from quantification, as were any inadequate cross-sections or glomeruli which appeared significantly disrupted by the tissue sectioning process. Cells in the glomerular tuft were deemed positive for β-gal if the signal intensity visually matched that of the PECs in Bowman's capsule of the same glomerulus, and if the β-gal staining demonstrated a cytoplasmic distribution around a nucleus (identified with DAPI staining). Glomeruli with equivocal β-gal staining in the tuft were counted as negative. For p57/β-gal staining, a double-positive cell was defined as a cell with a green nucleus surrounded by a red cytoplasm. Positive β-gal/podocin double staining was defined as a yellow-colored cell, which results from overlap between red and green signal respectively in the cytoplasm.

CD44 Staining—CD44 is considered a marker of activated PECs [30, 32, 42] Indirect immunoperoxidase staining was performed to determine CD44 expression in normal (n=6) and diseased (n=17) PEC reporter mice. Sections were rehydrated and antigen retrieval was performed as described above. Tissues were blocked using the steps listed above, with the additional suppression of endogenous peroxidase activity using 3% hydrogen peroxide. Sections were incubated overnight at 4°C with a rat monoclonal antibody to CD44 (1:100, BD Biosciences, San Jose, CA). A biotinylated mouse anti-rat IgG was used to detect the primary antibody (1:200, Jackson ImmunoResearch Laboratories, West Grove, PA) followed by Vectastain R.T.U. ABC reagent (Vector Labs, Burlingame, CA) and 3,3′-diaminobenzamidine (DAB) (Sigma-Aldrich, St Louis, MI, USA) was used as the chromogen. Counterstaining was performed with hematoxylin (Sigma-Aldrich, St Louis,

MI, USA). Sections were dehydrated in ethanol and mounted using Histomount mounting medium (National Diagnostics, Atlanta, GA, USA).

Immunofluorescence staining was performed with CD44, β-gal and podocin. Tissues were first stained for β-gal as described above, re-blocked, and incubated with rat anti-CD44 (1:10, BD Biosciences, San Jose, CA, USA). Staining was visualized using biotinylated mouse anti-rat secondary antibody (1:200, Jackson ImmunoResearch, WestGrove, PA, USA) and a streptavidin-conjugated AlexaFluor647 (1:100, Life Technologies, Eugene, OR). Tissue was re-blocked again, and then incubated with, rabbit anti-NPHS2 (1:400, Abcam, Cambridge, MA, USA). This staining was visualized using donkey anti-rabbit IgG conjugated with AlexaFluor488 (1:100, Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

Identifying proliferation in PEC reporter mice—Double immunostaining was performed with BrdU and β-gal on paraffin embedded tissue was prepared and blocked as described above. Tissue was incubated overnight at 4°C with a primary mouse anit-BrdU (1:1000, Amersham, GE Life Sciences, Buckinghamshire, UK). This was followed with an AlexaFluor488 conjugated donkey anti-mouse secondary (1:100, Jackson Immunoresearch Laboratories, Inc, West Grove, PA, USA). Tissue was then re-blocked and stained for β-gal as described above. Double positive cells for BrdU and β-gal were defined as those with a green nuclear signal and surrounding red cytoplasm. 100 glomeruli were quantified for each animal.

Detection of phospho-ERK activity—Phospho-ERK staining was markedly increased in cells lining Bowman's capsule in this FSGS model in other mouse strains.[44] Triple immunofluorescence was performed using the following primary antibodies: rat monoclonal antibody to CD44 (1:10, BD Biosciences, San Jose, CA, USA), rabbit polyclonal antibody to phospho-p44/42 MAPK (1:250, Cell Signaling Technology, Beverly, MA, USA), anti-NPHS2 and anti-β-gal as described above. P-p44/42 (to identify phosphorylated ERK 1/2) was used with an Alexa647 conjugated donkey anti-rabbit IgG-(1:100, Jackson, West Grove, PA, USA). Anti-NPHS2 was visualized with AlexaFluor488 conjugated goat anti-rabbit IgG- (1:100, Invitrogen, Eugene, OR, USA). The secondary antibody for CD44 was biotinylated mouse anti-rat IgG (1:200, Jackson ImmunoResearch, West Grove, PA), visualized with either Alexa488 or -Alexa647 conjugated streptavidin (1:100, Life Technologies, Eugene, OR, USA). Cells in these sections were considered double- or triple-positive using signal overlap in the cytoplasm.

Microscopy

For quantification, glomeruli were examined on a Leica DMRB microscope and an EVOS FL Cell Imaging System using 40× magnification. Images were collected using confocal microscopy on a Leica DMI400B. Scale bars were applied to each image by calibrating set scale at each magnification to a slide micrometer and applying with the scale bar tool in Image J 1.44o (NIH).

Statistical analysis

Groups were compared using a two-tailed unpaired students t-test, with minimum significance set at $p<0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Jefferson JA, Shankland SJ. The pathogenesis of focal segmental glomerulosclerosis. Advances in Chronic Kidney Disease. 2014; 21:408–416. [PubMed: 25168829]
2. Wharram BL, Goyal M, Wiggins JE, et al. Podocyte depletion causes glomerulosclerosis: diphtheria toxin-induced podocyte depletion in rats expressing human diphtheria toxin receptor transgene. Journal of the American Society of Nephrology : JASN. 2005; 16:2941–2952. [PubMed: 16107576]
3. Matsusaka T, Xin J, Niwa S, et al. Genetic engineering of glomerular sclerosis in the mouse via control of onset and severity of podocyte-specific injury. Journal of the American Society of Nephrology : JASN. 2005; 16:1013–1023. [PubMed: 15758046]
4. Marshall CB, Shankland SJ. Cell cycle regulatory proteins in podocyte health and disease. Nephron Experimental Nephrology. 2007; 106:e51–59. [PubMed: 17570940]
5. Appel D, Kershaw DB, Smeets B, et al. Recruitment of podocytes from glomerular parietal epithelial cells. Journal of the American Society of Nephrology : JASN. 2009; 20:333–343. [PubMed: 19092119]
6. Grahammer F, Wanner N, Huber TB. Podocyte regeneration: who can become a podocyte? The American Journal of Pathology. 2013; 183:333–335. [PubMed: 23727347]
7. Shankland SJ, Smeets B, Pippin JW, et al. The emergence of the glomerular parietal epithelial cell. Nature Reviews Nephrology. 2014; 10:158–173. [PubMed: 24468766]
8. Swetha G, Chandra V, Phadnis S, et al. Glomerular parietal epithelial cells of adult murine kidney undergo EMT to generate cells with traits of renal progenitors. Journal of Celluar and Molecular Medicine. 2011; 15:396–413.
9. Sagrinati C, Netti GS, Mazzinghi B, et al. Isolation and characterization of multipotent progenitor cells from the Bowman's capsule of adult human kidneys. Journal of the American Society of Nephrology : JASN. 2006; 17:2443–2456. [PubMed: 16885410]
10. Bariety J, Mandet C, Hill GS, et al. Parietal podocytes in normal human glomeruli. Journal of the American Society of Nephrology : JASN. 2006; 17:2770–2780. [PubMed: 16943305]
11. Appel D, Kershaw DB, Smeets B, et al. Recruitment of podocytes from glomerular parietal epithelial cells. Journal of the American Society of Nephrology : JASN. 2009; 20:333–343. [PubMed: 19092119]
12. Wanner N, Hartleben B, Herbach N, et al. Unraveling the role of podocyte turnover in glomerular aging and injury. Journal of the American Society of Nephrology : JASN. 2014; 25:707–716. [PubMed: 24408871]
13. Berger K, Schulte K, Boor P, et al. The regenerative potential of parietal epithelial cells in adult mice. Journal of the American Society of Nephrology : JASN. 2014; 25:693–705. [PubMed: 24408873]

14. Rizzo P, Perico N, Gagliardini E, et al. Nature and mediators of parietal epithelial cell activation in glomerulonephritides of human and rat. The American Journal of Pathology. 2013; 183:1769–1778. [PubMed: 24095923]
15. Ohse T, Vaughan MR, Kopp JB, et al. De novo expression of podocyte proteins in parietal epithelial cells during experimental glomerular disease. American Journal of Physiology Renal Physiology. 2010; 298:F702–711. [PubMed: 20007346]
16. Zhang J, Yanez D, Floege A, et al. ACE-inhibition increases podocyte number in experimental glomerular disease independent of proliferation. Journal of the Renin-Angiotensin-Aldosterone System : JRAAS. 2014
17. Zhang J, Pippin JW, Krofft RD, et al. Podocyte repopulation by renal progenitor cells following glucocorticoids treatment in experimental FSGS. American Journal of Physiology Renal Physiology. 2013; 304:F1375–1389. [PubMed: 23486009]
18. Zhang J, Pippin JW, Vaughan MR, et al. Retinoids augment the expression of podocyte proteins by glomerular parietal epithelial cells in experimental glomerular disease. Nephron Experimental Nephrology. 2012; 121:e23–37. [PubMed: 23107969]
19. Pichaiwong W, Hudkins KL, Wietecha T, et al. Reversibility of structural and functional damage in a model of advanced diabetic nephropathy. Journal of the American Society of Nephrology : JASN. 2013; 24:1088–1102. [PubMed: 23641056]
20. Sagrinati C, Netti GS, Mazzinghi B, et al. Isolation and characterization of multipotent progenitor cells from the Bowman's capsule of adult human kidneys. Journal of the American Society of Nephrology : JASN. 2006; 17:2443–2456. [PubMed: 16885410]
21. Ronconi E, Sagrinati C, Angelotti ML, et al. Regeneration of glomerular podocytes by human renal progenitors. Journal of the American Society of Nephrology : JASN. 2009; 20:322–332. [PubMed: 19092120]
22. Kietzmann L, Guhr SS, Meyer TN, et al. MicroRNA-193a Regulates the Transdifferentiation of Human Parietal Epithelial Cells toward a Podocyte Phenotype. Journal of the American Society of Nephrology : JASN. 2014
23. Sakamoto K, Ueno T, Kobayashi N, et al. The direction and role of phenotypic transition between podocytes and parietal epithelial cells in focal segmental glomerulosclerosis. American Journal of Physiology Renal Physiology. 2014; 306:F98–F104. [PubMed: 24154691]
24. Hackl MJ, Burford JL, Villanueva K, et al. Tracking the fate of glomerular epithelial cells in vivo using serial multiphoton imaging in new mouse models with fluorescent lineage tags. Nature Medicine. 2013; 19:1661–1666.
25. Miyazaki Y, Shimizu A, Ichikawa I, et al. Mice are unable to endogenously regenerate podocytes during the repair of immunotoxin-induced glomerular injury. Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association. 2014; 29:1005–1012.
26. Guhr SS, Sachs M, Wegner A, et al. The expression of podocyte-specific proteins in parietal epithelial cells is regulated by protein degradation. Kidney International. 2013; 84:532–544. [PubMed: 23615505]
27. Pippin JW, Sparks MA, Glenn ST, et al. Cells of renin lineage are progenitors of podocytes and parietal epithelial cells in experimental glomerular disease. The American Journal of Pathology. 2013; 183:542–557. [PubMed: 23769837]
28. Pippin JW, Glenn ST, Krofft RD, et al. Cells of renin lineage take on a podocyte phenotype in aging nephropathy. American Journal of Physiology Renal Physiology. 2014; 306:F1198–1209. [PubMed: 24647714]
29. Smeets B, Uhlig S, Fuss A, et al. Tracing the origin of glomerular extracapillary lesions from parietal epithelial cells. Journal of the American Society of Nephrology : JASN. 2009; 20:2604–2615. [PubMed: 19917779]
30. Fatima H, Moeller MJ, Smeets B, et al. Parietal epithelial cell activation marker in early recurrence of FSGS in the transplant. Clinical Journal of the American Society of Nephrology : CJASN. 2012; 7:1852–1858. [PubMed: 22917699]
31. Smeets B, Moeller MJ. Parietal epithelial cells and podocytes in glomerular diseases. Seminars in Nephrology. 2012; 32:357–367. [PubMed: 22958490]

32. Smeets B, Kuppe C, Sicking EM, et al. Parietal epithelial cells participate in the formation of sclerotic lesions in focal segmental glomerulosclerosis. Journal of the American Society of Nephrology : JASN. 2011; 22:1262–1274. [PubMed: 21719782]
33. Fanni D, Fanos V, Gerosa C, et al. CD44 immunoreactivity in the developing human kidney: a marker of renal progenitor stem cells? Renal Failure. 2013; 35:967–970. [PubMed: 23826724]
34. Schmits R, Filmus J, Gerwin N, et al. CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. Blood. 1997; 90:2217–2233. [PubMed: 9310473]
35. Sicking EM, Fuss A, Uhlig S, et al. Subtotal ablation of parietal epithelial cells induces crescent formation. Journal of the American Society of Nephrology : JASN. 2012; 23:629–640. [PubMed: 22282596]
36. Okamoto T, Sasaki S, Yamazaki T, et al. Prevalence of CD44-positive glomerular parietal epithelial cells reflects podocyte injury in adriamycin nephropathy. Nephron Experimental Nephrology. 2013; 124:11–18. [PubMed: 24401840]
37. Marshall CB, Krofft RD, Blonski MJ, et al. Role of smooth muscle protein SM22alpha in glomerular epithelial cell injury. American Journal of Physiology Renal Physiology. 2011; 300:F1026–1042. [PubMed: 21289056]
38. Ruggenenti P, Perna A, Benini R, et al. In chronic nephropathies prolonged ACE inhibition can induce remission: dynamics of time-dependent changes in GFR. Investigators of the GISEN Group. *Gruppo Italiano Studi Epidemiologici in Nefrologia*. Journal of the American Society of Nephrology : JASN. 1999; 10:997–1006. [PubMed: 10232685]
39. Macconi D, Sangalli F, Bonomelli M, et al. Podocyte repopulation contributes to regression of glomerular injury induced by ACE inhibition. The American Journal of Pathology. 2009; 174:797–807. [PubMed: 19164508]
40. Benigni A, Morigi M, Rizzo P, et al. Inhibiting angiotensin-converting enzyme promotes renal repair by limiting progenitor cell proliferation and restoring the glomerular architecture. The American Journal of Pathology. 2011; 179:628–638. [PubMed: 21718676]
41. Romagnani P, Lasagni L, Remuzzi G. Renal progenitors: an evolutionary conserved strategy for kidney regeneration. Nature Reviews Nephrology. 2013; 9:137–146. [PubMed: 23338209]
42. Smeets B, Stucker F, Wetzels J, et al. Detection of Activated Parietal Epithelial Cells on the Glomerular Tuft Distinguishes Early Focal Segmental Glomerulosclerosis from Minimal Change Disease. The American Journal of Pathology. 2014; 184:3239–48. [PubMed: 25307344]
43. Hakroush S, Cebulla A, Schaldecker T, et al. Extensive podocyte loss triggers a rapid parietal epithelial cell response. Journal of the American Society of Nephrology : JASN. 2014; 25:927–938. [PubMed: 24335975]
44. Chang AM, Ohse T, Krofft RD, et al. Albumin-induced apoptosis of glomerular parietal epithelial cells is modulated by extracellular signal-regulated kinase 1/2. Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association. 2012; 27:1330–1343.
45. Schulte K, Berger K, Boor P, et al. Origin of parietal podocytes in atubular glomeruli mapped by lineage tracing. Journal of the American Society of Nephrology : JASN. 2014; 25:129–141. [PubMed: 24071005]
46. Traykova-Brauch M, Schonig K, Greiner O, et al. An efficient and versatile system for acute and chronic modulation of renal tubular function in transgenic mice. Nature Medicine. 2008; 14:979–984.
47. Ohse T, Vaughan MR, Kopp JB, et al. De novo expression of podocyte proteins in parietal epithelial cells during experimental glomerular disease. American Journal of Physiology- Renal Physiology. 2010; 298:F702–711. [PubMed: 20007346]
48. Ohse T, Chang AM, Pippin JW, et al. A new function for parietal epithelial cells: a second glomerular barrier. American Journal of Physiology Renal Physiology. 2009; 297:F1566–1574. [PubMed: 19794110]
49. Marshall CB, Krofft RD, Pippin JW, et al. The CDK-inhibitor p21 is Pro-survival in Adriamycin(R)-Induced Podocyte Injury, in vitro and in vivo. American Journal of Physiology Renal Physiology. 2010; 298:F1140–51. [PubMed: 20130121]

Abbreviations

β-gal	β-galactosidase
FSGS	focal segmental glomerulosclerosis
GFP	green fluorescent protein
PAS	Periodic Acid Schiff
PEC	glomerular parietal epithelial cells

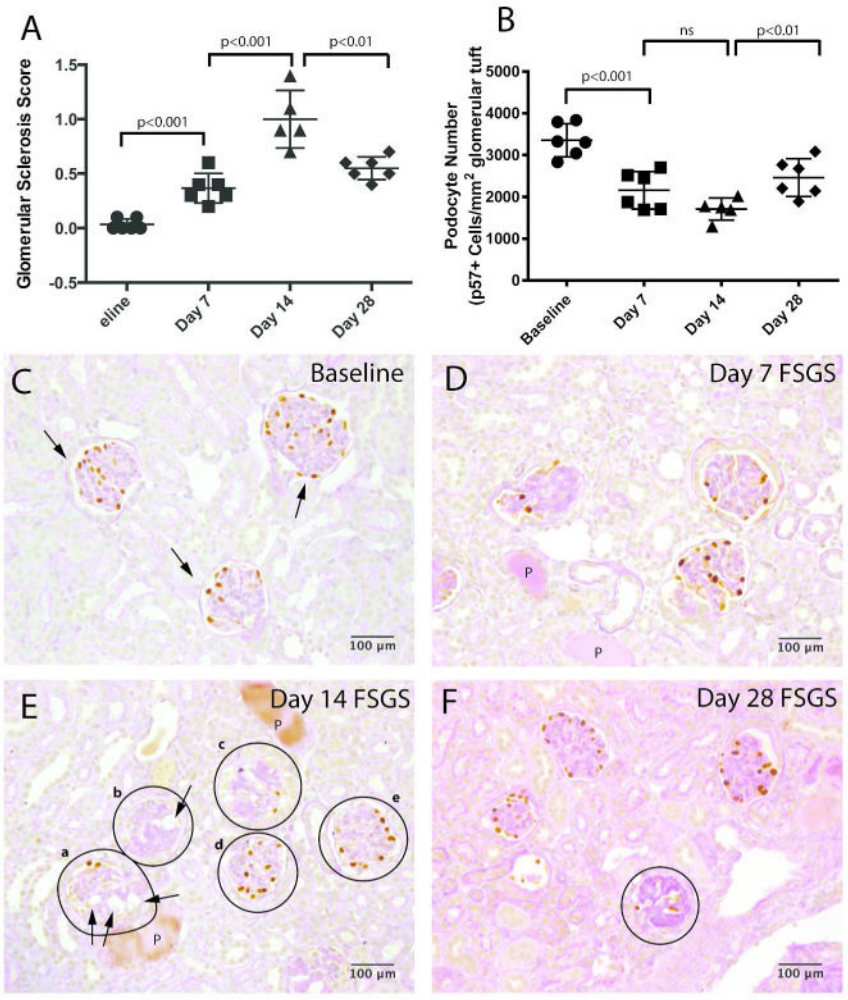

Figure 1. Podocyte depletion and glomerulosclerosis in PEC-Reverse Tetracycline-Transactivator (rtTA)/LC1/Rosa26 reporter mice

Podocytes were identified by p57 staining (brown, nuclear) and glomerulosclerosis by PAS staining. (**A**) Glomerulosclerosis increased at day 7, was maximal at day 14, and was lower by day 28. (**B**) Podocyte number was measured by quantitating the number of cells staining positive for p57 per glomerular tuft area. Podocyte number decreased abruptly at d7, with a nadir at d14, but was significantly higher at d28.

(**C**-**F**) *Representative p57 and PAS staining with scale bar*. (**C**) Baseline mice (d0) showing typical p57 staining (examples represented by arrows), without sclerosis. (**D**) At FSGS d7, p57 staining was reduced, consistent with podocyte depletion. Proteinacious material was noted in some tubules (examples denoted by P). (**E**) FSGS d14 kidney section with 5 glomeruli marked by circles, named a-e. p57 staining was markedly reduced in glomeruli a-c, consistent with significant podocyte depletion, which was accompanied by glomerulosclerosis (arrow) and dilated capillary loops (arrows). Glomeruli d and e had a normal podocyte complement, with no sclerosis. Proteinacious material is present in some tubules (marked by P) (**F**) At day 28, the majority of glomeruli had a normal or near normal

podocyte number; the glomerulus circled had continued podocyte depletion, accompanied by scarring.

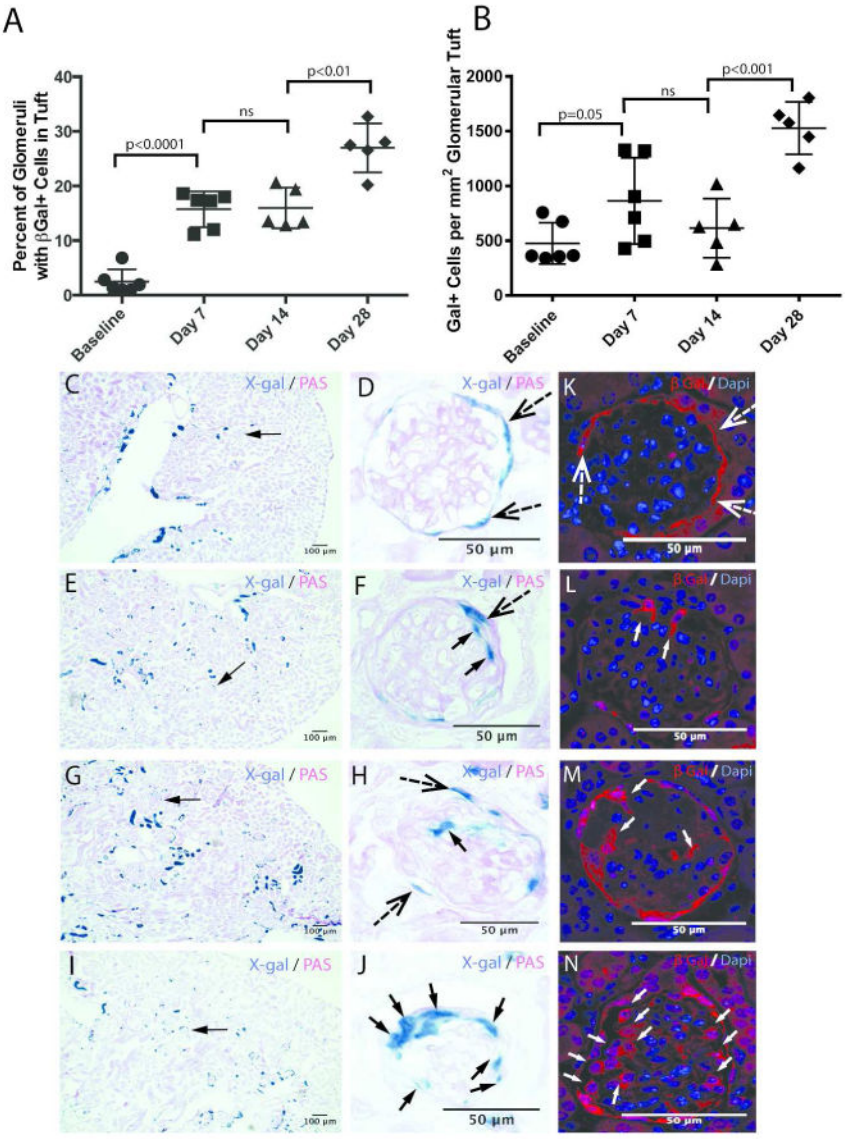

Figure 2. Labeled PECs move onto the glomerular tuft in diseased PEC-Reverse Tetracycline-Transactivator (rtTA)/LC1/Rosa26 reporter mice

PECs were permanently labeled with the β-galactosidase reporter, only when mice are given doxycycline. The reporter was detected by X-gal (blue color, bright field) in panels C-J, and by β-gal staining (red color, confocal) in panels K-N.

(**A**) Graph showing that compared to baseline, the percentage of glomeruli with one or more β-gal positive cells in the tuft was significantly increase at d7 (12.7±7.2%), d14 (16±3.2%) and d28 (26.75±5.4%) of FSGS. (**B**) Graph showing that within those glomeruli containing labeled PECs on the tuft, the absolute number of β-gal^{+}cells increased progressively in number during disease. (**C, E, G, I**) show low power views of *bright field micrographs of X-gal staining (blue, cytoplasmic) and PAS counterstain (pink), and the higher power views of the glomeruli marked by arrows are shown in panels D, F, H and J respectively*. (**D**) Representative image at baseline of X-gal positive cells following doxycyline induction,

restricted to Bowman's capsule within the glomerulus (dashed arrows). Following the onset of FSGS, there was a progressive increase in X-gal positive cells on the glomerular tuft at d7 (**F**), d14 (**H**) and d28 (**I**)(solid arrows indicate examples).

(**K-N**) *Confocal micrographs of β-gal (red, cytoplasmic) and DAPI (blue, nuclear) staining*. (**K**) Representative image at baseline of β-gal positive cells, restricted to Bowman's capsule within the glomerulus (dashed arrows). Following the onset of FSGS, there was a progressive increase in β-gal positive cells on the glomerular tuft at d7 (**L**), d14 (**M**) and d28 (**N**)(solid arrows indicate examples).

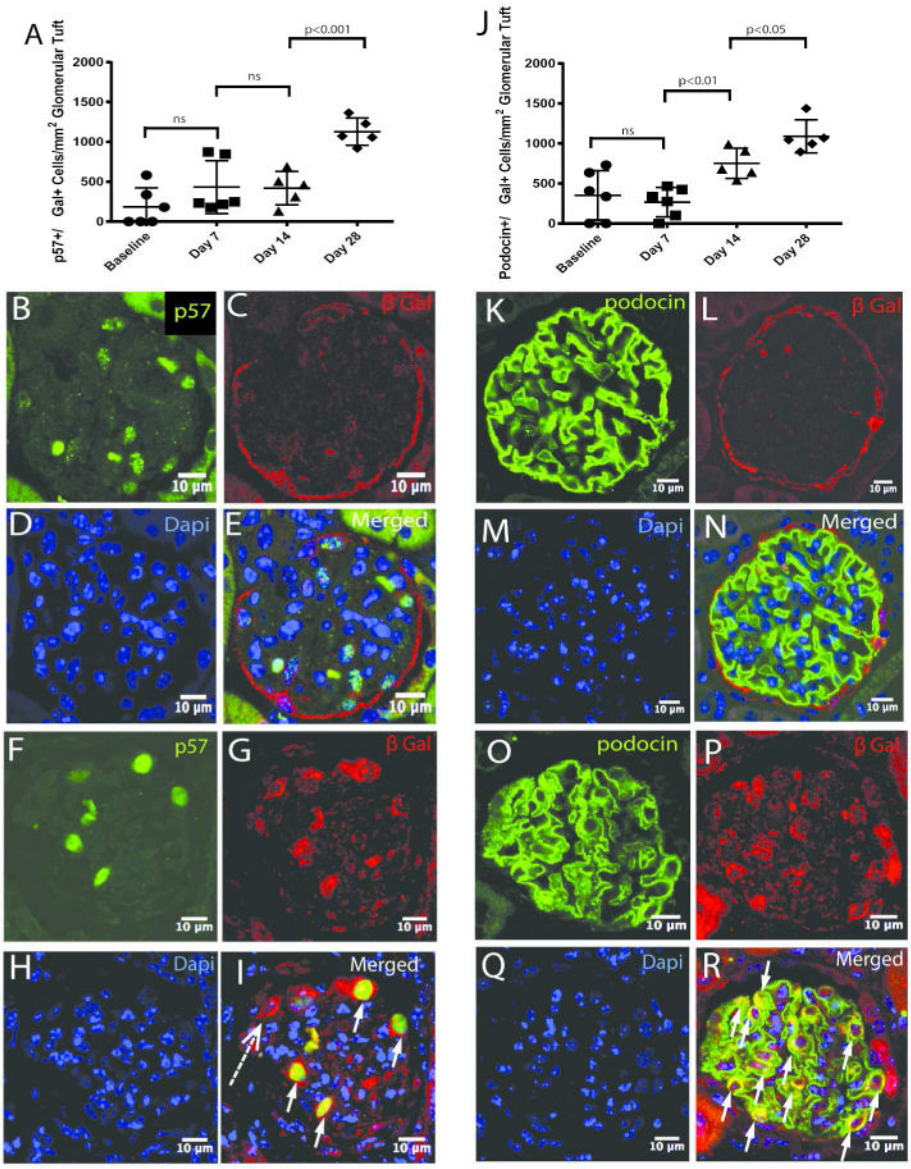

Figure 3. Following the onset of FSGS, a subset of labeled PECs that move onto the glomerular tuft co-express the podocyte markers p57 and podocin

Confocal microscopy was used to identify permanently labeled PECs by β-gal staining (red color), and podocytes by p57 or podocin staining (green colors).

(**A-I**) *β-Gal and p57 double-staining and quantitation*. (**A**) Graph showing the unbiased stereology data where a significantly higher number of β-gal^{+}/p57^{+} double-positive cells on the glomerular tuft at day 28 of FSGS.

(**B-E**) *Baseline mouse*. Staining for (**B**) p57 staining (green, nuclear) is limited to the glomerular tuft, (**C**) β-gal staining (labels PECs, red, cytoplasmic) is restricted to Bowman's capsule, and (**D**) DAPI (labels nuclei, blue) single channels and (**E**) merged images in a normal glomerulus at baseline. There is no overlap in staining, as PECs are restricted to Bowman's capsule.

(**F-I**) *FSGS day 28*. (**F**) p57 staining. (**G**) β-gal positive cells are detected on the glomerular tuft. (**H**) DAPI staining. (**I**) The merged image shows a subset of PECs that migrated to the glomerular tuft co-express p57 (yellow color, solid arrows). An example of a PEC on the tuft that does not co-express p57 is shown (red, dashed arrow).
(**J-R**) β-Gal and podocin double-staining and quantitation. (**J**) Graph showing that the number of β-gal$^+$/podocin$^+$ double-positive cells on the tuft was significantly higher on days 14 and 28 of FSGS.
(**K-N**) *Baseline mouse*. (**K**) Podocin staining (green, cytoplasmic) is detected in podocytes, (**L**) β-gal staining is restricted to cells lining Bowman's capsule, (**M**) DAPI staining. (**N**) merged images showns no overlap, as PECs are restricted to Bowman's capsule.
(**O-R**) *FSGS day 28*. (**O**) Podocin staining. (**P**) β-gal positive cells are detected on the glomerular tuft. (**Q**) DAPI staining. (**R**) The merged image shows a subset of PECs on the glomerular tuft co-express podocin (yellow color, arrows).
Taken together, these results show that a subset of PECs that have migrated to the glomerular tuft in disease co-express the podocyte proteins p57 and podocin.

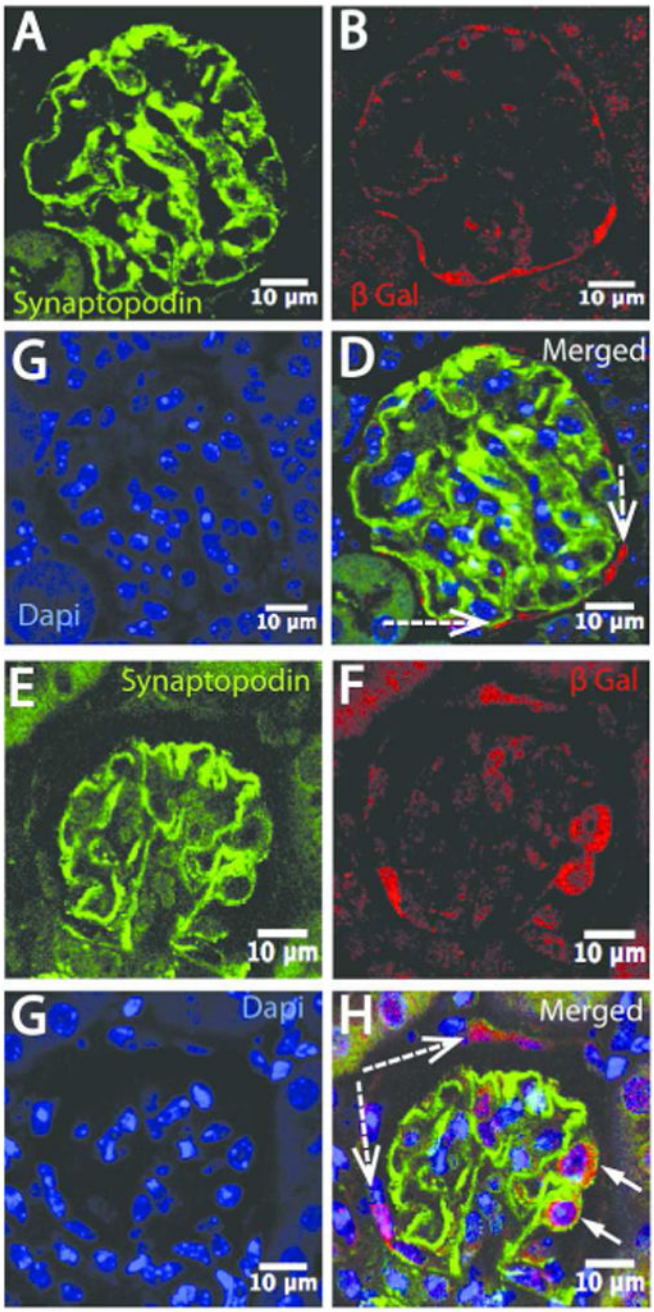

Figure 4. A subset of labeled PECs co-express the podocyte marker synaptopodin on the glomerular tuft in mice with FSGS

(**A-D**) Baseline mice. (A) Synaptopodin (green, membrane location in podocytes), (B) β-Gal (red, cytoplasmic, PEC label), (C) DAPI (blue, nuclear) and (D) Merge of all three stains, showing no colocalization, because PECs are restricted to Bowman's capsule (dashed arrows).

(**E-H**) FSGS mice. (**E**) Synaptopodin staining, (**F**) β-Gal staining is detected in cells lining Bowman's capsule and inside the tuft, (**G**) DAPI. (**H**) Merge of all three stains, showing a

subset of PECs on the tuft co-colocalize with synaptopodin (solid arrows). Because β-gal is restricted to the cell cytoplasm and synaptopodin is membrane bound, they do not merge to form a yellow color. Dashed arrows show no co-staining with synaptopodin in β-gal stained cells lining Bowman's capsule.

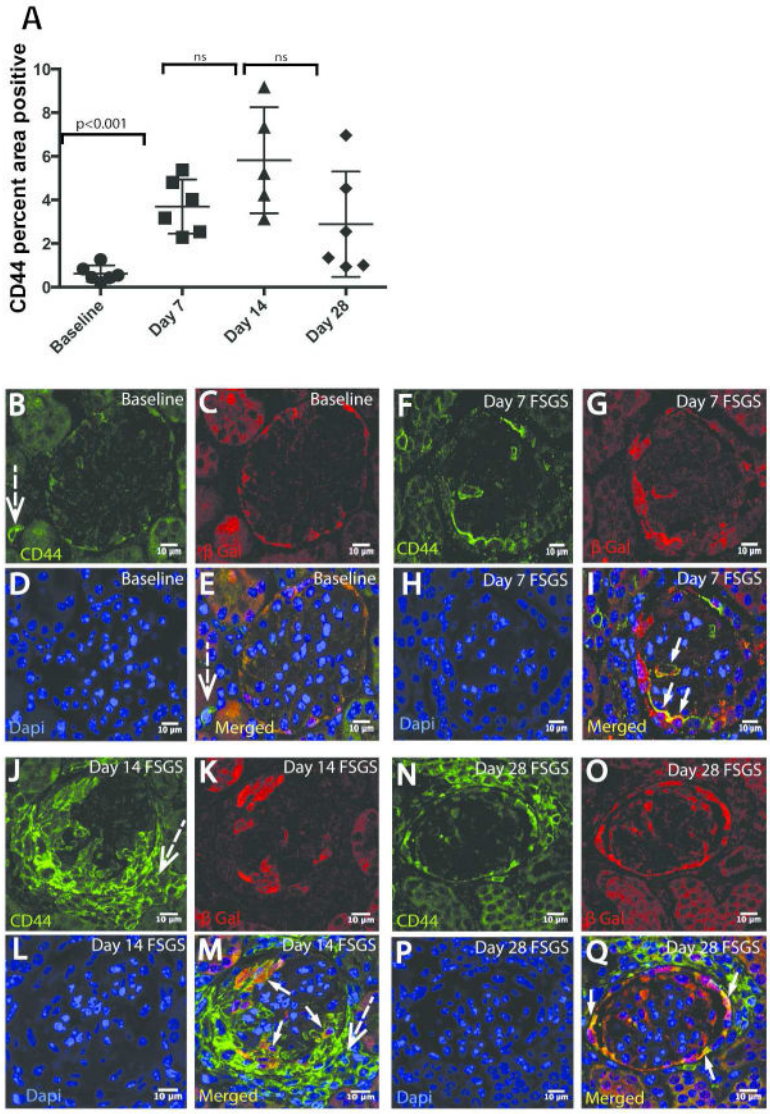

Figure 5. CD44, a marker of PEC activation, is detected in labeled cells along Bowman's capsule and on the tuft in PEC-Reverse Tetracycline-Transactivator (rtTA)/LC1/Rosa26 reporter mice with FSGS

Confocal images showing double-staining for CD44 (green color), β-gal (red color) and merge (yellow color).

(A) *Quantitation of CD44 staining in glomeruli*. Graph showing the percentage of the glomerular area that was $CD44^+$ was significantly higher on days 7 of FSGS, but was variable and did not reach significance on day 14 and 28.

(B-E) Baseline mice. (**B**) Staining for CD44 (green, cytoplasmic) is not detectable in glomerular cross sections, but is present in occasional cells outside of glomeruli (dashed arrow). (**C**) Within glomeruli, β-gal staining (red) is limited to PECs lining Bowman's capsule. (**D**) DAPI staining (blue, nuclei). (**E**) When merged, there is no overlap with CD44 and β-gal staining.

(**F-I**) FSGS day 7 (**F**) CD44 staining is detected in cells lining Bowman's capsule, and in cells in the glomerular tuft. (**G**) β-Gal staining shows PECs in the glomerular tuft. (**H**) DAPI

staining. (**I**) The merge shows a subset of PECs that have migrated to the glomerular tuft co-express CD44 (β-gal$^+$/CD44$^+$) (solid arrows).

(**J-M**) FSGS day 14 (**J**) The number of cells staining positive for CD44 on the glomerular tuft is substantially higher. CD44 stained cells are detected in the peri-glomerular distribution (dashed arrows). (**K**) β-Gal stained cells have migrated to the tuft. (**L**) DAPI staining. (**M**) The merge image shows a subset of PECs within the glomerular tuft co-express CD44 (β-gal$^+$/CD44$^+$, solid arrows).

(**N-Q**) FSGS day 28. (**N**) The number of cells staining positive for CD44 on the glomerular tuft is substantially lower. CD44 stained cells are detected in the peri-glomerular distribution. (**O**) β-Gal stained cells continue to be detected on the glomerular tuft. (**P**) DAPI staining. (**Q**) The merge image shows labeled PECs on the tuft no longer co-express CD44, however, a subset of PECs along Bowman's capsule continue to co-express CD44 (β-gal$^+$/CD44$^+$, solid arrows).

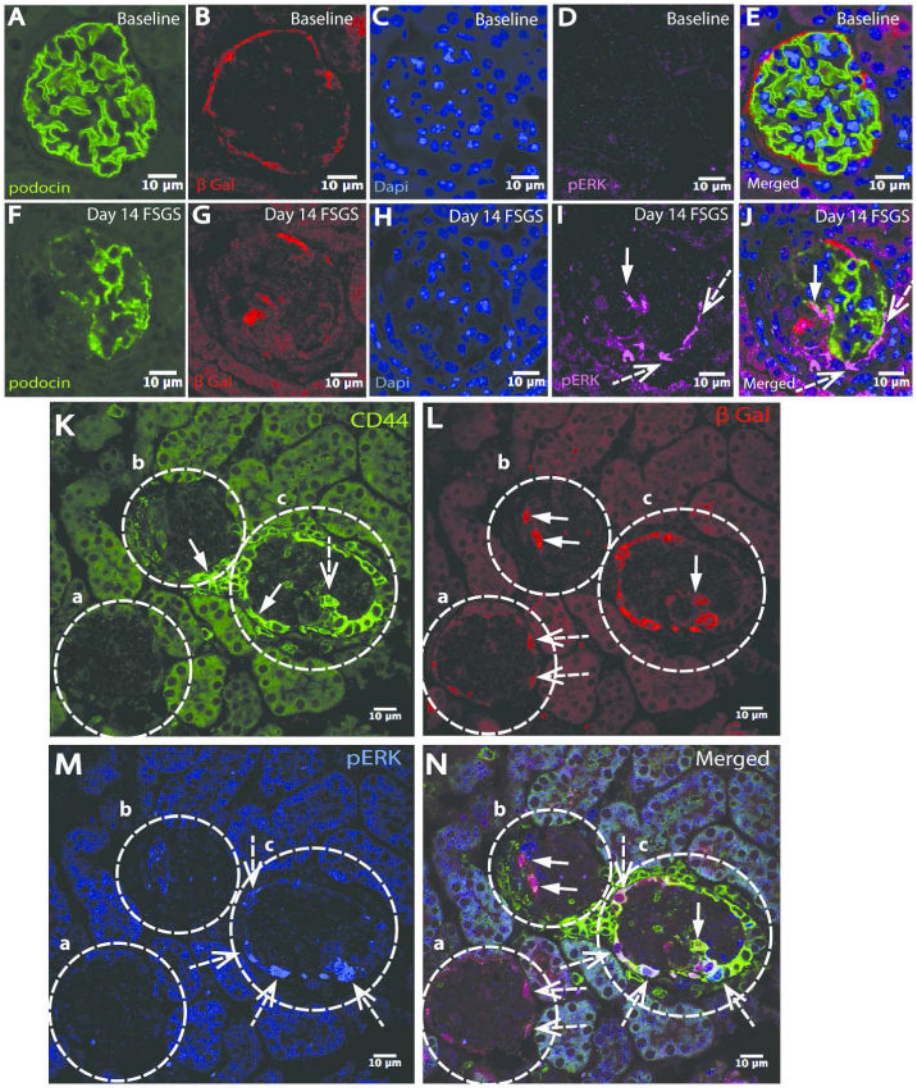

Figure 6. In reporter mice with FSGS, phospho-ERK co-localizes with PECs co-expressing CD44, but not with PECs co-expressing podocin

Confocal images of triple stains, used to determine which PECs co-expressed the active form of ERK (phospho-ERK).

(**A-J**) Triple staining for phosphor-ERK, podocin and β-gal.

(**A-E**) *Baseline mice*: (**A**) Podocin (green) stains podocytes; (**B**) β-gal staining (red) is restricted to PECs along Bowman's capsule; (**C**) DAPI staining (blue, nuclear); (**D**) Phospho-ERK (purple) is not detected; (**E**) Merged image showing that PECs and podocyte markers do not overlap, and neither co-express phospho-ERK.

(**F-J**) *FSGS mice D14*: (**F**) Podocin staining is reduced in the left lower quadrant of the glomerulus, consistent with reduced podocyte number; (**G**) β-gal stained PECs are detected on the tuft; (**H**) DAPI staining (blue, nuclear); (**I**) Phospho-ERK (purple) is detected in cells lining Bowman's capsule (dashed arrow) and in cells in the tuft (solid arrows); (**J**) Merged image showing that phospho-ERK staining is not co-expressed in PECs that stain for podocin.

(**K-N**) Triple staining for phospho-ERK, CD44 and β-gal in FSGS mice D7. Three glomeruli are shown in a mouse with FSGS, each labeled (hatched circle) and demarcated (a-c) for ease of identification.

(**K**) *CD44 staining* (green) is increased in cells lining Bowman's capsule (solid arrow) and in the glomerular tuft (dashed arrow) in glomeruli b and c. CD44 staining is not detected in glomerulus a.

(**L**) *β-gal staining* (red) is restricted to PECs lining Bowman's capsule in glomerulus a (arrows), is detected in the tuft in glomerulus b, and is in both locations in glomerulus c.

(**M**) *Phospho-ERK staining* (blue) is not detected in glomerulus a, is in the tuft in glomerulus b, and in both locations in glomerulus c.

(**N**) *Merge of triple stains*: shows that β-gal staining is restricted to PECs lining Bowman's capsule in glomerulus a (dashed arrows), but CD44 staining, β-gal staining and Phospho-ERK staining co-localize in cells within the tuft in glomerulus b (solid arrows) and with cells lining Bowman's capsule (dashed arrows) as well as within the tuft (solid arrow) of glomerulus c.

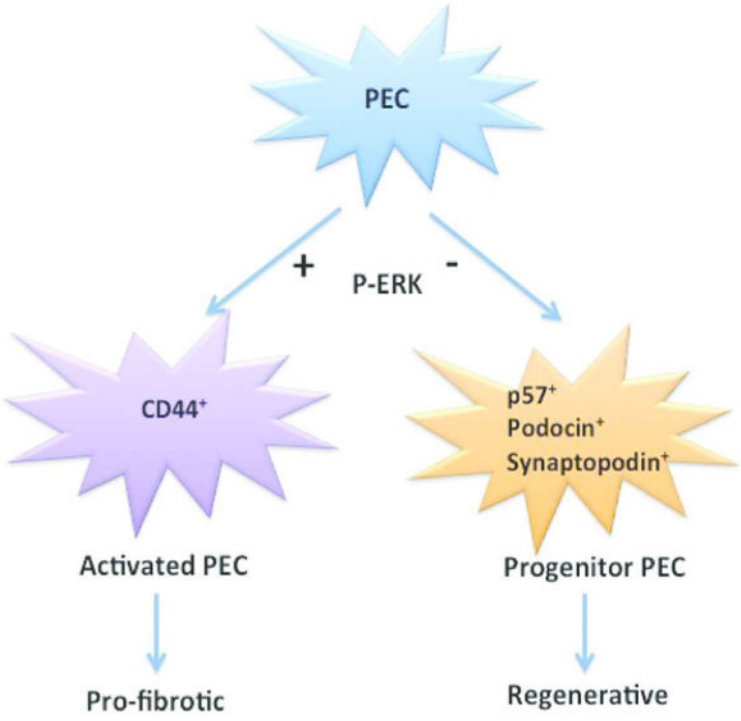

Figure 7. Overall proposed schema based on results of the current studies

In experimental FSGS characterized by abrupt podocyte loss and subsequent repletion, a subset of adult PECs invade the glomerular tuft, and can participate in either a pro-fibrotic or regenerative pathway. We propose that activated PECs ($CD44^+$) co-express phospho-ERK and are pro-fibrotic. However, PECs that have migrated to the glomerular tuft that do not express phospho-ERK are podocyte progenitors (express p57, podocin and synaptopodin) which are regenerative.