

Detection of renin lineage cell transdifferentiation to podocytes in the kidney glomerulus with dual lineage tracing

Diana G. Eng^{1,4}, Natalya V. Kaverina^{1,4}, Remington R.S. Schneider¹, Benjamin S. Freedman¹, Kenneth W. Gross², Jeffrey H. Miner³, Jeffrey W. Pippin¹ and Stuart J. Shankland¹

¹Division of Nephrology, University of Washington, Seattle, Washington, USA; ²Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, New York, USA; and ³Division of Nephrology, Washington University School of Medicine, St. Louis, Missouri, USA

Understanding of cellular transdifferentiation is limited by the technical inability to track multiple lineages *in vivo*. To overcome this we developed a new tool to simultaneously fate map two distinct cell types in the kidney, and genetically test whether cells of renin lineage (CoRL) can transdifferentiate to a podocyte fate. *Ren1cCreER/tdTomato/Nphs1-FLPo/FRT-EGFP* mice (CoRL-PODO mice) were generated by crossing *Ren1c-CreER/tdTomato* CoRL reporter mice with *Nphs1-FLPo/FRT-EGFP* podocyte reporter mice. Following tamoxifen administration in these animals, CoRL were labeled with red fluorescence (tdTomato) and co-localized with renin. Podocytes were labeled green (enhanced green fluorescent protein) and co-localized with nephrin. Following podocyte loss by nephrotoxic antibody and subsequent enalapril-enhanced partial replacement, tdTomato-EGFP-labeled CoRL were detected as yellow-colored cells in a subset of glomerular tufts, without the use of antibodies. Co-localization with podocin indicated that these cells are podocytes, derived from CoRL origin. Thus, our novel study shows that two distinct cell types can be simultaneously labeled in the mouse kidney and provide strong genetic evidence *in vivo* that lost podocytes can be replaced in part by CoRL.

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Correspondence: Stuart J. Shankland, Division of Nephrology, Department of Medicine, University of Washington School of Medicine, Box 358058, 750 Republican Street, Seattle, Washington 98109, USA. E-mail: stuartjs@uw.edu

⁴These authors contributed equally.

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Site-specific recombinase (SSR) is an essential tool for lineage-tracing cells in mice.¹ DNA recombinases from the *P1* bacteriophage (Cre) and *Saccharomyces cerevisiae* (flippase, FLP), combined with cell-specific promoters and reporters silenced by *loxP*- or *FRT*-flanked STOP sequences, allow for permanent expression of either fluorescent or enzymatic reporters in specific cell populations.² Combined with ligand-dependent activation, such as the tamoxifen-responsive CreER^{T2} recombinase, SSR can be achieved within specific temporal periods.^{3,4} This provides a noninvasive method of mapping cell fate throughout development or tracing cell lineages during disease and/or regeneration.

SSR techniques have been used extensively in the study of kidney regeneration, and have led to several significant findings regarding the ability of distinct cell populations to undergo repair.⁴ Yet, despite these genetic advances, simultaneous lineage tracing of 2 distinct cell types has been a challenge, in part because the majority of SSR in the kidney utilizes only Cre-*loxP* recombination.^{5,6} Expression of Cre recombinase, even under the control of distinct promoters and utilizing distinct reporters, cannot uniquely label 2 cell types. This has posed a barrier in our ability to observe transdifferentiation events, in which 1 cell type adopts the characteristics of another. The limited numbers of studies that have been performed that utilize Cre and FLP systems simultaneously, outside of the kidney, are intersectional or subtractive genetic fate mapping that do not allow for the continuous tracing of 2 distinct populations and require enzymatic or immunohistochemical staining of the β -gal reporter.^{1,7}

Here, we have succeeded in simultaneously labeling 2 distinct kidney cell populations by combining Cre-*lox* and FLP-*FRT* recombination strategies in the same mouse, regardless of sex, with the use of directly observable fluorescent reporters that we are calling dual lineage tracing. We utilized this methodology to genetically demonstrate that cells of renin lineage (CoRL) transdifferentiate toward a podocyte fate following podocyte depletion.

RESULTS

Figure 1 shows a schema of our dual transgenic approach. Unlabeled CoRL activate the *Ren1c* promoter inducing CreER expression; however, CreER remains sequestered in the

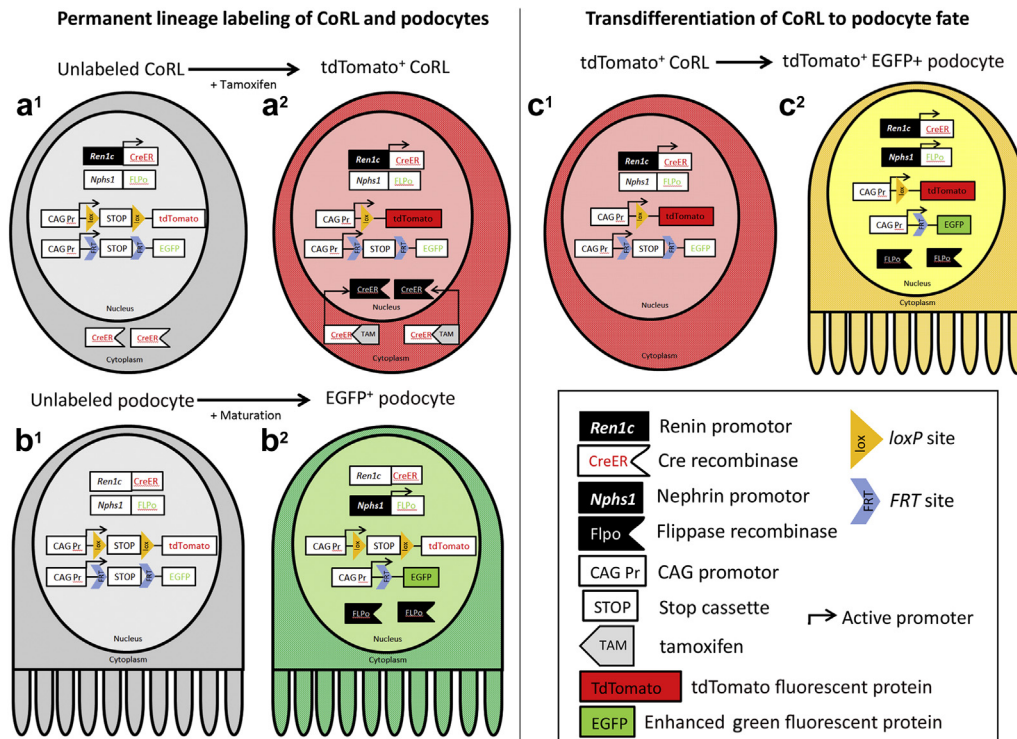


Figure 1 | Schema of dual lineage tracing transgenic approach. (a¹) Unlabeled CoRL activate the *Ren1c* promoter (black box, white type) inducing CreER (white flag, red type). CreER remains sequestered in the cytoplasm and unable to bind *loxP*. (a²) Tamoxifen (gray flag, black type) binds to the ligand-binding domain of the Cre-estrogen receptor fusion protein, resulting in translocation to the nucleus (black flag, white type) and recombination of *loxP* sites (orange triangle, black type) and the intervening STOP cassette (white box, black type), inducing permanent tdTomato (red box, black type) expression. (b¹) Immature unlabeled podocytes have not yet activated the *Nphs1* promoter (white box, black type). (b²) During podocyte maturation, the *Nphs1* promoter is activated (black box, white type), driving FLPase (black flag, white type) expression, which recombines the FLPase recognition targets (blue chevron, black type) and STOP cassette (white box, black type) to induce permanent enhanced green fluorescent protein (green box, black type) expression. (c¹) tdTomato⁺ CoRL have not yet activated the *Nphs1* promoter (white box, black type). (c²) Upon transdifferentiation to a podocyte fate, the *Nphs1* promoter is activated (black box, white type), driving FLPase (black flag, white type) expression; FLPase recombines the FRT sites (blue chevron, black type) and STOP cassette (white box, black type), inducing permanent enhanced green fluorescent protein (green box, black type), resulting in a yellow color.

cytoplasm and unable to bind *loxP* (Figure 1a¹). Following tamoxifen, CreER translocates to the nucleus and recombines the *loxP* sites to remove the STOP cassette, thus inducing permanent tdTomato expression (Figure 1a²). As podocytes mature (Figure 1b¹), the *Nphs1* promoter is activated to drive FLP expression, which recombines the FRT sites to remove the STOP cassette, thus inducing permanent EGFP expression (Figure 1b²). During transdifferentiation to a podocyte fate, tdTomato⁺ CoRL (Figure 1c¹) activate the *Nphs1* promoter, inducing FLP expression. FLP removes the FRT-flanked STOP cassette, inducing permanent EGFP expression and turning the originally red cell yellow (Figure 1c²).

Figure 2 shows validation of our dual lineage tracing approach. Following tamoxifen, young adult *Ren1cCreER/tdTomato/Nphs1-FLPo/FRT-EGFP* mice (herein called CoRL-PODO mice) expressed tdTomato specifically in cells in the juxta-glomerular compartment (JGC) (Figure 2a, arrow). As shown previously, tdTomato overlapped with 95% of renin⁺ cells (Figure 2b, arrow). When CoRL-PODO mice were given the tamoxifen vehicle corn oil, tdTomato was not detected in the JGC (Figure 2c, arrow). Similarly, in mice lacking the *Ren1cCreER* transgene, tdTomato was not

detected following tamoxifen administration (Figure 2d, arrow).

In the same mouse, EGFP (green) was detected within the glomerular tuft in a podocyte distribution pattern (Figure 2a) and overlapped with staining for the podocyte marker nephrin in 98% of EGFP⁺ cells (Figure 2e, arrow). However, in CoRL-PODO mice lacking the *Nphs1-FLPo* transgene, EGFP was not detected (Figure 2f, arrow). There was no overlap between tdTomato and EGFP under normal nonstressed conditions (Figure 2a). These results show distinct inducible tdTomato labeling of CoRL in the JGC and constitutive EGFP labeling of podocytes in the glomerular tuft, in the same kidney. As this approach enabled simultaneous tracking of 2 cell types *in vivo*, we called it dual lineage tracing.

To genetically demonstrate that a subset of CoRL can replace lost podocytes in disease, experimental focal segmental glomerulosclerosis (FSGS), characterized by podocyte loss and subsequent partial replacement, was induced in CoRL-PODO mice with a nephrotoxic antibody.^{8,9} Mice were treated with enalapril, which we have previously shown increases podocyte repletion.^{8,9} Dual lineage tracing was performed to follow migration and transdifferentiation (Figure 3). At baseline,

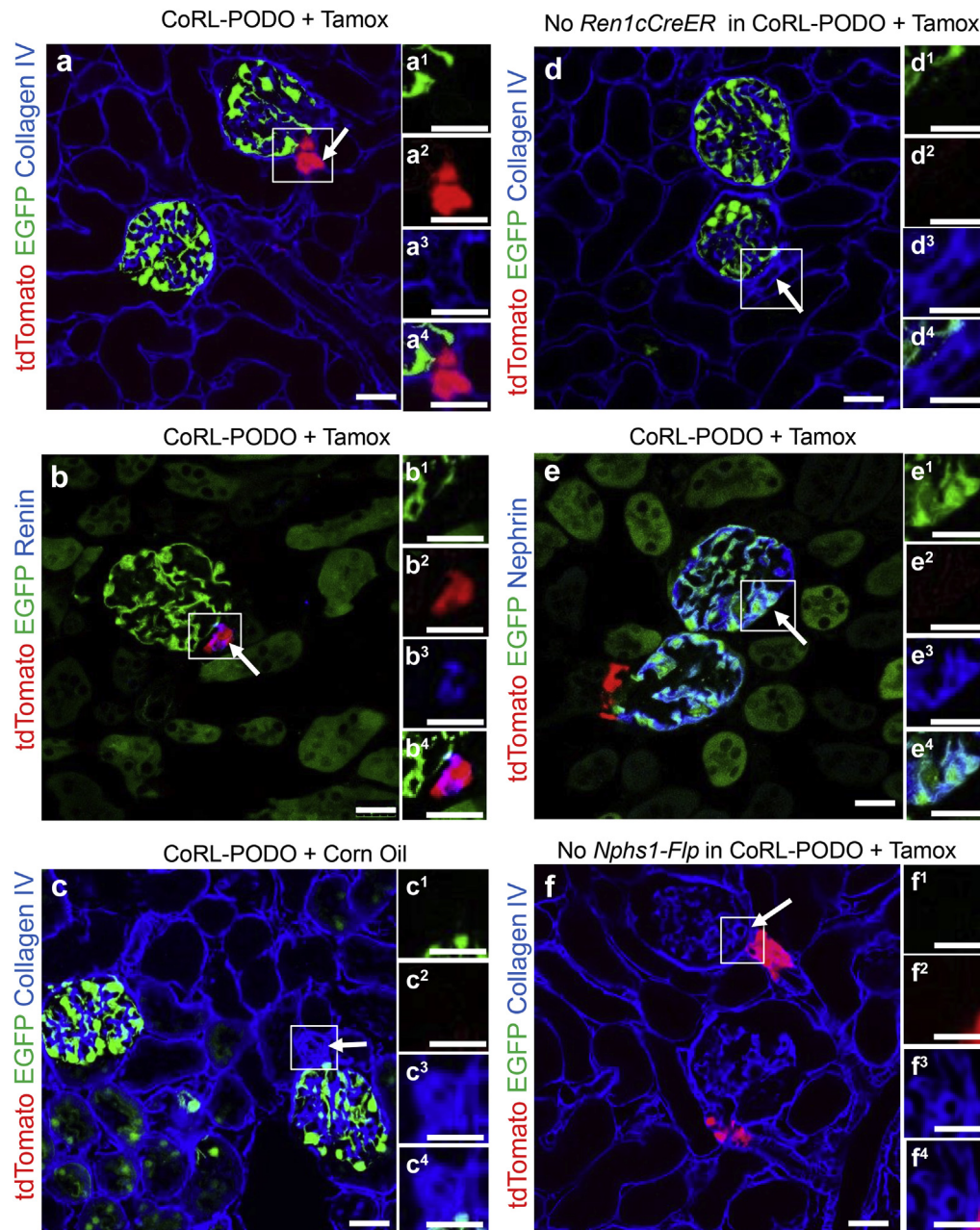


Figure 2 | CoRL-PODO mice specifically and inducibly label renin expressing cells and constitutively label nephrin expressing cells (podocytes). *Ren1cCreER/tdTomato* mice were crossed with *Nphs1-FLPo/FRT-EGFP* mice to generate dual-reporting *Ren1cCreER/tdTomato/Nphs1-FLPo/FRT-EGFP* (CoRL-PODO) mice. Immunohistochemistry for collagen IV is used to easily identify glomeruli in panels **a**, **c**, **d**, and **f**. Higher magnification images of the areas of interest outlined by the white boxes are shown to the right in superscript 1-4 for green, red, far red and merged fluorescence channels respectively. **(a)** Confocal direct fluorescence of CoRL-PODO mice administered tamoxifen (Tamox) express red fluorescence (tdTomato) in cells of renin lineage (CoRL) in the juxta-glomerular compartment (JGC; arrow) and enhanced green fluorescent protein (EGFP) in podocytes in the glomerular tuft. **(b)** Immunohistochemistry for renin in tamoxifen-induced CoRL-PODO mice shows colabeling of renin (blue) and tdTomato in CoRL in the JGC (purple color, arrow). **(c)** When CoRL-PODO mice are given the vehicle corn oil, there is no induced expression of tdTomato (arrow). The constitutive *Nphs1-FLPo/FRT-EGFP* transgene allows for the expression of EGFP. **(d)** In CoRL-PODO mice given tamoxifen that lack the *Ren1c-CreER* transgene, there is no inducible labeling of tdTomato CoRL (arrow), but the constitutive *Nphs1-FLPo/FRT-EGFP* transgene allows for the expression of EGFP. **(e)** Immunohistochemistry for the podocyte protein nephrin in tamoxifen-induced CoRL-PODO mice shows colabeling of nephrin and EGFP in podocytes (arrow). **(f)** In CoRL-PODO mice given tamoxifen who do not have the *Nphs1-FLPo* transgene, there is no labeling of EGFP in podocytes, but the induction of the *Ren1c-CreER* transgene allows for the expression of tdTomato in CoRL in the JGC (arrow). Bar = 25 μ m. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

tdTomato⁺ CoRL were restricted to the JGC. However, following podocyte depletion and enalapril treatment, tdTomato⁺ CoRL were visualized in the glomerular tuft. While a subset of CoRL that migrated remained only red, another

subset expressed both tdTomato and EGFP, indicating trans-differentiation to podocytes occurred (Figure 3).

Collagen IV staining was performed to better visualize and define the boundaries of glomeruli (Figure 3). Again,

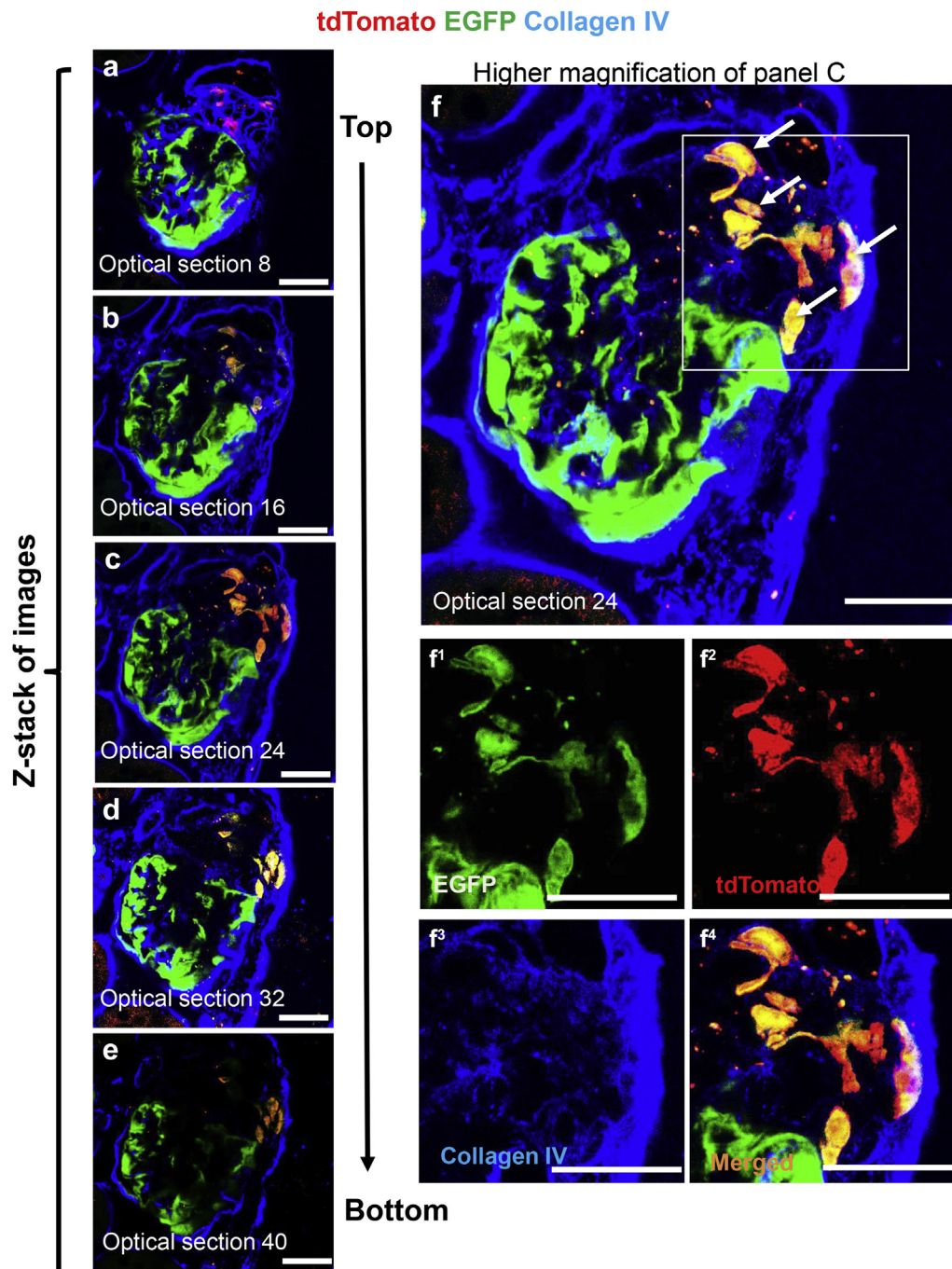


Figure 3 | A subset of red fluorescence (tdTomato)⁺ cells of renin lineage (CoRL) migrate onto the glomerular tuft and begin to coexpress enhanced green fluorescent protein (EGFP). Immunohistochemistry for collagen IV is used to easily identify glomeruli. (a–e) Fifty 0.4-μm optical sections were taken by confocal microscopy through a 20-μm thick section of a glomerulus from CoRL-PODO mice with focal segmental glomerulosclerosis. Five equally distributed representative optical sections are shown. (f) Higher magnification of (d) optical section 32 showing tdTomato⁺ EGFP⁺ colabeled cells on the glomerular tuft, which appear yellow in merged images (arrows). (f^{1–4}) Images of the areas of interest outlined by the white boxes are shown below in superscript 1 through 4 for green (EGFP), red (tdTomato), far red (Collagen IV), and merged fluorescence channels, respectively. Bar = 25 μm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

colocalization was observed between the tdTomato and EGFP reporters. Notably, optical sectioning by confocal microscopy through a 20- μ m kidney section identified tdTomato-EGFP-labeled CoRL in the glomerular tuft that would have been missed by simply looking at a single 4- μ m section (Figure 3). EGFP⁺ cells were not detected in the JGC.

To further confirm that a subset of tdTomato⁺ EGFP⁺ CoRL (Figure 4a) transdifferentiated toward a podocyte fate, staining for podocin was performed. Indeed, a subset of CoRL in the glomerular tuft coexpressed tdTomato (red), EGFP (green), and podocin (blue) (Figure 4b, arrows). Quantitation showed that after induction with tamoxifen, there were no RFP⁺ cells on the glomerular tuft. However, at D28 of FSGS, 26.3% \pm 4.2% of glomeruli per kidney section still showed

signs of podocyte injury, evidenced by a decrease in podocyte density, similar to what we have previously reported in this model.^{8,10,11} Of these glomeruli, 45.1% \pm 3.1% contained at least 1 RFP⁺ cell. Furthermore, in glomeruli with EGFP⁺ CoRL, there was an average of 6.7 \pm 1.2 individual RFP⁺ CoRL, and 53.8 \pm 0.7% were EGFP⁺.

DISCUSSION

We set out to lineage-trace 2 distinct cell types in the kidney and use dual SSR as genetic proof that tdTomato⁺ CoRL transdifferentiate to the fate of podocytes. By combining inducible Cre- and constitutive FLP-mediated recombinase systems with separate *loxP*-flanked STOP tdTomato and *FRT*-flanked STOP EGFP reporter systems, we could use tamoxifen

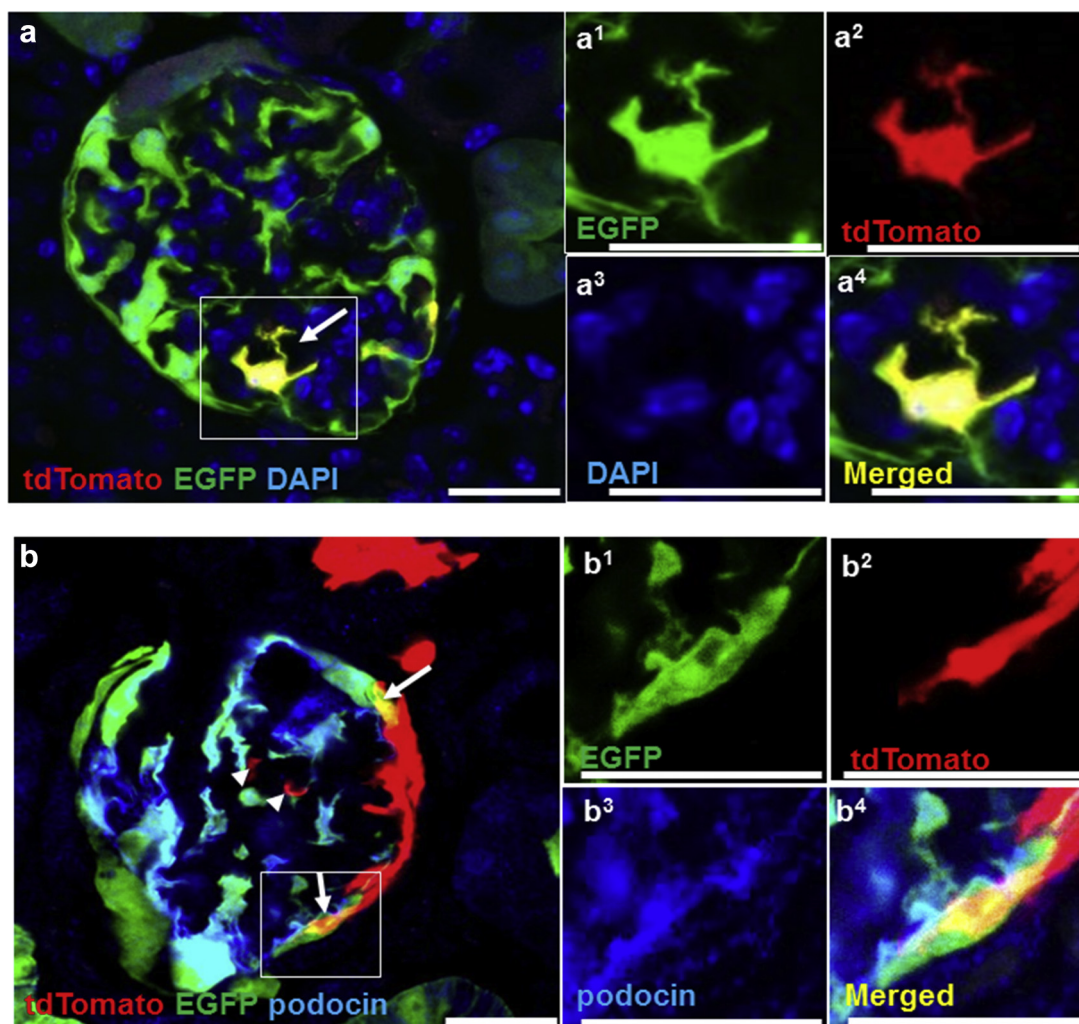


Figure 4 | Red fluorescence (tdTomato)⁺ cells of renin lineage (CoRL) on the glomerular tuft coexpress enhanced green fluorescent protein (EGFP) and stain for podocyte marker podocin. (a) Another clear example of a single tdTomato⁺ EGFP⁺ CoRL on the glomerular tuft (arrow). (b) Immunohistochemistry for the podocyte protein podocin (blue) shows colabeling with tdTomato⁺ EGFP⁺ CoRL on the glomerular tuft (arrows). (a¹⁻⁴, b¹⁻⁴) Images of the areas of interest outlined by the white boxes are shown to the right in superscript 1 through 4 for green (EGFP), red (tdTomato), far red (DAPI or podocin), and merged fluorescence channels, respectively. It should be noted that the tdTomato and EGFP reporters are not targeted to any particular cellular compartment, whereas staining for native proteins such as podocin are localized to specific structures, such as the slit diaphragms of foot processes in normal podocytes.²⁴ Furthermore, not all tdTomato⁺ CoRL that migrate onto the tuft (arrowheads) express podocyte markers.⁹ Bar = 25 μ m. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

to induce permanent labeling of CoRL with tdTomato using the *Ren1c* promoter and constitutively and permanently label podocytes with EGFP using the *Nphs1* promoter. The 2 reporters did not overlap under normal (no disease) conditions. These results support that dual lineage tracing is feasible in the kidney. The possibility of any off-target effects of Cre expression is highly unlikely given the extended washout period used in the current studies.

A similar dual SSR approach utilized dual *loxP*-flanked STOP and *FRT*-flanked STOP cassettes upstream of the *EGFP* gene. After removal of the flanked STOP cassettes via Cre- and FLPo-mediated recombination, EGFP reporter was expressed.¹² This system utilized 2 unique promoter-driven SSR systems, but labeled the cell of interest with EGFP only when both promoters were active. Therefore, it did not label 2 distinct cell types with 2 distinct reporters.

The second finding was genetic evidence that a subset of lineage traced CoRL transdifferentiate toward an adult podocyte fate following podocyte depletion in experimental FSGS. Previously, our group^{11,13,14} and others^{15,16} have relied on the *de novo* coexpression of cell-specific markers in reporter-labeled cells as evidence of transdifferentiation. For example, labeled CoRL coexpressing podocin suggests a podocyte fate, claudin-1 a parietal epithelial cell fate, $\alpha 8$ integrin a mesangial cell fate, and PDGFR β -receptor/NG2 a pericyte fate.^{11,13–16} Here we show tdTomato⁺ CoRL track into the glomerulus following podocyte depletion, and express EGFP under the control of nephrin promoter activity, a podocyte-specific gene. These findings are consistent with genetic evidence of CoRL transdifferentiation toward a podocyte fate. Furthermore, *de novo* coexpression of podocyte-specific markers were observed in dual reporter⁺ CoRL.

We acknowledge that there is some debate regarding the replenishment of adult podocytes following disease-induced loss. Elegant studies by Wanner *et al.* used a diphtheria toxin podocyte injury model and showed a 38% renewal of ablated podocytes in that model.¹⁷ In the adriamycin nephropathy model of podocyte injury, Lasagni *et al.* showed a 5% to 10% regeneration of podocyte by renal progenitor cells.¹⁵ Our data showed that in a subset of individual glomeruli with reduced podocyte density, yellow cells were detected, which in the current study are the genetic readout for CoRL transdifferentiating into podocytes. While CoRL themselves are clearly not adequate to fully replace podocytes, together with other progenitor sources they may be able to tip the balance enough to reduce scarring and improve proteinuria, as demonstrated in these studies. In addition to the current model, we have demonstrated CoRL migration into the glomerulus following chronic podocyte depletion in the experimental remnant kidney model.¹⁴

We considered using the mTmG system, which also utilizes dual reporters, where membrane-targeted tandem dimer Tomato (mT) is expressed ubiquitously prior to Cre-mediated excision and membrane-targeted green fluorescent protein (mG) after excision. Such dual reporters allow for genetic evidence of transdifferentiation but do not allow for active dual lineage tracing.^{18,19}

We recognize that a limitation to the current study is the use of constitutive podocyte labeling rather than inducible labeling. To our knowledge, ligand-inducible *FLP-FRT* systems are limited at this time. Furthermore, the constitutive *FLP-FRT* system in this study proved helpful, as we were able to show strong genetic evidence of CoRL transdifferentiation to podocytes without requiring the continuous presence of ligand during the 28 days of study. Taken together, these are not a major weakness to the current study.

Others have shown that the use of 4- μ m sections, particularly when observing glomeruli, is problematic due the relatively small sampling of the ≈ 80 - μ m glomerulus (4 μ m = 5%) and the inherent heterogeneity in glomerular size both normally and in disease.²⁰ In the current study, we utilized 20- μ m sections and optical sectioning by confocal microscopy. We noted a marked increase in the ability to detect reporter⁺ CoRL compared to a single 4- μ m section. We therefore recommend that lineage tracing in glomerular studies be performed by optically sectioning thick sections or utilizing multiple physical thin sections.

In conclusion, we have successfully shown the effective use of dual lineage tracing in the kidney, which enables tracking 2 different cell types *in vivo*. We have furthermore utilized dual lineage tracing to demonstrate the transdifferentiation of a subset of CoRL into podocytes in the kidney glomerulus after injury. As dual lineage tracing is highly flexible and could be used for a variety of different promoters and genes, it provides a powerful new tool to monitor the cellular changes and transitions during homeostasis and regeneration.

METHODS

Animals

Dual reporting *Ren1cCreER/tdTomato/Nphs1-FLPo/FRT-EGFP* (CoRL-PODO) were bred by crossing our previously generated inducible *Ren1cCreER/tdTomato* mice, utilizing the tdTomato clone Ai14 (Jackson Laboratory stock no. 007914)^{21,22} with *Nphs1-FLPo/FRT-EGFP* mice. The *Nphs1-FLPo* mouse expresses an optimized FLP recombinase (FLPo) under the control of a 4.2-kb fragment of the mouse nephrin promoter and has been characterized and previously described by Goldberg *et al.*²³ The *FRT-EGFP* mice, also known as RCE:FRT (stock no. 010812), were purchased from the Jackson Laboratory (Bar Harbor, ME). In *Nphs1-FLPo/FRT-EGFP* mice, the *FRT*-flanked STOP cassette is excised, allowing for specific and permanent EGFP reporter expression in podocytes. Control mice comprised triple transgenic mice without the Cre or FLP. Both male and female mice were utilized, and no differences between sexes were observed in these studies. Mice were housed in the animal care facility of the University of Washington under specific pathogen-free conditions. Studies were reviewed and approved by the University of Washington Institutional Animal Care and Use Committee (2968-04).

Reporter induction

Permanent labeling of CoRL with td-Tomato was induced in young adult (8-week-old) male and female CoRL-PODO mice by delivering 100 mg/kg tamoxifen (Sigma-Aldrich, St. Louis, MO), in corn oil (Sigma-Aldrich), 4 times i.p. every other day. This was followed by a washout period of 4 weeks. Control for tamoxifen was vehicle (corn oil) alone. No drugs were required for the induction of the EGFP reporter.

Podocyte depletion

Experimental FSGS was induced in CoRL-PODO mice by 2 i.p. injections, 24 hours apart, of sheep anti-glomerular antibody at 12 mg/20 g body weight.^{9,11,14} Animals were given enalapril (75 mg/l drinking water, Sigma-Aldrich) starting on day 3 of FSGS through day 28 to augment podocyte regeneration.⁹

Tissue collection

Mice were killed with an overdose of ketamine and xylazine, cardiac perfused with 10 to 15 ml of ice-cold phosphate-buffered saline (PBS) at 25 inches of gravity pressure through a 21-gauge butterfly infusion set. Kidneys were removed when blanched and split lengthwise or “butterflied.” Kidneys were then placed into 4% paraformaldehyde in PBS (PFA; Affymetrix, Santa Clara, CA) for 45 minutes, washed briefly in PBS, placed in 30% sucrose in PBS (Sigma-Aldrich) overnight, blotted dry, embedded in Tissue-Tek O.C.T. Compound (VWR, Radnor, PA), and frozen in a 100% ethanol and dry ice bath.

Visualization of dual (tdTomato/EGFP) reporter

In order to visualize tdTomato and EGFP, 20- μ m cryosections were rinsed in PBS (pH 7.4) to remove OCT compound and mounted with Vectashield with DAPI (Vector Labs, Burlingame, CA). No antibodies were required for visualization of the dual tdTomato and EGFP reporters. Confocal images were acquired with a Leica TCS SPE II laser scanning confocal microscope (Solms, Germany) with $\times 40$ (1.3 NA) oil objective, at 1024 \times 1024 pixel format with 12-bit intensity resolution. Sets of 50 serial images were collected at 0.4- μ m step size. The acquisition wavelengths were set as follows: EGFP 488 nm excitation and 497 to 510 nm emission, and tdTomato 561 nm excitation and 575 to 654 nm emission.

Multicolor immunofluorescence staining

Indirect immunofluorescence staining was performed on 20- μ m sections from 4% PFA-fixed frozen tissue processed as described above, and as previously performed.^{11,14} Frozen sections were warmed from -80°C to room temperature and allowed to air-dry. All sections were equilibrated in PBS (pH 7.4) then blocked with Background Buster (Accurate Chemical & Scientific Corporation, Westbury, NY) for 30 minutes to minimize nonspecific protein interactions. Endogenous biotin activity was quenched with the avidin and biotin blocking kit (Vector Laboratories). After blocking, sections were incubated overnight at 4°C with the appropriate primary antibodies: to identify podocytes, guinea pig antibody to nephrin, dilution 1:500 (Fitzgerald Industries International, Inc., 20R-NP002, Concord, MA), and rabbit antibody to podocin, dilution 1:4000 (Abcam, ab 50339, Cambridge, MA), were used. The appropriate biotinylated secondary antibodies (Vector Laboratories) were applied and followed by Streptavidin-AlexaFluor 647 (Life Technologies - Molecular Probes, Grand Island, NY). To demarcate the glomerular compartment, biotinylated collagen IV antibody, dilution 1:100 (Southern Biotechnology, 1340-08, Birmingham, AL) and Streptavidin-AlexaFluor 647 (Life Technologies) were used. Biotinylated anti-renin antibody, dilution 1:100 (Innovative Research, IASMPREN-GF-HT-BIO, Novi, MI) and Streptavidin-AlexaFluor 647 were used to detect renin. All immunofluorescence samples were mounted using Vectashield with DAPI. As a negative control, all staining was performed without primary antibodies.

DISCLOSURE

All the authors declared no competing interests.

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