Cells of renin lineage are adult pluripotent progenitors in experimental glomerular disease

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Pippin JW, Kaverina NV, Eng DG, Krofft RD, Glenn ST, Duffield JS, Gross KW, Shankland SJ. Cells of renin lineage are adult pluripotent progenitors in experimental glomerular disease. Am J Physiol Renal Physiol 309: F341–F358, 2015. First published June 10, 2015; doi:10.1152/ajprenal.00438.2014.—Modified vascular smooth muscle cells of the kidney afferent arterioles have recently been shown to serve as progenitors for glomerular epithelial cells in response to glomerular injury. To determine whether such cells of renin lineage (CoRL) serve as progenitors for other cells in kidney disease characterized by both glomerular and tubulointerstitial injury, permanent genetic cell fate mapping of adult CoRL using Ren1CreER X Rs-tdTomato-R reporter mice was performed. TdTomo-tabeled CoRL were almost completely restricted to the juxtaglomerular compartment in healthy kidneys. Following 2 wk of antibody-mediated focal segmental glomerulosclerosis (FSGS) or 16 wk of 5/6 nephrectomy-induced chronic kidney diseases, tdTomato-mapped CoRL were identified in both interstitial and glomerular compartments. In the interstitium, PDGFβ receptor (R)-expressing cells significantly increased, and a portion of these expressed tdTomato. This was accompanied by a decrease in native pericyte number, but an increase in the number of tdTomato cells that coexpressed the pericyte markers PDGFβ-R and NG2. These cells surrounded vessels significantly increased, and a portion of these expressed tdTomato. This was accompanied by a decrease in native pericyte number, but an increase in the number of tdTomato cells that coexpressed the pericyte markers PDGFβ-R and NG2. These cells surrounded vessels and coexpressed the pericyte markers CD73 and CD146, but not the endothelial marker ERG. Within glomeruli of reporter mice with the 5/6 nephrectomy model, a subset of labeled CoRL migrated to the glomerular tuft and coexpressed podocin and synaptopodin. By contrast, labeled CoRL were not detected in glomerular or interstitial compartments following uninephrectomy. These observations indicate that in addition to supplying new adult podocytes to glomeruli, CoRL have the capacity to become new adult pericytes in the setting of interstitial disease. We conclude that CoRL have the potential to function as progenitors for multiple adult cell types in kidney disease.

FSGS: remnant kidney; podocyte; pericyte; regeneration; tubulointerstitium; PDGFβ-R

Proteinuric glomerular diseases are the leading cause of chronic and end stage kidney diseases. The number of podocytes covering the glomerular capillaries decreases in progressive focal segmental glomerulosclerosis (FSGS) and following a significant reduction in kidney mass in the remnant kidney, and is widely accepted to be responsible for glomerular leak of plasma proteins. In both disease settings, the tubulointerstitial compartment is secondarily involved during the progressive phase of disease, characterized by tubular cell atrophy, peritubular capillary loss, and interstitial fibrosis. Recent studies have shown that pericytes are a major contributor to the interstitial profibrotic processes in glomerular diseases (10, 31).

Adult podocytes exhibit many similarities to adult pericytes. First, they structurally wrap around and support underlying microvessels, namely, the glomerular (69) and peritubular capillaries, respectively (7). Second, they synthesize and secrete angiogenic factors such as VEGF, angiopoietin-1, and ephrins (18, 53, 58, 59, 63, 68). Third, both synthesize high levels of capillary basement membrane proteins (9, 42). Fourth, they express several genes traditionally considered restricted to neurons or associated glial cells such as the glial-derived neurotropic factor and nerve growth factor receptor (37, 67). Finally, they have similar developmental origins during which they expressed similar developmental transcription factors (32). Podocytes arise from cap mesenchyme progenitors and require activation of SIX2, PAX2, and WT1 (28, 32). Thus these cells undergo a mesenchymal-to-epithelial transition during early nephrogenesis. Podocytes subsequently activate the transcription factors FOXD1 and TCF21 (30, 34, 38). Pericytes arise from a population of FOXD1 transcription factor-expressing progenitor cells in the metanephric mesenchyme from gestational day E11.5 onward during mouse nephrogenesis (21).

Because adult podocytes do not have the ability to proliferate and replace their numbers following disease-induced depletion, several groups have attempted to determine how adult podocytes might regenerate by identifying putative local stem/progenitor cells that might participate in their regeneration (reviewed in Ref. 16). The neighboring glomerular parietal epithelial cell (PEC) might serve such a role in humans (reviewed in Refs. 36 and 51) although such a role has been disputed in mice (17, 35, 52, 54). More recently, we focused on cells of renin lineage (CoRL) in adult animals as candidate adult podocyte progenitors. CoRL are normally restricted to the kidney’s extraglomerular vascular smooth muscle compartment, where they are the sole source of renin under normal states. However, we showed that following acute or chronic podocyte depletion in experimental FSGS and aging nephropathy, respectively, a subset of CoRL enter the glomerulus and acquire a podocyte phenotype, defined as the de novo expression of four different proteins considered to be “podocyte specific.” This was accompanied by ultrastructural changes characteristic of podocytes, including foot processes and slit diaphragms. Following the induction of experimental FSGS in four different strains of CoRL reporter mice, we showed that a subpopulation of CoRL that moved to the intraglomerular compartment also coexpressed proteins considered specific to glomerular parietal epithelial cells. Other groups have also shown that CoRL exhibit marked stemness/plasticity, includ-
ing adult CoRL transdifferentiating into erythropoietin-producing cells (29), smooth muscle cells (55), and mesangial cells (20, 55, 61).

Although it has been suggested that CoRL can also give rise to kidney pericytes, this has not been shown in disease states to our knowledge. Included in the definition of a progenitor cell is the ability of a dormant cell to replace cells lost in cases of tissue injury. In the current study, we aimed to determine whether the progenitor nature of CoRL was extended to both the glomerular and tubulointerstitial compartments in the same disease process by studying the FSGS model of abrupt podocyte depletion, and the remnant kidney model of chronic kidney disease in inducible CoRL reporter mice.

METHODS

Animals

To study the fate of CoRL within specific temporal windows in different kidney compartments following a progressive decrease in nephron number accompanied by chronic kidney disease, we introduced Cre recombinase fused to the human estrogen receptor (ER) ligand-binding domain (LBD) (13) into exon one of the Ren1 gene residing within a 227-Kb BAC (abbreviated RenCreER) using recently described methods (45). Crossing the Ren1 CreER transgenic line with the reporter Gt (ROSA) 26Sortm9(CAG-tdTomato) Hze (45) yields Ren1CreER × Rs-tdTomato mice with a mixed C57 BL/10/C3H background, which allows for inducible and permanent tagging of CoRL with tomato red protein within temporal windows defined by the administration of tamoxifen. Accordingly, 7- to 10-wk-old bigenic mice were given tamoxifen (100 mg/kg), or vehicle (controls), by IP injection on alternate days for 6 days, as we have previously reported (45). A significant washout period of 8–11 wk ensued before one of three disease models was induced.

Experimental Models of Acute and Chronic Kidney Diseases

All three models described below were conducted in inducible Ren1CreER × Rs-tdTomato mice following a significant washout period after tamoxifen administration, chosen to specifically and permanently label a subset of CoRL with the tdTomato reporter.

Acute FSGS model. We have previously reported that following the administration of a cytotoxic anti-podocyte antibody, podocyte number is abruptly depleted, accompanied by glomerulosclerosis (45, 70, 71). Similar to other proteinuric glomerular diseases, secondary tubulointerstitial injury occurs in this model, manifest by patchy interstitial fibrosis, tubular dilatation, and atrophy of tubular necrosis. Archival tissue from our recent publication (45) was used for the purpose of the studies described herein.

Remnant kidney model of chronic kidney disease. Surgically reducing kidney mass to one-sixth of the original mass results in chronic kidney disease, characterized by reduced podocyte number, glomerulosclerosis, and tubulointerstitial scarring (33). This model was induced in tamoxifen-labeled Ren1CreER × Rs-tdTomato mice by first performing a left-sided uninephrectomy, and following full recovery, a second surgical reduction in renal mass was performed on the remaining kidney to induce the remnant kidney model. To accomplish the latter, the upper and lower poles of the right kidney were surgically excised. The hemostatic agent Gelfoam (Baxter Healthcare, Hayward, CA) was applied to stop bleeding. This resulted in a total reduction to 15% of the original kidney mass, measured by wet kidney weight. Remnant kidney mice were euthanized 16 wk later, where the remaining remnant kidney was removed and fixed for analysis as described below. Importantly, the kidney obtained following the uninephrectomy served as the baseline, so that individual mice could be studied over time.

Uninephrectomy model. In contrast to the remnant kidney, a uninephrectomy is not associated with features of chronic kidney disease, podocyte number is normal, and glomerular and tubulointerstitial fibrosis are absent. Uninephrectomy therefore serves as a good control to determine the impact of reducing nephron number on CoRL movement, and on their movement in the absence of kidney fibrosis. To surgically reduce renal mass by 50%, the left renal artery, vein, and ureter were ligated using suture material in labeled Ren1CreER × Rs-tdTomato aged 18 wk. The left kidney was decapsulated and removed, leaving the capsule, adrenal gland, and ligatures in place. The entire kidney tissue was fixed in 10% buffered formalin and served as the baseline control for individual animals followed temporally. Following recovery, animals (n = 8) were monitored for 16 wk, at which time kidney biopsies were obtained from the remaining right kidney (at 34 wk of age).

Inducible reporter mice for all models were housed in a specific pathogen-free facility. Surgical procedures were performed under anesthesia, and postsurgical analgesics and monitoring were performed in accordance with the University of Washington Institutional Animal Care and Use Committee’s approval.

Glomerular and Tubulointerstitial Injury

Glomerulosclerosis was determined on periodic acid-Schiff- and silver-stained sections on an average of 50 ± 5 glomeruli/animal and was graded quantitatively based on the percentage of glomerular tuft area involvement as follows: grade 0 = 0% (normal glomerulus with no abnormalities); grade 1 = <25% (glomerulus contains a few capillaries with dilation); grade 2 = <25–50% (glomerulus contains multiple capillaries with dilation); grade 3 = 50–75% (glomerulus contains multiple capillaries with dilation and some synechial attachments); and grade 4 = <75–100% (glomerulus contains multiple synechial attachments with FSGS) as previously reported (4, 23, 64, 70).

Tubular and interstitial injury was graded quantitatively as we have reported (41, 65) based on the degree of interstitial fibrosis, tubular dilatation, and degree of tubular cell atrophy as follows: 0 = normal tubules with no dilatation or tubular cell atrophy and no interstitial fibrosis; grade 1 = minor tubular dilatation or atrophy and interstitial fibrosis (<25% of field involved); grade 2 = mild tubular dilatation or atrophy and interstitial fibrosis (<25–50% of field involved); grade 3 = moderate tubular dilatation or atrophy and interstitial fibrosis (<50–75% of field involved); and grade 4 = severe tubular dilatation or atrophy and interstitial fibrosis (<75–100% of field involved).

Immunostaining

p57 Staining to measure podocyte number. To quantitate podocyte number, single immunostaining for p57 was performed as we have reported (64). Rabbit antibody to p57 (Santa Cruz Biotechnology, Santa Cruz, CA) was followed with a biotin-conjugated mouse anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA). The ABC kit (Vector Laboratories, Burlingame, CA) was used for signal amplification and 3,3′-diaminobenzamidine (DAB; DAB; Sigma, St. Louis, MO) was used as a chromogen. Slides were counterstained with hematoxylin (Sigma-Aldrich), dehydrated, and mounted in Histomount (National Diagnostics, Atlanta, GA). The number of cells in the glomerular tuft staining for p57 was measured on 37–50 glomeruli/cross section. Because of differences in glomerular size, the tuft area was measured and used as a denominator.

Identifying pericytes. Pericytes can be identified by the expression of several markers, including the coexpression of PDGFß-R with NG2, CD73, and/or CD146. To identify pericytes that derived from CoRL, triple immunostaining was performed. To detect the reporter (tdTomato red), a Dylite 594-conjugated red fluorescent protein (RFP) rabbit antibody (Rockland Immunochemicals, Gilbertsville, PA) was used. To detect stromal cells, either a biotinylated rabbit antibody to PDGFß-R (R&D Systems, Minneapolis, MN) or an unconjugated

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rabbit antibody to PDGFβ-R (Cell Signaling, Danvers, MA) was used followed by either Alexa 488-conjugated streptavidin or Alexa 647-conjugated donkey anti-rabbit IgG. Rat antibody to mouse CD73 (R&D Systems) or rabbit antibody to NG2 or CD146 (Millipore, Billerica, MA) were used to identify pericytes, followed by either DyLight 405-conjugated donkey anti-rat IgG, Alexa 594-, or Alexa 488-conjugated streptavidin (Jackson ImmunoResearch).

**Renin staining.** To confirm that renin protein expression was confined to cells in the extraglomerular compartment, and that renin expression did not occur de novo during disease, staining was performed with biotinylated sheep antibody to renin (Innovative Research, Novi, MI), using the methods described below.

**Identifying the tomato red reporter.** No antibody is required to visualize the tomato red reporter, the expression of which is induced

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**Fig. 1.** Increase in glomerular and interstitial scarring and peritubular microvascular damage in Ren1cCreER × RcdTomato-R mice with focal segmental glomerulosclerosis (FSGS). Representative images show periodic acid-Schiff staining at baseline (A) and in a FSGS kidney (B). Arrows indicate dilated tubules, and proteinaceous material within dilated tubules is indicated by p. Quantification of the percentage of area staining for PDGFβ-R receptor (R) significantly increased in FSGS (C). Representative images show PDGFβ-R staining (green) at baseline (D), which is increased in FSGS (E). Quantification of the percentage of area staining for CD31 significantly decreased in FSGS (F). Representative images show CD31 staining (green) at baseline (G) and in FSGS (H).
by tamoxifen to permanently label a subset of CoRL during that temporal window. To visualize Tomato red in Ren1cCreER × Rs-tdtTomato-R mice, kidneys were perfusion fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4, followed by emersion fixation for 60 min. Biopsies were then transferred to 30% sucrose, left at 4°C overnight, and frozen in Tissue-Tek Cryo-OCT Compound (VWR). Four-micrometer cryosections were utilized. Immunofluorescent staining for the tomato red reporter was also performed as described below.

**Identifying podocytes derived from CoRL.** Immunofluorescent double staining for the tomato red reporter was performed in combination (podocin and synaptopodin) as we have reported (45). To detect the reporter (tdTomato red), a DyLight 594-conjugated RFP rabbit antibody (Rockland Immunochemicals) was used. To detect podocyte proteins, a rabbit antibody to podocin (Abcam, Cambridge, MA) or mouse monoclonal antibody to synaptopodin (Fitzgerald Industries, Concord, MA) were used. The appropriate biotinylated secondary antibody (Vector Laboratories) was applied followed by streptavidin, Alexa Fluor 488 conjugate (Molecular Probes, Eugene, OR). Because the RFP antibody was directly conjugated, this eliminated any chance for cross-reactivity of the reporter antibody with the other antibodies or the detecting reagents used to visualize them. Negative controls

Fig. 2. Interstitial pericyte number decreased in Ren1cCreER × Rs-tdtTomato-R mice with FSGS. Triple staining identified pericytes as negative for α-smooth muscle actin (SMA) and positive for PDGFβ-R and NG2 (αSMA- PDGFβ-R + NG2+). A–C: single-channel images at baseline for α-SMA (A, green), PDGFβ-R (B, blue) and NG2 (C, red). When merged (D), αSMA- PDGFβ-R + NG2+ cells have a purple/pink color and are indicated by the numbers shown. αSMA- PDGFβ-R + NG2+ contained white/green color and were not considered pericytes. E–G: single-channel images at day 14 FSGS for α-SMA (E), PDGFβ-R (F), and NG2 (G). When merged (H), αSMA- PDGFβ-R + NG2+ cells have a purple/pink color and are indicated by the numbers shown. αSMA- PDGFβ-R + NG2+ contained white/green color and were not considered pericytes. Quantification showed that the number of pericytes (αSMA- PDGFβ-R + NG2+) was significantly lower in FSGS (I).

* p=0.0035 vs Baseline

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**Note:** This text is a continuation of the description of the experimental methods and results related to the study of pericyte and podocyte progenitors in the renal system. The images and figures illustrate the staining procedures and the outcomes of the experiments, showing the decrease in interstitial pericyte number with FSGS, and the quantification of pericyte numbers. The results are supported by statistical analysis, indicating significant differences in pericyte numbers between baseline and FSGS conditions.
consisted of omitting the primary antibody and using tissue from Ren1cCreER × Rs-tdTomato-R mice where Cre was absent, which confirmed both the specificity of the RFP antibody and that there was no cross-reactivity (45).

The number of reporter-positive vs. the number of reporter-podocin or -synaptopodin double-positive cells were counted to determine what percentage of reporter-positive cells within the glomerular tuft were expressing either podocin or synaptopodin.

Identifying endothelium. To determine whether adult endothelial cells also derived from CoRL during disease, and to demonstrate that CoRL-derived pericytes surrounded the peritubular microvascular endothelium, double immunostaining was performed. To detect the reporter (tdTomato red), a DyLight 594-conjugated RFP rabbit antibody (Rockland Immunochemicals) was used. To detect endothelial cells, a rabbit monoclonal (EPR3864) to ERG (ets related gene; Abcam) and a rat antibody to CD31/PECAM (Dianova/Jackson ImmunoResearch) were used.

Immunostaining for potential stem/progenitor markers. Mouse mesenchymal stem cells (MSCs) can be identified by positive expression of a panel of markers: CD105, CD106, CD73, CD29, CD44, Sca-1, and negative expression of CD45 and CD11b (2, 25, 26, 43, 46, 47, 62, 66). To determine whether CoRL expressed any of these stem/progenitor markers, double immunostaining was performed. For two-dimensional evaluation, 4-μm fresh-frozen sections were stained with a panel of antibodies against MSC markers (CD105, CD106, CD73, CD29, CD44, and Sca-1, R&D Systems) according to the manufacturer’s instructions. Alexa 488-labeled species-specific IgG secondary antibodies (Invitrogen, Life Technologies, Grand Island, NY) were applied for visualization of primary antibodies. To detect the tdTomato red reporter, a DyLight 594-conjugated RFP rabbit antibody (Rockland Immunochemicals) was used. As a negative control, all staining was performed without primary antibodies.

Immunoelectron microscopy. To better visualize the morphology of CoRL when in a glomerular location, immunoperoxidase staining was performed with a rabbit antibody to RFP (Rockland Immunochemicals). The tomato red reporter was then visualized with electron-dense DAB that was reacted with 2% OsO₄, dehydrated, and infiltrated with a 50/50 mixture of PolyBed (PolySciences, Warrington, PA) and propylene oxide. Ultrathin sections were prepared, mounted on grids, and examined by transmission electron microscopy as previously described (1).

Fig. 3. Cells of renin lineage (CoRL) were detected in the interstitium in FSGS mice, and a subset coexpress the pericyte marker PDGFβ-R. A: Ren1cCreER × Rs-tdTomato-R mice given tamoxifen had tdTomato-labeled cells (arrows, red color) in the juxtaglomerulus surrounding an arteriole (a) but not in the glomerulus (g). B: quantification showed the number of red fluorescent protein (RFP) reporter-positive and PDGFβ-R- positive cells significantly increased in FSGS. Representative images of FSGS show PDGFβ-R (C, green), Tdtomato reporter (D, red), and merge (E, yellow). F–H: higher magnification images (×1,000) of the areas shown in the dashed insets in C–E (arrows indicate the cells of particular interest).
A

* p=0.0031 vs Baseline

E

* p=0.0003 vs Baseline

B

% pericytes RFP+

F

1000x

C

400x

D

400x

G

400x

H

1000x

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Microscopy and statistical analysis. PDGFβ-R staining, CD31 staining, reporter-positive cells, and pericytes were quantified on 20 images of kidney cortex using ×200 total magnification using the EVOS FL Cell Imaging System (Life Technologies). Reporter-positive cells within glomerular cross sections were quantified on an average of 56 ± 6 glomeruli/animal and were categorized as reporter positive alone or reporter and podocyte protein positive. All fluorescent images presented were collected by confocal laser microscopy on a Leica DMI400B. One-way ANOVA was calculated, and a P value <0.05 was considered significant. Two-tailed t-tests were used to calculate significance, and data are presented as means ± SE. All data were analyzed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

RESULTS

CoRL Were Detected in the Tubulointerstitial Compartment in the FSGS Model

The abrupt podocyte-depletion model of FSGS was initiated by antibody-mediated cytotoxicity to podocytes (40, 45, 64, 70, 71). As occurs in other experimental and human proteinuric diseases, glomerular changes were accompanied by secondary tubulointerstitial injury, characterized by proteinaceous casts within tubular lumens, patchy areas of interstitial fibrosis, periglomerular hypercellularity, and patches of tubular atrophy and dilation (Fig. 1). Similar to other interstitial fibrotic states (6, 49), the number of interstitial cells expressing PDGFβ-R was markedly increased in this model, accompanied by dilatation and loss of the peritubular microvascular endothelium (Fig. 1).

To determine whether the secondary tubulointerstitial injury was accompanied by a decrease in the number of pericytes, triple immunostaining was performed. When identified by negative staining for α-smooth muscle actin (α-SMA) and positive staining for both PDGFβ-R and the chondroitin sulfate proteoglycan NG2 as previously described by others (22), the number of pericytes (α-SMA− PDGFβ-R− NG2+) was significantly lower in FSGS mice (10.5 ± 1.1, FSGS vs. 24.1 ± 1.7, baseline, P = 0.003) (Fig. 2).

We next mapped the fate of renin-producing juxtaglomerular cells, referred to as CoRL, by treating Ren1cCreER × Rs-tdTomato-R mice with tamoxifen 8–11 wk before the induction of FSGS. Following labeling of CoRL, we detected these cells by red fluorescence restricted to the juxtaglomerular apparatus (JGA) as expected (Fig. 3). Labeled CoRL were not detected in the glomerular tuft and only extremely rarely in the tubulointerstitial compartment (Fig. 2). Fourteen days after induction of FSGS, labeled CoRL were detected in the interstitial compartment of the kidney cortex (Fig. 3). Temporally, this followed the appearance of labeled CoRL in glomeruli (45). Labeled CoRL in the interstitium were typically located surrounding capillaries. Thus, in addition to moving into the glomerular compartment (45), CoRL also moved to the interstitial compartment in the FSGS model of acute podocyte depletion.

CoRL Migrated to the Tubulointerstitium and Coexpress Pericyte Markers

Pericytes cover peritubular capillaries, yet are profibrotic in progressive kidney diseases by differentiating into myofibroblasts (10), and little is known about the repopulation of pericytes in disease (21). To determine whether a subset of adult pericytes derive from fate-mapped CoRL (red color), CoRL-derived interstitial cells were labeled for pericyte markers PDGFβ-R, NG2, CD73, and CD146 (Figs. 3 and 4). A subset of the tdTomato reporter-labeled CoRL coexpressed PDGFβ-R (green) in disease, creating a yellow color (Fig. 3). When quantitated, there was a significant increase in the number of reporter+PDGFβ-R+ cells in the interstitium following FSGS (P = 0.0172 vs. baseline). A minority of tdTomato-labeled cells in the interstitium did not express PDGFβ-R, despite being in the same location as those that coexpressed PDGFβ-R. These observations suggest a subset of CoRL migrate from their normal juxtaglomerular location to the interstitium, and transdifferentiate into pericytes during FSGS.

To validate this further following FSGS, colabeling for the reporter with the pericycle markers NG2 (green) and PDGFβ-R (blue) identified an average of 3.4 ± 0.45 reporter-positive pericytes/mm² vs. 0.63 ± 0.07 at baseline (P = 0.003 vs. baseline). This number indicated that reporter-positive pericytes accounted for 25% of all pericytes at 14 days post-induction of FSGS. Two additional pericycle markers, CD73 (blue) and CD146 (green), identified 50% of the tdTomato reporter−, PDGFβ-R+ interstitial cells coexpressed CD73, whereas 70% of these double-positive cells coexpressed CD146 (Fig. 4). Therefore, following a decrease in the number of native pericytes in FSGS, a subset of labeled CoRL present in the interstitium acquired several markers of pericytes during the fibrotic phase of disease.

To rule out the possibility that labeled CoRL in the interstitium were endothelial cells, sections were colabeled for PDGFβ-R (green) and the endothelial marker ERG (blue nuclei) (19, 50) (Fig. 5). Mapped CoRL in the interstitium coexpressed PDGFβ-R, but not ERG. As expected, ERG expression was limited to endothelial cells. CoRL were also colabeled with the endothelial marker CD31. As expected, reporter-positive CoRL could clearly be seen surrounding afferent arterioles, but following induction of FSGS, CoRL could also be observed surrounding the peritubular microvascular endothelium, a typical feature of pericytes. Taken together, these results show that a subset of mapped CoRL move to the interstitium during disease, acquire markers of pericytes, but not endothelial cells, and surrounded the peritubular microvascular endothelium. A minority of mapped CoRL did not express pericyte markers after migration to the interstitium. In the
same diseased kidneys, a subset of CoRL also migrate to the glomerulus and acquire features of both podocytes and parietal epithelial cells (45).

Pericytes Derived From CoRL Do Not Produce Renin

Studies have shown that kidney pericytes in young mice can express renin, although this decreases with age (3). Although the studies described herein were performed in Ren1cCreER × RstTomato mice where a subset of adult CoRL are only labeled following tamoxifen administration in adulthood, pericytes derived from CoRL were interrogated for renin production (Fig. 6). In baseline kidneys, renin staining was restricted to cells in the JGA before the induction of disease, and a subset of these cells coexpressed the tdTomato fate map reporter. In disease, renin staining remained confined to cells in the JGA and was not detected in the interstitium or glomerulus in experimental FSGS.

CoRL Migrate to Both Glomerular and Interstitial Compartments in the Remnant Model of Chronic Kidney Disease

To understand whether migration of CoRL from the JGA to glomerular capillaries as well as peritubular capillaries is a
Similar to published studies (57, 60), 5⁄6 nephrectomy in these teen weeks after renal mass reduction, kidneys were analyzed. (83%) of the total kidney mass was surgically removed. Six-administering tamoxifen. Eight to 11 wk later, five-sixths (83%) surgery), our data showed the following. First, when every Rs-tdTomato-R was markedly increased. Therefore, in tamoxifen-activated −R was markedly increased. Therefore, in Glomerular tuft section, P < 0.001 vs. baseline) (Fig. 7). Synechial attachments were also noted in glomeruli with segmental glomerulosclerosis. In addition to the expected glomerular changes, many tubular lumens contained proteinaceous casts, accompanied by tubular atrophy and dilation. Patchy areas of interstitial fibrosis were seen (Fig. 7). Moreover, the number of interstitial cells staining for PDGFβ-R was markedly increased. Therefore, in tamoxifen-activated Ren1cCreER × Rs-tdTomato mice, % nephrectomy resulted in features of chronic kidney disease of both glomeruli and the tubulointerstitium and was accompanied by podocyte depletion and expansion of pericyte-derived cells in the interstitium. To determine whether a subset of CoRL that migrated to the remnant glomeruli coexpressed a podocyte protein.

A Subset of CoRL That Migrated to the Glomerular Tuft Coexpress Podocyte Proteins

To determine whether a subset of CoRL that migrated to the remnant glomerular tufts coexpressed podocyte proteins, double-staining was performed for tdTomato and podocin, and for tdTomato and synaptopodin (Fig. 8). The data showed double labeling for the CoRL reporter and podocyte proteins in 0.1 ± 0.02 cells/glomerular cross section vs. 0.0 at baseline, P < 0.001. Stated differently, 34% of labeled CoRL that moved to the glomerular tuft, and not along Bowman’s capsule. Third, glomerulosclerosis was typically absent or barely detected in individual glomeruli containing fate-mapped positive CoRL (P < 0.001 vs. glomeruli with no CoRL) (Fig. 8). Fourth, within the same kidneys where mapped CoRL were detected in glomeruli, a subset of mapped CoRL were also detected in the interstitium, and these were restricted anatomically to peritubular capillaries. Therefore, mapped CoRL migrated to both glomerular and interstitial compartments in the remnant model of chronic kidney disease.

### Baseline

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<tr>
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<td>C</td>
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### FSGS

**Table:**

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Fig. 6. CoRL in the interstitium exhibit ultrastructural features consistent with pericytes and cated for PDGFβ-R, but not for renin. A: representative image of triple staining at baseline showing reporter (RFP, red), PDGFβ-R (green), and renin (blue). As expected, reporter and renin staining colocalized in cells in the juxtaglomerular surrounding the arteriole (a) but neither were detected in the glomerulus (g). B: representative image of triple staining in FSGS. The reporter (red) and PDGFβ-R (green) colocalized in cells in the interstitium (yellow color, arrow). Renin staining (blue) was not detected in the interstitium. A glomerulus is indicated (g) C: a higher magnification (×1,000) of the area shown in the dashed inset in B. These data show that a subset of interstitial pericytes derive from CoRL.
detected in the CoRL surrounding the arterioles outside the glomerulus, and renin granules were also observed, which served as a positive control (Fig. 8G). The majority of podocytes lacked electron-dense (black) staining in their cell bodies and foot processes (Fig. 8H). However, cells with reporter (RFP) staining contained electron-dense black material within their cell bodies and foot processes in remnant kidney (Fig. 8I). These results are consistent with a subset of CoRL within the glomerular tuft having ultrastructural features of podocytes.

Because acute depletion in podocyte number in the FSGS model was accompanied by CoRL coexpressing PEC markers (45), expression of the PEC marker claudin-1 was assessed, but in the remnant kidney model no CoRL were found to coexpress claudin-1 at the time point analyzed (not shown).

A Subset of Labeled CoRL in the Interstitium of Remnant Kidneys are Pericytes

Mapped CoRL (tdTomato+) were readily seen in the interstitium of remnant kidneys, and they coexpressed PDGFβ-R, but not renin (Fig. 9). More than 90% of mapped CoRL that were in the interstitium were closely associated with capillaries and coexpressed the pericyte marker CD146 (Fig. 10). However, none of the interstitial mapped CoRL expressed the endothelial marker ERG (blue nuclei) or CD31 (19, 50) (Fig. 10). Therefore, in the remnant model of chronic kidney disease, a subset of CoRL moved to the glomerulus and coexpress podocyte proteins, whereas another subset of CoRL moved to the interstitium, coexpress pericyte markers, and become closely integrated with the peritubular microvascular endothelium.
Differential Staining for Mesenchymal Stem Markers in CoRL That Migrate to the Glomerulus and Interstitium

Increasing evidence suggests MSCs are a subpopulation of pericytes.(2, 25, 26, 43, 46, 47, 62, 66). To test whether CoRL express markers consistent with MSCs, we labeled kidneys with a panel of MSC markers including CD29, CD105, CD106, CD44, and Sca-1. CD29 was expressed by tdTomato-labeled CoRL in the JGA at baseline (Fig. 11). The majority of tdTomato-mapped CoRL that migrated to the glomerulus in FSGS also coexpressed CD29 (Fig. 11), as well as labeled CoRL that migrated to the interstitium. By contrast, CD105 was not detected in CoRL in the JGA at baseline although the receptor was readily detected on the endothelium (15). In the setting of disease, the majority of mapped CoRL that migrated to the interstitium coexpressed CD105 (Fig. 11), whereas those CoRL that migrated to the glomerulus in FSGS did not express CD105. CD106, CD44, and anti-Sca-1 were not detected in labeled CoRL at baseline or in disease.

CoRL Were Restricted to the Juxtaglomerular Compartment Following Uninephrectomy

In the two proteinuric models described, CoRL served as progenitors for glomerular (podocytes) and interstitial (pericytes) cell types, both of which derive from cells of mesenchymal origin during development. To ensure that these events were not simply events that occurred when kidneys are perturbed, the uninephrectomy model was performed in tamoxifen-labeled Ren1cCreER × Rs-tdTomato mice. As expected, despite a 50% decrease in nephron mass, the remaining kidney does not develop glomerular or interstitial fibrosis (data not shown), and absolute podocyte density remains unchanged (2.74 ± 0.2 vs. 2.73 ± 0.14, P = 0.5). Twelve weeks following uninephrectomy, no labeled CoRL were detected in glomeruli or the interstitium; they were restricted to the JGA and were indistinguishable from those in the excised kidney from each experimental mouse. Therefore, although kidney mass is reduced by 50% and renal growth occurs, in the absence of kidney injury, mapped CoRL remain restricted to their juxtaglomerular location.

DISCUSSION

Here, we show that renin-expressing vascular smooth muscle cells of the JGA (known as CoRL) migrate from the arteriolar wall during acute and chronic kidney disease and differentiate into glomerular podocytes and non-glomerular pericytes. Therefore, CoRL are a pool of latent progenitor cells that have multilineage potential in animal models of adult kidney disease that ensues after injury.

Acute and progressive glomerular diseases, such as FSGS and the remnant kidney model of chronic kidney disease, respectively, are typically accompanied by changes in the tubulointerstitium (11). These include tubular atrophy and dilatation, peritubular vascular damage, and interstitial fibrosis. The integrity of peritubular vessels is dependent on neighboring interstitial pericytes. However, in the tubulointerstitial damage that occurs secondary to glomerular diseases, interstitial pericytes change from their normal supportive role of blood vessels to profibrogenic and deleterious cells (10). This begs the question of whether adult interstitial pericytes can be replaced to maintain their number and thus function. The JGA has been thought of as a site of cellular plasticity from previous studies (14, 55). To explore this possibility, we used an inducible fate-mapping approach in Ren1cCreER × Rs-tdTomato-R reporter mice. The strength of this approach is that only a cohort of CoRL are permanently labeled during a period of tamoxifen induction. The fate of these adult cells in disease can therefore be accurately and confidently mapped.

The first major finding from these studies is that in both the FSGS and remnant chronic kidney disease models, a subset of CoRL migrate to perivascular locations in the kidney interstitium in both cortical and corticomedullary regions. These cells express pericyte markers and appear closely integrated into the vascular wall, and appear to replace pericytes lost through differentiating into scar-forming myofibroblasts and migrate into the expanding interstitial spaces. To date, the source of new adult pericytes to replenish pericyte loss is unknown, although it has been speculated from a number of studies that resident progenitor cells likely exist that can serve this function (10). The current studies provide the first evidence of an adult pericyte progenitor and show that the JGA is a progenitor source for new pericytes in a disease state.

We have reported that a subset of CoRL migrate from the juxtaglomerular compartment to the intraglomerular compartment following an abrupt decrease in podocyte number by 30–40% (45). The purpose of the current study was to determine whether similar events occurred in a chronic glomerular disease characterized by reduced podocyte number. Similar to other reports in the % remnant kidney model, results from the current study in an inducible CoRL reporter mouse show a...
30% decrease in podocyte number in this model. The second major finding is that remnant kidney glomeruli have recruited CoRL to the glomerular tuft following 16 wk of progressive kidney disease. A proportion of the CoRL that have moved to the glomerular tuft coexpress proteins functionally important, and typically restricted to, podocytes and also demonstrate ultrastructural features of podocytes. In combination with their new location (i.e., attached to glomerular capillary loops), these results are consistent with a subset of glomerular CoRL differentiating into adult podocytes in disease characterized by chronic podocyte depletion.

An important observation is that CoRL movement to the glomerulus is closely and temporally associated with a decrease in podocyte number. This reasoning follows the results of these studies, where the decrease in nephron mass by 50% following uninephrectomy does not trigger migration of CoRL after 12 wk. Podocyte number is unchanged following uninephrectomy. However, when individual mice are followed longitudinally following the “conversion” from uninephrectomy to the remnant kidney model, podocyte number decreases, and CoRL are now detected in a subpopulation of glomeruli. Thus, similar to the model of abrupt podocyte depletion of ≥30% (45), a “tipping point” may be reached when >80% of the kidney mass is removed (% remnant kidney model) (8, 57), which leads to a chronic decrease in podocyte number, also by 30%. The implication from this comparison is that the JGA receives signals from injured cells, either in glomerular tufts, where endothelial cells have lost podocyte coverage, or in the interstitium, where capillaries have lost pericyte coverage.

When all glomeruli in the remnant kidney tissue are carefully examined, the decrease in podocyte number in remnant glomeruli greatly exceeds the increase in glomerular number of labeled CoRL after 16 wk of disease. Thus podocyte loss clearly exceeds any regenerative mechanisms attempted by CoRL. However, within the subset of individual glomeruli where CoRL migrated and coexpressed podocyte proteins,
glomerulosclerosis was not detected. The authors acknowledge CoRL have a limited adult podocyte regenerative capacity in chronic glomerular disease. We can only speculate that certain therapies that limit scarring might augment CoRL regeneration of adult podocytes. Such studies are needed.

In these studies, adult CoRL were reprogrammed to transdifferentiate into both adult podocytes and adult pericytes in two disease models. These studies beg the question of whether regeneration of these cells in adult kidneys might derive from a common mesenchymal progenitor. CoRL have been reported as progenitors for vascular smooth muscle cells and mesangial cells (61). Vascular smooth muscle, including cells of the JGA, pericytes, and mesangial cells, derive from Foxd1+/Tcf21+ progenitor cells of the metanephric mesenchyme (21, 32, 39). Podocytes and PECs, on the other hand, derive from a separate pool of Six2+/Cited1+ progenitor cells that form the epithelium of the kidney. In late development through the neonatal period, however, podocyte and PEC progenitors activate transcription factors that regulate mesenchymal differentiation, including Foxd1 and Tcf21 (38, 48). This coincides with differentiation of these glomerular epithelial cells into cells with more mesenchyme characteristics and indicates similiar-

Fig. 10. CoRL in the interstitium in remnant kidneys are pericytes and not endothelial cells. A: representative image of triple staining showing the CoRL reporter (RFP, red), PDGFβ-R (blue), and the additional pericycle marker CD146 (green) colocalizing (purple and white) in the interstitium of remnant kidneys (arrows). B: higher magnification of image from A showing colocalization. C–E: to ensure that CoRL in the interstitium were not differentiating into endothelial cells, triple staining was performed for the CoRL reporter (RFP, red), PDGFβ-R (green), and the endothelial cell marker ERG (blue). C: there was no staining overlap at baseline (no purple and white color). PDGFβ-R (green) localized to interstitial cells and mesangial cells in the glomerular tuft (g) and did not colocalize with ERG. Reporter localized to PDGFβ-R staining cells in the juxtaglomerularus surrounding (yellow color, arrow) an arteriole (a), but did not colocalize with ERG staining. D: in the remnant kidney, triple staining showed that the reporter (RFP, red) and PDGFβ-R (green) colocalized (yellow, arrow), but neither colocalized with ERG (blue; no purple and white colors detected). E: higher magnification of the area shown in the dashed inset in D.
Fig. 11. CoRL differentially express mesenchymal stem cell (MSC) markers in FSGS. A: representative image of double staining showing reporter (RFP, red) and CD29 (green) colocalize in cells in the juxtaglomerular surrounding an arteriole (a) at baseline. Double staining showing reporter (RFP, red) and CD29 (green) colocalize (yellow) in cells in the glomerular tuft (g; B) and perivascular cells in the interstitium (C) in FSGS. D: representative image of triple staining showing reporter (RFP, red), CD105 (green), and PDGFβ-R (blue) do not colocalize in cells in the juxtaglomerular surrounding an arteriole (a) at baseline. E: triple staining showing reporter (RFP, red), CD105 (green), and PDGFβ-R (blue) colocalize (purple) in interstitial cells in FSGS. F: higher magnification of the area shown in the dashed inset in D.
ties between podocytes and pericytes. In addition, podocytes share characteristics of pericytes such as high production of angiogenic and endothelial survival factors, synthesis of capillary basement membrane, and local regulation of flow (12, 24). Therefore, unlike the tubular epithelium, the podocyte (and PEC) harbors both epithelial as well as mesenchymal (perivascular) characteristics.

Our finding that CoRL can serve to replenish podocytes under conditions of injury in adult mice would appear to be ostensibly at odds with reports on the mechanism underlying specification of the respective cell lineages during kidney development. In an extensive series of studies, Kobayashi et al. (27) have elegantly delineated that a strict nephron and stromal lineage boundary appears to be established early during kidney organogenesis. Fate mapping of stromal lineages, as demarcated by Foxd1 expression, and epithelial/nerphron lineages, as demarcated by Six2 expression, indicated that while there is a low transient reassignment from the Foxd1 to Six2 compartments at the onset of kidney development, thereafter distinct multipotent self-renewing progenitor populations for the two compartments appear. As shown by Brunskill et al. (5) and noted by Kobayashi et al. (27), there is a curious robust expression of Foxd1 that characterizes the adult podocyte expression profile. Moreover, Sequeira-Lopez et al. (56), while studying the relationship between the Foxd1 progenitor compartment and Ren1-expressing compartment, noted a hierarchical relationship such that Foxd1 progenitors gave rise to other mural cell lineages (smooth muscle cells, perivascular fibroblasts and pericytes) in addition to the Ren-expressing cells. Of interest, under conditions of injury induced by Foxd1-driven diphtheria toxin expression, they observed increased ectopic renal tubular expression of Foxd1 lineage markers (56). They interpreted this finding as indicative of either an attempt to increase the expression of Fox1 within a common mesenchymal progenitor cell, resulting in additional differentiation to Six2 lineage cells, or de novo expression of Foxd1 within the epithelial compartment in an attempt to compensate for a lack of Foxd1 in stromal derivatives. Kobayashi et al. (27) also noted occasional labeling of renal tubular structures during Foxd1 lineage tracing. These and our findings in the current study and recent published reports (45) raise the question of whether there may be enhanced plasticity under conditions of injury in adult cells. Finally, under certain conditions such as VHL deletion, they have been reported to undergo transdifferentiation into EPO-producing cells (29).

These studies also build on previously published work, which has shown CoRL supply new adult podocytes in aging nephropathy (44), supply PECs in FSGS models (45), as well as recently published work that CoRL supply new mesangial cells in states of mesangiolysis (61). It is likely therefore that the JGA provides a niche for latent progenitor cells in many forms of kidney disease. At this time, however, we have little understanding of the signals and molecular pathways that sense and regulate the differentiation steps, nor do we know whether such a mechanism could be enhanced for therapeutic benefits to patients with kidney disease.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


