



WT1 Is Necessary for the Proliferation and Migration of Cells of Renin Lineage Following Kidney Podocyte Depletion

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SUMMARY

Wilms' tumor suppressor 1 (WT1) plays an important role in cell proliferation and mesenchymal-epithelial balance in normal development and disease. Here, we show that following podocyte depletion in three experimental models, and in patients with focal segmental glomerulosclerosis (FSGS) and membranous nephropathy, WT1 increased significantly in cells of renin lineage (CoRL). In an animal model of FSGS in *RenWT1^{f/f}* reporter mice with inducible deletion of WT1 in CoRL, CoRL proliferation and migration to the glomerulus was reduced, and glomerular disease was worse compared with wild-type mice. To become podocytes, CoRL undergo mesenchymal-to-epithelial transformation (MET), typified by reduced staining for mesenchymal markers (MYH11, SM22, α SMA) and *de novo* expression of epithelial markers (E-cadherin and cytokeratin18). Evidence for changes in MET markers was barely detected in *RenWT1^{f/f}* mice. Our results show that following podocyte depletion, WT1 plays essential roles in CoRL proliferation and migration toward an adult podocyte fate.

INTRODUCTION

Kidney podocytes are terminally differentiated glomerular epithelial cells unable to self-renew due to their inability to proliferate (Lasagni et al., 2013; Wanner et al., 2014), and replacement following loss is dependent on local kidney progenitors (Ronconi et al., 2009; Lasagni et al., 2015; Starke et al., 2015). Glomerular parietal epithelial cells (PECs) (Romagnani, 2011; Zhang et al., 2013; Eng et al., 2015; Kuppe et al., 2015; Lazzeri and Romagnani, 2015) and cells of renin lineage (CoRL) (Pippin et al., 2013, 2014; Lichtnekert et al., 2016) are considered likely adult podocyte progenitors. CoRL serve as progenitors for several kidney cell types (Sequeira Lopez et al., 2004), including podocytes (Grahammer et al., 2013; Pippin et al., 2013, 2014, 2015; Shankland et al., 2014; Lichtnekert et al., 2016), PECs (Pippin et al., 2013; Eng et al., 2015), mesangial cells (Thoma, 2014; Starke et al., 2015), and pericytes (Stefanska et al., 2013; Pippin et al., 2015). RAAS inhibition augments the CoRL reservoir in the juxta-glomerular compartment (JGC) through proliferation, enhances migration to the glomerulus, and increases transdifferentiation toward a podocyte fate (Lichtnekert et al., 2016). CoRL and podocytes derive from mesenchymal origins (Costantini and Kopan, 2010; Matsushita et al., 2010; Little and McMahon, 2012; Wang et al., 2013). CoRL maintain mesenchymal characteristics, whereas adult podocytes exhibit epithelial characteristics (Filipovic et al., 2017). Therefore, for CoRL to serve as podo-

cyte progenitors, they must undergo changes resembling a mesenchymal-to-epithelial transition (MET).

The transcription factor Wilms' tumor suppressor protein 1 (WT1) is required for podocyte development and homeostasis (Kann et al., 2015). Missense mutations and conditionally deleting WT1 from podocytes leads to glomerular scarring in humans and mice (Pelletier et al., 1991; Gao et al., 2004; Chau et al., 2011). WT1 is closely linked to the mesenchymal-epithelial balance in development and disease (Miller-Hodges and Hohenstein, 2012), and the homeostasis of different cell types (Ozdemir and Hohenstein, 2014), including the plasticity and cell fate of certain cells (Karki et al., 2014; Wen et al., 2016). WT1 drives MET at the start of nephron development (Essaf et al., 2011; Berry et al., 2015), although in the developing epicardium, WT1 controls the reverse, i.e., epithelial-to-mesenchymal transition (Martinez-Estrada et al., 2010). WT1 enhances proliferation and migration in certain cell types in a context-dependent manner (Wagner et al., 2008; Brett et al., 2013; Graziano et al., 2017). Finally, WT1 and renin co-localize in a fraction of cells in the afferent arterioles. Moreover, WT1 (−KTS) can negatively modulate renin gene transcription through interaction at a binding site within the renin enhancer (Steege et al., 2008).

These reports provided the rationale to test the hypothesis that WT1 is required for CoRL proliferation, migration, and transdifferentiation toward a podocyte fate in the context of podocyte depletion.

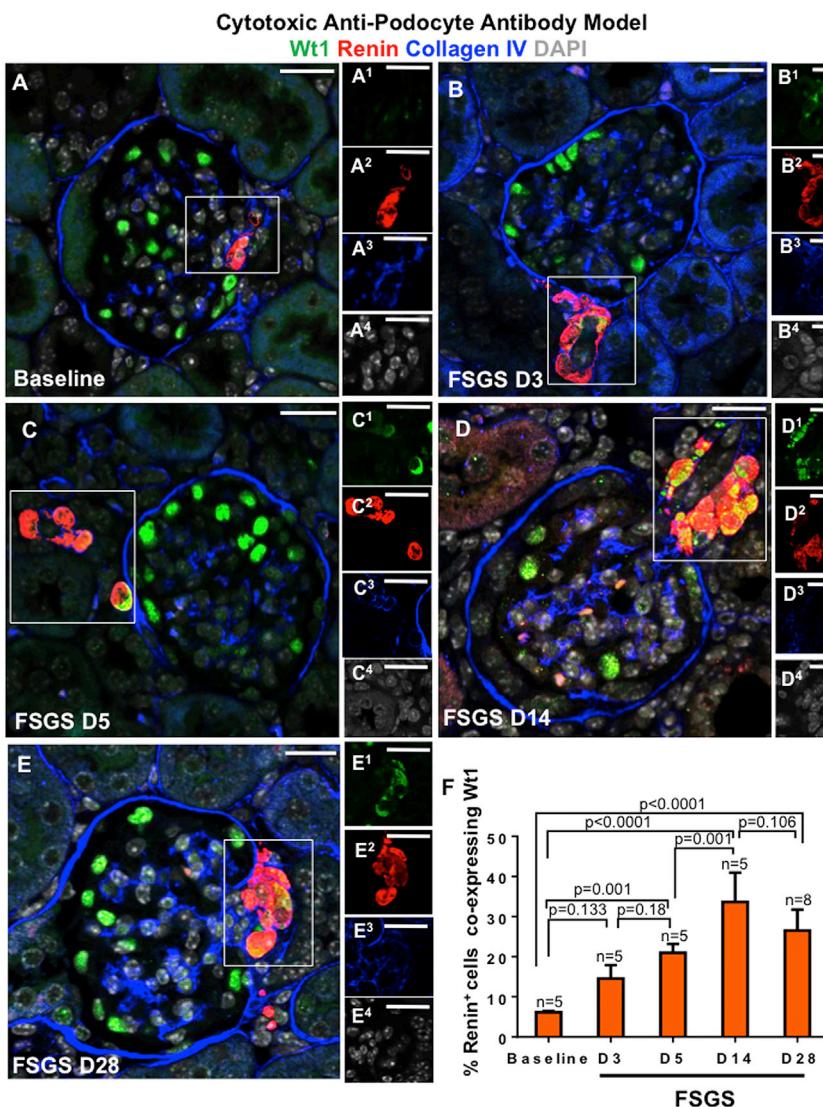


Figure 1. WT1 Increases in Renin-Stained Cells in the Juxta-Glomerular Compartment Following Podocyte Depletion in the Cytotoxic Anti-podocyte Antibody Model of FSGS

Confocal microscopy images marked (A–E) show the merge for WT1 (green), renin (red), collagen IV (blue), and DAPI (gray). Collagen IV demarcates glomeruli. Insets show the JGC for each channel, labeled as superscripts. Scale bars, 20 μ m.

(A) Baseline ($n = 5$), WT1 (A1) is limited to podocytes and not detected in renin-stained cells (A2).

(B) D3 ($n = 5$), WT1 (B1) is detected in the cytoplasm of renin-stained cells (B2).

(C) D5 ($n = 5$), WT1 (C1) increases in JGC, overlapping with renin (C2), creating a yellow color.

(D) D14 ($n = 5$), renin-stained cells (D2) co-staining with WT1 (D1) increase in the JGC.

(E) D28 ($n = 8$), WT1 (E1) and renin (E2) co-staining persists.

(F) Quantitation of the percentage of renin-stained cells in the JGC co-staining for WT1.

RESULTS

WT1 Expression Increases in Renin-Stained Cells Following Podocyte Depletion in Mice and Humans

We began by measuring the temporal expression of WT1 in renin-stained cells in the JGC in *RenWt1^{+/+}* mice following podocyte depletion induced by the administration of a cytopathic anti-podocyte antibody. Following podocyte depletion, the percentage of renin cells co-staining for WT1 increased as follows (Figures 1A–1F): from a baseline to D3 of FSGS ($6.16\% \pm 0.3\%$ versus $14.5\% \pm 3.35\%$; $p = 0.133$; Figures 1A and 1B), from baseline to D5 of FSGS ($6.16\% \pm 0.3\%$ versus $20.99\% \pm 2.20\%$; $p = 0.001$; Figures 1A and 1C), from D5 to D14 of FSGS ($20.99\% \pm 2.20\%$ versus $33.63\% \pm 7.3\%$; $p = 0.001$; Figures 1C and 1D), and from baseline to

D28 of FSGS ($6.16\% \pm 0.3\%$ versus $26.5\% \pm 5.24\%$; $p < 0.0001$; Figures 1A and 1E).

Podocyte loss in podocyte TGF β -Receptor1 transgenic (*PodTgfb1*) mice (Figures S1A–S1C) and membranous nephropathy rats (passive Heymann nephritis [PHN] model) (Figures S1D and S1E) was also accompanied by increased WT1 in renin-stained JGC cells. In contrast, WT1 was not detected in renin cells post uninephrectomy in mice, where absolute podocyte number remained normal (Figure S1F).

We confirmed the experimental results in human diseases. In normal human kidney, WT1 was mostly limited to podocytes (Figure 2A). However, *de novo* WT1 was detected in renin-stained JGC cells in human FSGS (Figure 2B) and membranous nephropathy (Figure 2C). Similar results were obtained with different WT1 antibodies, and no

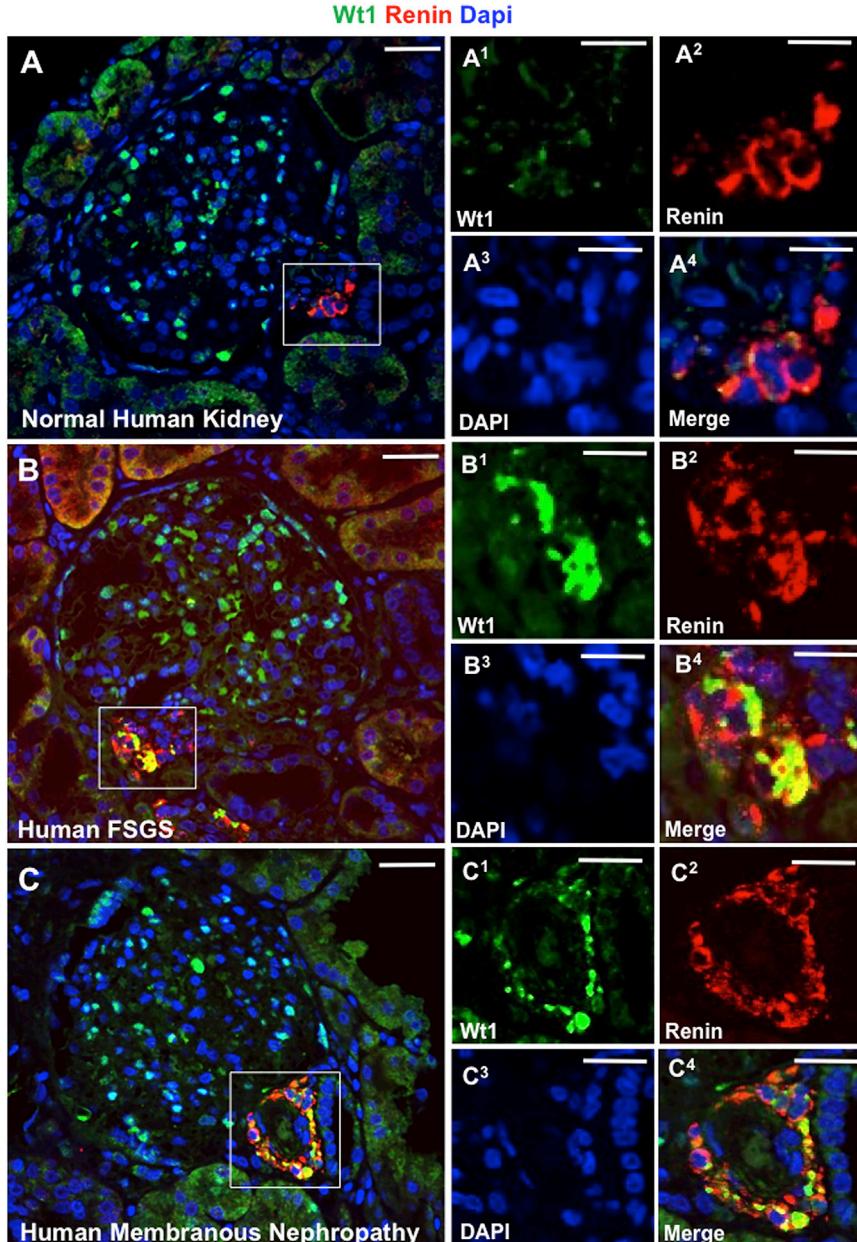


Figure 2. WT1 Increases in Renin-Stained Cells in the JGC in Human FSGS and Membranous Nephropathy

Confocal microscopy for WT1, renin, and DAPI in human kidneys. Insets show the JGC for each channel: WT1 (1, green), renin (2, red), DAPI (3, blue), and merged (4, yellow). Scale bars, 20 μ m.

(A) Normal human kidney ($n = 5$), WT1 is limited to podocytes (A1), and not detected in renin-stained cells (A2), hence no merge (A4).

(B) Human FSGS ($n = 5$), WT1 in the JGC (B1) overlaps with renin-stained cells (B2) creating a yellow color (B4).

(C) Human membranous nephropathy ($n = 5$), WT1 (C1) and renin in the JGC (C2) produce a yellow color (C4).

staining was detected when the primary antibody was omitted (not shown).

These results show that WT1 protein increases in renin-stained cells in the JGC in both experimental and human FSGS and membranous nephropathy in the context of podocyte loss.

Selective Deletion of WT1 in Cells of Renin Lineage Has No Impact on Podocytes or Kidney Function under Non-diseased Conditions

To test if *de novo* expression of WT1 in CoRL has relevance for their progenitor role to replace lost podocytes, *RenCreER*

tdTomato mice (Pippin et al., 2013, 2015) were crossed with *Wt1^{f/f}* mice (Martinez-Estrada et al., 2010; DeFilippis and Wagner, 2014) to generate *RenCreER tdTomato Wt1^{f/f}* mice (abbreviated *RenWt1^{f/f}*) so that WT1 could be conditionally and selectively deleted in *tdTomato*-labeled CoRL (Figure S2). CoRL and their descendants permanently express *tdTomato* dependent on Cre-mediated recombination, which allows lineage tracing of CoRL. Glomerular cells do not ectopically express renin (and hence Cre) under injury conditions. *RenCreER tdTomato Wt1^{+/+}* (abbreviated *RenWt1^{+/+}*) served as controls, where *tdTomato* was expressed in CoRL but WT1 remained (Figures S2A, S2F, and



S2H). Genotyping discerned the lox and wild-type *Wt1* alleles (Figure S2A1), and a flox deletion primer distinguished *RenWt1^{f/f}* mice given tamoxifen versus corn oil (Figure S2A2). Following tamoxifen, *Wt1* mRNA from RFP⁺CoRL isolated using laser capture microscopy was significantly lower in *RenWt1^{f/f}* compared with *RenWt1^{+/+}* mice (Figure S2B), while *renin* mRNA levels were similar (Figure S2C). Cre was restricted to the JGC in mice containing the *CreER* transgene but not in Cre-negative mice (Figures S2D and S2E). TdTomato, detected without the use of an antibody, confirmed similar CoRL labeling efficiency in *RenWt1^{+/+}* and *RenWt1^{f/f}* mice given tamoxifen (Figures S2F and S2G). tdTomato was absent in *RenCre*-negative mice (not shown). Ninety-five percent of renin⁺ CoRL stained with red fluorescent protein (RFP) antibody, which detected tdTomato in *RenWt1^{+/+}* and *RenWt1^{f/f}* mice (not shown). Mice that did not report were excluded, accounting for <1% of all mice given tamoxifen. At baseline, WT1 was in a typical podocyte distribution in *RenWt1^{f/f}* mice, indistinguishable from *RenWt1^{+/+}* mice, proving deletion in CoRL had no impact on podocytes (Figures S2H and S2I). WT1 was detected in <5% of CoRL in non-diseased *RenWt1^{+/+}* mice but was not detected in CoRL in non-diseased *RenWt1^{f/f}* mice (Figures S2H and S2I). These results show that *Wt1* mRNA and WT1 protein were selectively reduced in tdTomato⁺ CoRL of *RenWt1^{f/f}* but not in *RenWt1^{+/+}* mice, with no consequences on podocyte health under normal conditions.

Increased WT1 Was Not Seen in JGC of Wt1 Conditional Knockout Mice Following Podocyte Depletion

WT1 overlap with RFP⁺ was not present in the vast majority of CoRL in the JGC in normal *RenWt1^{+/+}* and *RenWt1^{f/f}* mice (7 ± 2.5 versus 5.6 ± 2.0 , respectively; $p = 0.99$; Figures 3A, 3D, and 3G). In *RenWt1^{+/+}* mice at D28 following podocyte loss, RFP⁺CoRL in the JGC co-stained with WT1 increased compared with baseline (103 ± 12.36 versus 7 ± 2.5 ; $p < 0.001$; Figures 3B and 3G). By contrast, in *RenWt1^{f/f}* mice at D28, only 5.8 ± 1.8 of RFP⁺ CoRL in the JGC co-stained with WT1 (Figures 3E and 3G). Because enalapril increases RFP⁺CoRL in the JGC (Lichtnekert et al., 2016), *RenWt1^{+/+}* mice were given enalapril for 25 days following podocyte loss. RFP⁺CoRL in the JGC co-expressing WT1 further increased at D28 following enalapril compared with baseline (419.5 ± 117.79 versus 7 ± 2.5 ; $p < 0.0001$; Figures 3A, 3C, and 3G). An increase in WT1 expression was not observed in JGC of *RenWt1^{f/f}* mice after enalapril (Figures 3F and 3G). Similarly, the number of RFP⁺WT1⁻CoRL increased in *RenWt1^{+/+}* mice following podocyte depletion (Figures S3B, S3C), while the number of RFP⁺WT1⁻CoRL in *RenWt1^{f/f}* mice did not increase (Figures S3E, S3F).

These results show the following: (1) although WT1 was not detected in the majority of cells in the JGC under normal conditions, the number of RFP⁺CoRL co-expressing WT1 increases progressively following podocyte loss in *RenWt1^{+/+}* mice; (2) WT1 did not increase in RFP⁺CoRL in diseased *RenWt1^{f/f}* mice, consistent with successful targeted ablation of WT1 in CoRL; (3) enalapril increased the number of CoRL co-expressing WT1 in *RenWt1^{+/+}* mice following podocyte loss but not in *RenWt1^{f/f}* mice.

CoRL Migration to the Glomerulus Is Reduced in *RenWt1^{f/f}* Mice Following Podocyte Depletion

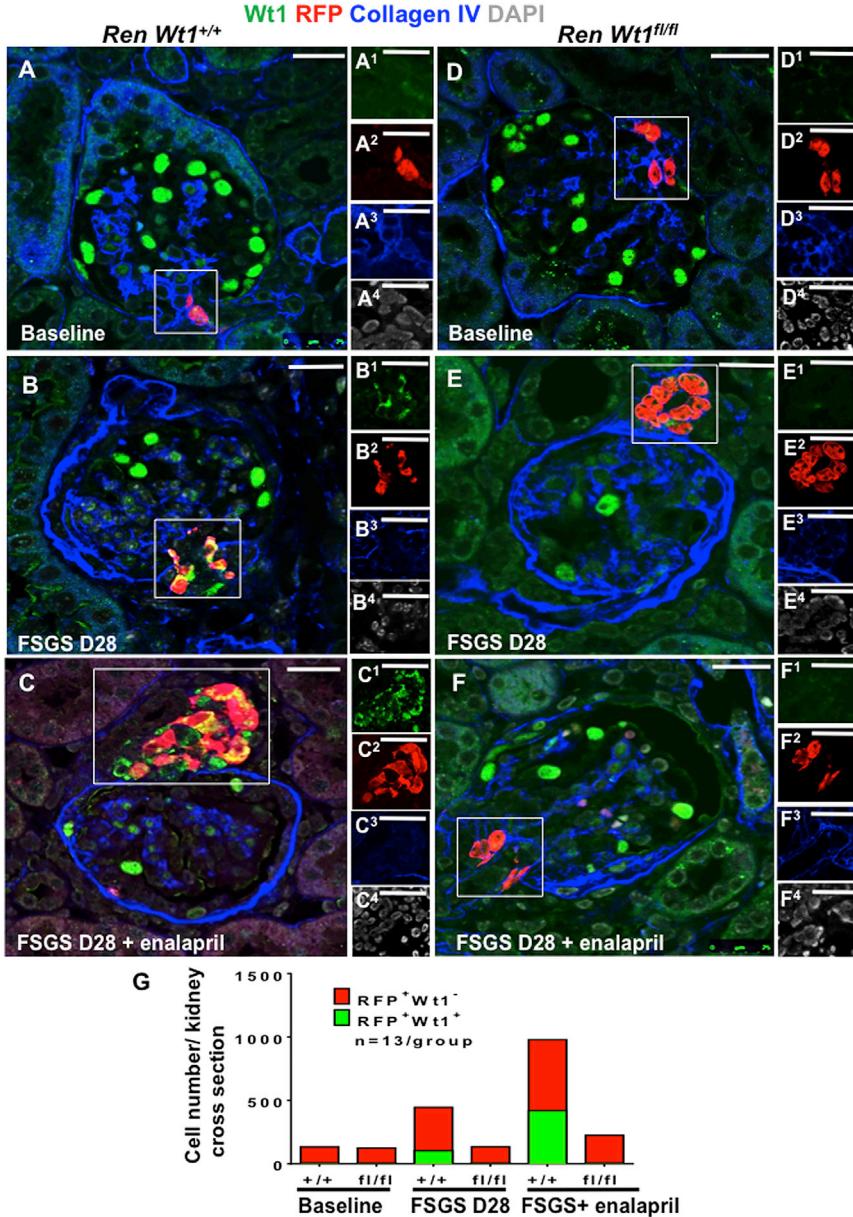
Triple staining was performed for p57 (podocytes), RFP (tdTomato⁺, CoRL), and collagen IV (demarcates glomeruli) to determine if deleting WT1 reduced CoRL migration from the JGC to the glomerulus following abrupt podocyte loss. RFP⁺CoRL were rarely detected in glomeruli at baseline of either *RenWt1^{+/+}* or *RenWt1^{f/f}* mice (Figures 4A, 4D and 4G). At D28 FSGS in *RenWt1^{+/+}* mice, RFP⁺CoRL were detected in $3.5\% \pm 2.4\%$ of glomeruli compared with baseline ($0.43\% \pm 0.2\%$; $p = 0.013$; Figures 4B and 4G). At D28 FSGS in *RenWt1^{f/f}* mice, RFP⁺CoRL were barely detected in glomeruli compared with *RenWt1^{+/+}* mice ($p = 0.001$; Figures 4E and 4G). Consistent with our recent report (Lichtnekert et al., 2016), administering enalapril to *RenWt1^{+/+}* mice following podocyte depletion increased the migration of RFP⁺CoRL to $12.95\% \pm 3.07\%$ of glomeruli compared with untreated ($p < 0.001$; Figures 4C and 4G). However, enalapril had little impact on RFP⁺CoRL migration to glomeruli in *RenWt1^{f/f}* mice (Figures 4F and 4G). Staining for renin and Cre was not detected in glomerular cells in disease, consistent with CoRL migration to the glomerulus rather than ectopic renin expression in disease. These results were similar to our previous reports (Pippin et al., 2013).

Figures S4 and 4C show that once RFP⁺CoRL migrated from the JGC to the glomerulus, a subset *de novo* co-express podocyte markers P57 (Figure 4C) podocin (Figure S4A–S4A4) and synaptopodin (Figure S4B–S4B4), consistent with CoRL transdifferentiating toward a podocyte fate.

These results show that WT1 is critical for CoRL migration to the glomerulus following podocyte loss and their transdifferentiation. In the absence of WT1, enalapril has reduced efficacy on CoRL migration and transdifferentiation.

WT1 Is Important for CoRL Proliferation Following Podocyte Depletion

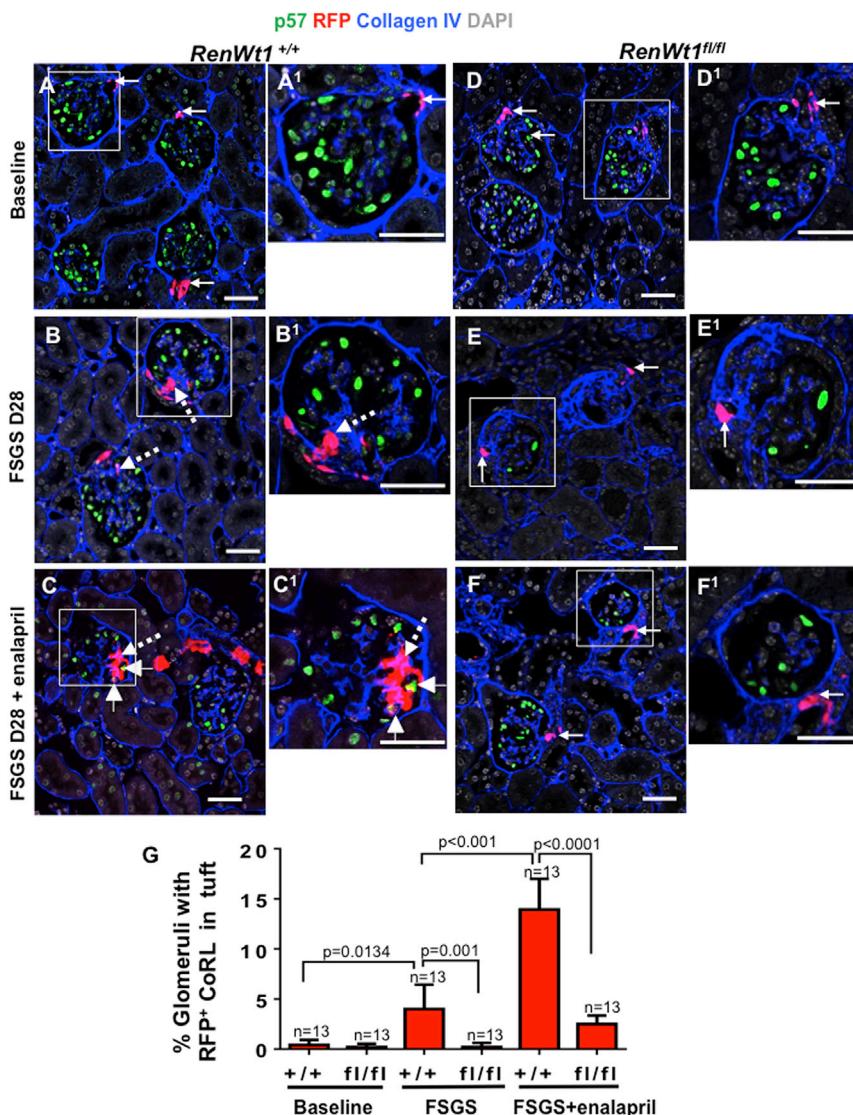
To test the role of WT1 in CoRL proliferation, bromodeoxyuridine (BrdU) was administered via intraperitoneal injections every 48 hr, starting at D2. Triple staining was performed for BrdU, WT1, and RFP to determine if proliferation was restricted to RFP⁺CoRL that co-expressed WT1,



and if WT1 is necessary for CoRL proliferation? BrdU was absent in RFP⁺CoRL at baseline in *RenWt1^{+/+}* and *RenWt1^{fl/fl}* mice (Figures 5A, 5D, and 5G). This was not a false negative, as BrdU was detected in neighboring tubules. Following podocyte depletion in *RenWt1^{+/+}* mice, BrdU increased in RFP⁺WT1⁺ cells compared with baseline (25.8 ± 9.2 versus 0.8 ± 0.5 ; $p < 0.01$; Figures 5B and 5G). In contrast, BrdU was barely detected in RFP⁺CoRLs in *RenWt1^{fl/fl}* mice (Figures 5E and 5G). In enalapril-treated *RenWt1^{+/+}* FSGS mice, RFP⁺WT1⁺BrdU⁺ cells in the JGC increased compared with untreated mice (131.3 ± 51.7 versus 25.8 ± 9.2 ; $p = 0.0001$; Figures 5C and 5G). In enalapril-treated *RenWt1^{fl/fl}* FSGS mice, RFP⁺WT1⁺BrdU⁺ cells did

not increase (Figures 5F and 5G). To test WT1 independent proliferation, we counted RFP⁺BrdU⁺WT1[−] cells. BrdU was only observed in a small portion of RFP⁺WT1[−] cells in *RenWt1^{+/+}* FSGS mice compared with baseline (5 ± 0.8 versus 0.3 ± 0.5 ; $p < 0.05$; Figures 5B, 5C, and 5G).

Triple staining was also performed for the proliferation marker Ki67, WT1, and RFP (Figure S7). Ki67 was not detected in RFP⁺CoRL at baseline in *RenWt1^{+/+}* and *RenWt1^{fl/fl}* mice (Figures S7A and S7D) but was detected in neighboring tubules. Following podocyte depletion, Ki67 increased in RFP⁺ WT1⁺CoRL (Figure S7B), which was augmented by enalapril (Figure S7C). In *RenWt1^{fl/fl}* mice, Ki67 was barely detected in RFP⁺CoRL (Figures S7E and S7F).



These results show that after podocyte depletion, the following occurs: (1) proliferation (BrdU⁺ or Ki67⁺) of CoRL (RFP⁺) occurs in cells co-expressing WT1; (2) CoRL proliferation was reduced in *RenWt1^{fl/fl}* mice; (3) enalapril increased CoRL proliferation in diseased *RenWt1^{+/+}* but not in *RenWt1^{fl/fl}* mice.

Following Podocyte Loss, CoRL that Migrate to the Glomerulus Lose Mesenchymal Markers and *De Novo* Acquire Epithelial Markers

CoRL and podocytes derive from mesenchymal lineages (Boyle et al., 2008; Kobayashi et al., 2008; Costantini and Kopan, 2010; Little and McMahon, 2012). To determine if WT1 in CoRL is necessary for changes in mesenchymal and epithelial cell markers, and if these changes were augmented by enalapril, additional studies were per-

formed (Figures 6 and S6). Staining for the mesenchymal marker myosin heavy chain 11 (MYH11) was detected in $90\% \pm 7.5\%$ and $84.1\% \pm 8.2\%$ of RFP⁺CoRL in the JGC in baseline *RenWt1^{+/+}* and *RenWt1^{fl/fl}* mice, respectively (Figures 6A, 6D, and 6G). Following podocyte depletion in *RenWt1^{+/+}* mice, the percentage of MYH11⁺RFP⁺ cells decreased to $67.5\% \pm 5.3\%$ compared with baseline ($p = 0.0059$; Figures 6B and 6G). The percentage of MYH11⁺RFP⁺ cells did not decrease in *RenWt1^{fl/fl}* mice with FSGS compared with baseline ($82.6\% \pm 10.6\%$ versus $84.1\% \pm 8.2\%$; $p = 0.99$; Figures 6E and 6G). In enalapril-treated FSGS *RenWt1^{+/+}* mice, MYH11⁺RFP⁺CoRL decreased (44.3% versus 67.5% ; $p = 0.008$; Figures 6C and 6G). Giving enalapril to *RenWt1^{fl/fl}* mice did not reduce MYH11⁺RFP⁺CoRL (78.7% versus 82.6% ; $p > 0.99$; Figures 6F and 6G). CoRL that migrated to

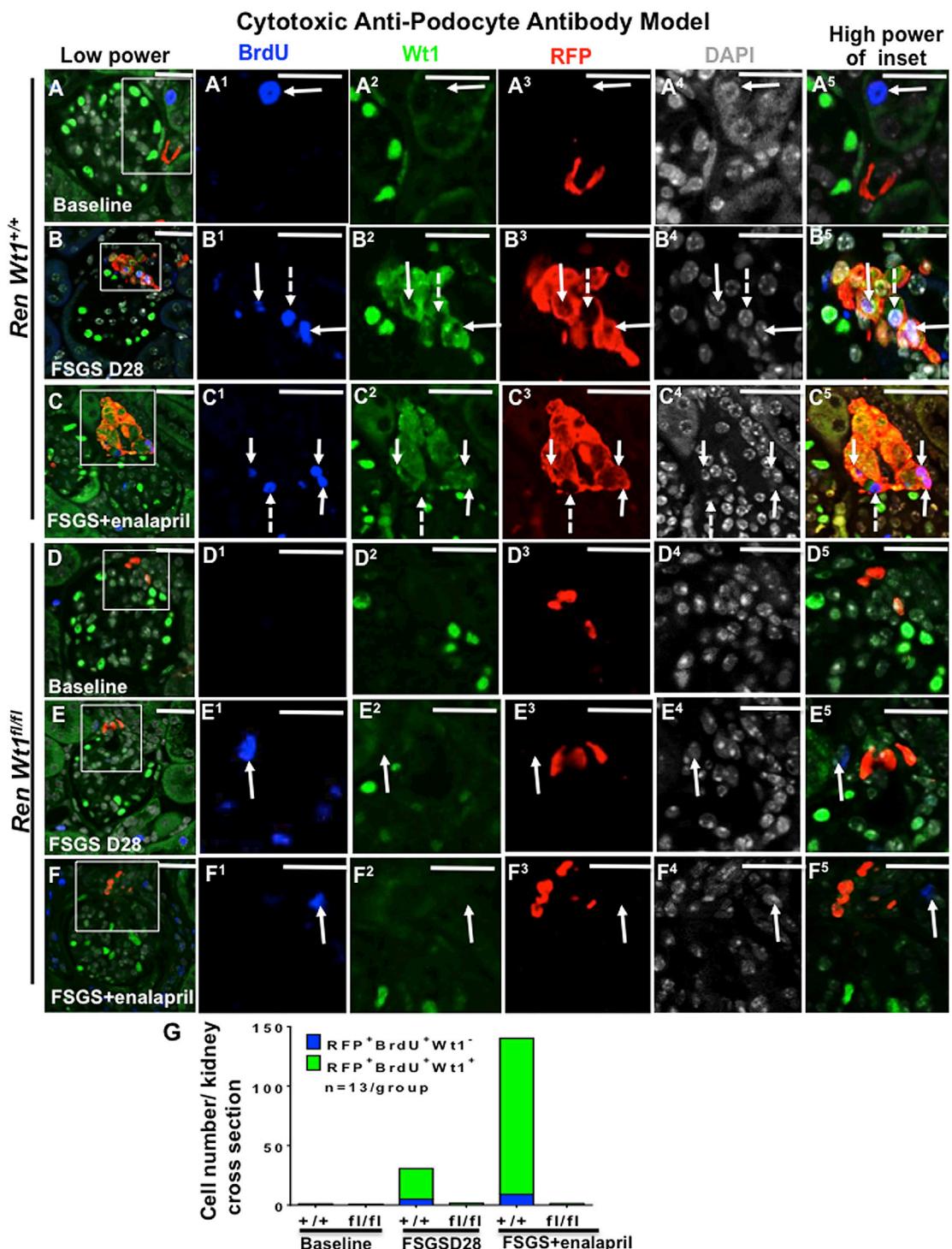


Figure 5. RFP⁺CoRL Proliferation Is Significantly Reduced in Diseased *RenWt1^{f/f}* Mice Compared with Diseased *RenWt1^{+/+}* Mice
 Confocal microscopy for BrdU (blue), WT1 (green), RFP (red), and DAPI (gray) in experimental FSGS in *RenWt1^{+/+}* and *RenWt1^{f/f}* mice. Insets show the JGC for each channel, labeled as superscripts. Scale bars, 20 μ m.

(A–C) *RenWt1^{+/+}* mice. (A) At baseline, a BrdU⁺ cell (A1) is detected in the adjacent tubule. BrdU is not detected in the JGC. WT1 (A2) is not detected in RFP⁺ cells (A3). (B) At FSGS D28, BrdU (B1) co-localizes with WT1 (B2) and RFP (B3), creating a white/purple color (B5, solid

(legend continued on next page)



glomeruli did not stain for MYH11 with either strain (data not shown).

In RFP⁺CoRL in the JGC, the epithelial cell marker cytokeratin 18 (CK18) was barely detected at baseline in either *RenWt1^{+/+}* or *RenWt1^{f/f}* mice (Figures 6H, 6K, and 6N). At D28 FSGS in *RenWt1^{+/+}* mice, the percentage of RFP⁺CK18⁺CoRL in the JGC increased compared with baseline (16.8% ± 2.4% versus 1.5% ± 0.9%; p < 0.0001; Figures 6I and 6N). The percentage of RFP⁺CK18⁺CoRL was further increased by enalapril compared with baseline (27.25% ± 6.08% versus 16.75% ± 2.36%; p < 0.0001; Figures 6J and 6N). In contrast, in *RenWt1^{f/f}* mice, the percentage of RFP⁺CK18⁺CoRL did not increase (2.8% ± 3.5% versus 1.5% ± 0.8%; p = 0.606; Figures 6K, 6L, and 6N), and enalapril had no impact (3.7% ± 3.1% versus 2.8% ± 3.5% versus FSGS without enalapril; p > 0.99) (Figures 6K, 6M, and 6N).

Figure S6 confirms these results using additional mesenchymal and epithelial cell markers. The mesenchymal markers αSMA (Figures S6A–S6F) and smooth muscle protein 22 (SM22) (Figures S6G–S6L) were abundant in RFP⁺CoRL in the JGC at baseline in both strains (Figures S6A, S6D, S6G, and S6J). Co-staining for both mesenchymal markers decreased in *RenWt1^{+/+}* mice with FSGS (Figures S6B and S6H), and was further decreased by enalapril (Figures S6C and S6I). No RFP⁺CoRL in glomeruli co-expressed αSMA or SM22 (Figures 6A and 6C). In contrast, αSMA⁺/RFP⁺CoRL and SM22⁺/RFP⁺CoRL did not decrease in *RenWt1^{f/f}* mice with FSGS, or with enalapril (Figures S6E, S6F, S6K, and S6L).

The epithelial cell marker E-cadherin was not detected in RFP⁺ cells in the JGC at baseline in either *RenWt1^{+/+}* or *RenWt1^{f/f}* mice (Figures S6M and S6P). Following podocyte depletion in *RenWt1^{+/+}* mice, RFP⁺ cells in the JGC *de novo* expressed E-cadherin, which was augmented by enalapril (Figures S6N and S6O). In contrast, E-cadherin staining was not detected in the JGC in *RenWt1^{f/f}* mice following podocyte depletion, and enalapril had no effect (Figures S6Q and S6R).

Following podocyte depletion in *RenWt1^{+/+}* mice, a subset of CoRL that migrated from the JGC (Figures S5A1–S5A4, S5B1–S5B4, and S5C1–S5C4) to the glomerular tuft no longer expressed αSMA (Figure S5A5–S5A8). There was *de novo* expression in CoRL in glomeruli of the epithelial markers E-cadherin (Figure S5B5–S5B8) and cytokeratin

18 (Figure S5C5–S5C8), coincident with loss of mesenchymal markers.

These results show that following abrupt podocyte depletion, mesenchymal markers (MYH11, αSMA, SM22) decrease in CoRL, coincident with *de novo* staining for the epithelial cell markers cytokeratin 18 and E-cadherin (Figure S6S). These events were augmented by enalapril in *RenWt1^{+/+}* mice, supporting the notion that CoRL undergo MET in *RenWt1^{+/+}* mice with FSGS, but these events are significantly reduced when WT1 is deleted.

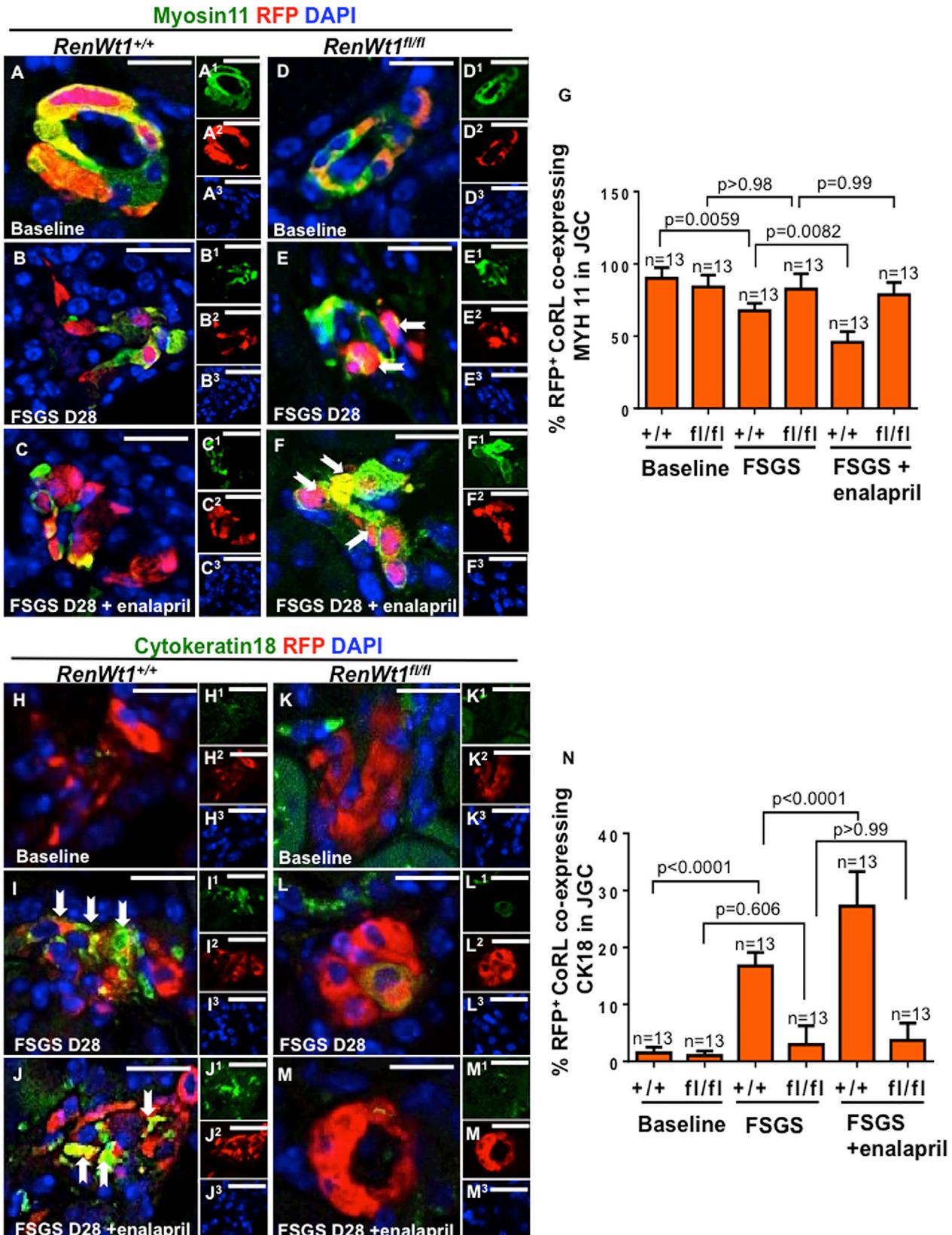
Podocyte Replacement Is Lower in Diseased *RenWt1^{f/f}* Mice

The binding of the podocyte-depleting antibody was similar in *RenWt1^{+/+}* and *RenWt1^{f/f}* mice (Figures S2J and S2K). Podocyte density, measured by quantitating p57, was similar at baseline in *RenWt1^{+/+}* and *RenWt1^{f/f}* mice (Figures 7A, 7B, and 7E). Podocyte density decreased in *RenWt1^{+/+}* mice with FSGS compared with baseline (185.2 ± 17.1 × 10⁶ versus 250.7 ± 10.0 × 10⁶ podocytes/μm³ glomerular tuft volume; p < 0.0001; Figures 7C and 7E). Moreover, podocyte density was significantly lower in diseased *RenWt1^{f/f}* mice compared with diseased *RenWt1^{+/+}* mice (109.3 ± 10.09 × 10⁶ versus 185.2 ± 17.1 × 10⁶ podocytes/μm³; p < 0.001; Figures 7D and 7E). Similar findings were shown for podocin (Figures 7G–7J). Glomerulosclerosis increased in diseased *RenWt1^{+/+}* mice but was significantly higher in diseased *RenWt1^{f/f}* mice (Figure 7F). The urinary albumin to creatinine ratio was also significantly higher in diseased *RenWt1^{f/f}* mice (Figure 7K). In summary, podocyte density was lower in *RenWt1^{f/f}* mice compared with *RenWt1^{+/+}* mice, accompanied by higher glomerulosclerosis and albuminuria.

DISCUSSION

Adult podocytes cannot proliferate and are unable to self-renew following depletion (Barisoni et al., 2000; Pavone et al., 2003). Their replacement in glomerular diseases is reliant on progenitors (Romagnani et al., 2013; Shankland et al., 2014). CoRL plasticity has been reported in podocytes, PECs, kidney pericytes, and mesangial cells (Gomez et al., 2014; Pippin et al., 2015; Starke et al., 2015; Kaverina et al., 2016; Stefanska et al., 2016). To serve

arrows). A few BrdU⁺ cells do not express WT1 (B1–B5, dashed arrow) (C) At D28 after enalapril, BrdU⁺ (C1), WT1⁺ (C2), RFP⁺ (C3) cells increase (C5, solid arrows). A few BrdU⁺ cells do not express WT1 (C1–C5, dashed arrow). (D–F) *RenWt1^{f/f}* mice. (D) At baseline, neither BrdU (D1) nor WT1 (D2) is detected in RFP⁺ (D3) cells in the JGC. (E) At D28, a BrdU⁺ cell (E1, solid arrow) is detected in the adjacent tubule and does not overlap with WT1 (E2) or RFP (E3), hence no merge (E5). (F) At D28 after enalapril, a BrdU⁺ cell (F1, solid arrow) does not overlap with WT1 (F2) or RFP (F3), hence no merge (F5). (G) Stacked bar graph shows the number of RFP⁺BrdU⁺WT1[−] (blue) and RFP⁺BrdU⁺WT1⁺ (green) cells in the JGC. RFP⁺BrdU⁺WT1⁺ cells increase progressively in diseased *RenWt1^{+/+}* mice but not in *RenWt1^{f/f}* mice (n = 13/group).



(legend on next page)



as progenitors for podocytes, CoRL must increase in number, migrate from the JGC to the glomerulus, and reprogram from a mesenchymal to an epithelial fate. To better understand the mechanisms underlying these events, we selectively genetically deleted WT1 in CoRL in transgenic mice. Reducing podocytes with a cytopathic anti-podocyte antibody resulted in a significant reduction in CoRL proliferation, migration, and MET, suggesting a critical role for WT1.

We show that WT1 staining is detected in <5%–7% of renin-expressing cells in the JGC in normal mice and rats, and only occasionally in the JGC in normal human kidneys, similar to previous reports (Puelles et al., 2015). The first major result of the studies was that following podocyte depletion in three experimental models, WT1 increased up to 5.5-fold from baseline in renin-expressing cells in the JGC. WT1 also increased in renin-expressing cells in human FSGS and membranous nephropathy. WT1 did not increase following uninephrectomy, serving as a negative control because podocyte number was unchanged.

The role for WT1 is well described in podocyte health (Ozdemir and Hohenstein, 2014; Mazzei and Manucha, 2016), in non-kidney cell transdifferentiation (Ijpenberg et al., 2007), and progenitor function (Martinez-Estrada et al., 2010; Bandiera et al., 2013). To test the hypothesis that WT1 is necessary for CoRL to function as podocyte progenitors, we generated a transgenic mouse where WT1 was inducibly deleted specifically in CoRL. Under non-stressed conditions, deleting WT1 in CoRL had no impact on kidney or podocyte function and morphology.

CoRL migrate from the JGC to the glomerulus or tubulo-interstitium during kidney development and in disease (Sequeira Lopez et al., 2004; Pippin et al., 2013, 2015; Starke et al., 2015; Zhang et al., 2015; Lichtnekert et al., 2016). Here, RFP⁺CoRL were rarely detected in glomeruli

of non-diseased *RenWt1*^{+/+} and *RenWt1*^{f/f} mice. However, RFP⁺CoRL were readily detected in glomeruli in *RenWt1*^{+/+} mice following podocyte depletion, similar to previous reports (Pippin et al., 2013; Lichtnekert et al., 2016). The second major finding was that following podocyte depletion, RFP⁺CoRL were barely detected in glomeruli of *RenWt1*^{f/f} mice, and migration to the glomerulus was not augmented by enalapril in *RenWt1*^{f/f} mice. These results are consistent with WT1 being necessary for CoRL migration and the enhanced migratory effects of enalapril. Similar results in the cancer field suggest that WT1 enhances cell invasion and migration (Jomgeow et al., 2006; Brett et al., 2013).

CoRL increase in the JGC following podocyte depletion (Pippin et al., 2013, 2014), increased glomerular volume (Hodgin et al., 2015), hypotension (Castellanos Rivera et al., 2011), and following angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (Lichtnekert et al., 2016). BrdU and Ki67 staining showed that following podocyte depletion in *RenWt1*^{+/+} mice, RFP⁺CoRL proliferated, which was augmented by enalapril (Duim et al., 2015; Lichtnekert et al., 2016). A third major finding was no increase in CoRL proliferation in *RenWt1*^{f/f} mice, and enalapril had no effect. This was of interest, as several groups have shown that WT1 enhances proliferation of tumor cells and cardiac endothelial cells (Li et al., 2015; Wu et al., 2015).

Like podocytes (Boyle et al., 2008; Kobayashi et al., 2008), CoRL derive from mesenchymal origins (Sequeira Lopez et al., 2001; Matsushita et al., 2010; Wang et al., 2013; Ciampi et al., 2016). To become podocytes, CoRL need to decrease mesenchymal proteins and acquire epithelial and podocyte proteins. At baseline, RFP⁺CoRL in the JGC in both *RenWt1*^{+/+} and *RenWt1*^{f/f} mice express mesenchymal markers (MYH11, α SMA, SM22) but not

Figure 6. RFP⁺CoRL MET Is Significantly Reduced in Diseased *RenWt1*^{f/f} Mice Compared with Diseased *RenWt1*^{+/+} Mice

Confocal microscopy for myosin11 (MYH11, green, mesenchymal marker), RFP (red, CoRL), and DAPI (blue, nuclei) in the JGC (A–F). Insets show each channel, labeled as superscripts. Scale bars, 20 μ m.

(A–C) *RenWt1*^{+/+} mice. (A) At baseline, MYH11 (A1) merges with RFP (A2) in the JGC, creating a yellow color. (B) At FSGS D28, MYH11 (B1) decreases in RFP⁺ (B2) cells. (C) At D28 after enalapril, MYH11 (C1) decreases, despite an increase in RFP⁺ cells (C2).

(D–F) *RenWt1*^{f/f} mice. (D) At baseline, MYH11 (D1) merges with RFP (D2). (E) At FSGS D28, MYH11 (E1) in RFP⁺ cells (E2, solid arrows) persists. (F) At D28 after enalapril, MYH11 (F1) in RFP⁺ cells (F2, solid arrows) also persists.

(G) The percentage of RFP⁺CoRL expressing MYH11 decreases in *RenWt1*^{+/+} mice with FSGS and is augmented by enalapril. *RenWt1*^{f/f} mice show no significant decrease in MYH11 ($n = 13$ /group).

(H–M) Confocal microscopy for cytokeratin 18 (CK18, green, epithelial marker), RFP (red, CoRL), and DAPI (blue, nuclei) in the JGC (H–M). Insets show each channel, labeled as superscripts. Scale bars, 20 μ m.

(H–J) *RenWt1*^{+/+} mice. (H) At baseline, CK18 (H1) is barely detected in RFP⁺ cells (H2). (I) At FSGS D28, CK18 (I1) increases in RFP⁺ cells (I2), creating a yellow color (solid arrows). (J) At D28 after enalapril, CK18 (J1) further increases in RFP⁺ cells (J2), creating a yellow color (solid arrows).

(K–M) *RenWt1*^{f/f} mice. (K) At baseline, CK18 (K1) is not detected in RFP⁺ cells (K2). At FSGS D28 (L) and after enalapril (M), CK18 (L1, M1) does not increase in RFP⁺ cells (L2, M2).

(N) The percentage of RFP⁺CK18⁺ cells increases in diseased *RenWt1*^{+/+} mice, which is augmented by enalapril. CK18 does not increase significantly in diseased *RenWt1*^{f/f} mice ($n = 13$ /group).

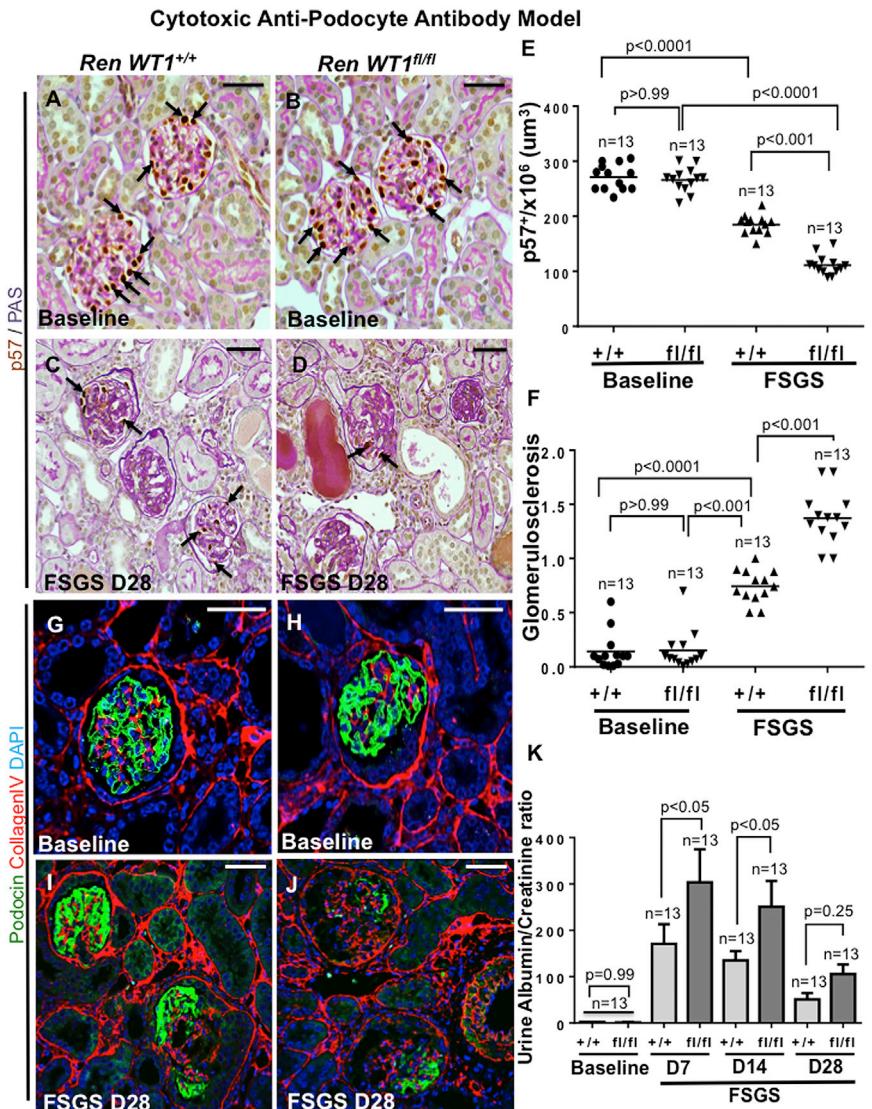


Figure 7. Podocyte Depletion in the Cytotoxic Anti-podocyte Antibody Model of FSGS Is Higher in Diseased *RenWt1*^{f/f} Mice than in Diseased *RenWt1*^{+/+} Mice

(A–F) Podocytes are identified by p57 (brown, nuclear, arrows) and matrix (pink) in a baseline biopsy from *RenWt1*^{+/+} (A) and *RenWt1*^{f/f} (B) mice, and a serial biopsy in the same mice at FSGS D28 (C and D). (E) Podocyte density was similar at baseline but lower in diseased *RenWt1*^{f/f} compared with diseased *RenWt1*^{+/+} mice. (F) Glomerulosclerosis is higher in diseased *RenWt1*^{f/f} compared with diseased *RenWt1*^{+/+} mice. (G–J) Podocin staining (green) is similar in healthy podocytes in baseline *RenWt1*^{+/+} (G) and *RenWt1*^{f/f} (H) mice. Podocin decreases at FSGS D28 in *RenWt1*^{+/+} mice (I) and is more pronounced in diseased *RenWt1*^{f/f} mice (J). Scale bars, 20 μ m. (K) Urinary albumin to creatinine ratio in *RenWt1*^{f/f} mice (dark bars) is higher than *RenWt1*^{+/+} mice (light bars) ($n = 13$ /group).

epithelial markers (cytokeratin 18, E-cadherin). Following podocyte depletion in *RenWt1^{+/+}* mice, RFP⁺CoRL in the JGC expressing these mesenchymal markers decreased significantly. This coincided with the *de novo* expression of cytokeratin 18 and E-cadherin. The MET-like changes in diseased *RenWt1^{+/+}* mice were augmented by enalapril. When in the glomerulus, a subset of CoRL co-expressed the podocyte markers p57, podocin, and synaptopodin. The fourth major finding was that following podocyte depletion in *RenWt1^{f/f}* mice, mesenchymal markers in RFP⁺ CoRL did not decrease, nor did they begin to express epithelial markers, and enalapril had no impact.

These findings are consistent with our previous reports on the importance of disruption of MET underlying the origin of Wilms' tumors (Berry et al., 2015; Hohenstein et al., 2015), the role of *Wt1* in this MET programming (Da-

vies et al., 2004; Essafi et al., 2011), and that loss of WT1 blocks MET in the cap mesenchyme (Essafi et al., 2011).

The fifth major finding was that following abrupt podocyte loss, podocyte density remained lower in diseased *RenWt1^{f/f}* compared with diseased *RenWt1^{+/+}* mice, accompanied by more glomerular scarring and albuminuria. We recognize that CoRL transdifferentiation alone is not sufficient to explain the differences, and other factors including different kidney progenitor populations need to be considered.

Finally, our observation of intra-glomerular CoRL with coincident downregulation of renin expression would be consistent with the negative regulation previously described (Steege et al., 2008). It will be of interest to assess the specific isoform(s) of WT1 expressed and retained in CoRL and the kinetics of renin downregulation and other



cell differentiation features altered as a function of this interaction.

In summary, WT1 expression in CoRL is important for the glomerular response to damage. When WT1 is selectively deleted in CoRL in the setting of podocyte loss, their proliferation and migration to the glomerulus, and to some extent their transdifferentiation toward a podocyte fate through MET changes, are markedly reduced.

EXPERIMENTAL PROCEDURES

RenCreER tdTomato Wt1^{+/+} and Wt1^{f/f} Reporter Mice

Ren1cCreERxRs-tdTomato-R mice (Pippin et al., 2013) were crossed with a *Wt1* conditional knockout mouse (*Wt1^{f/f}*) (Martinez-Estrada et al., 2010). *Wt1* was inactivated in *RenCreER tdTomato Wt1^{f/f}* mice (abbreviated *RenWt1^{f/f}*) by administration of 100 mg/kg tamoxifen on four occasions. Mouse genotype was identified by PCR (Martinez-Estrada et al., 2010). Kidney biopsies were performed to assess CoRL labeling as described in *Supplemental Experimental Procedures*. *RenWt1^{f/f}* and *RenWt1^{+/+}* were housed in the animal care facility of the University of Washington (UW) under specific pathogen-free conditions with food and water available ad libitum. These studies were reviewed and approved by the UW Institutional Animal Care and Use Committee (2968-04).

Experimental Models of Glomerular Disease

Accompanied by Podocyte Depletion

The following experimental models of podocyte depletion were used:

- (1) A cytotoxic sheep anti-glomerular antibody model of experimental FSGS was induced in *RenWt1^{f/f}* and *RenWt1^{+/+}* mice as previously described (Kaverina et al., 2016; Lichtnekert et al., 2016). On D3, when podocyte number was decreased by 30%–40%, mice were randomized into two groups: group 1 received drinking water; group 2 received the ACE inhibitor enalapril (75 mg/mL). Mice were killed on D28. Urine was collected at baseline, and on days 7, 14, and 28. Amersham Cell Proliferation Labeling Reagent (GE Healthcare Life Sciences, Little Chalfont, UK) was administered to quantitate cell proliferation (Kaverina et al., 2016). Urines were collected for albumin measurements by radial immunodiffusion assay (RID) (Marshall et al., 2011); urine creatinine was measured by colorimetric micro-plate assay (Cayman Chemical Company, Ann Arbor, MI).
- (2) Podocyte TGFβ-Receptor1 transgenic (*PodTgfb1*) mice given doxycycline underwent TGFβR1-induced podocyte apoptosis and loss of 25% (D7) and 40% (D14), accompanied by glomerulosclerosis (Daehn et al., 2014).
- (3) A PHN model of membranous nephropathy induced as previously described (Ohse et al., 2010) underwent progressive podocyte depletion (Petermann et al., 2003).
- (4) A uninephrectomy model served as a negative control for podocyte depletion (Pippin et al., 2015).

Human Glomerular Disease

Kidney biopsies from patients with FSGS and membranous nephropathy were obtained from the University of Chicago (UC). The study protocol was approved by the UC Institutional Review Board. Further details are described in *Supplemental Experimental Procedures*.

Laser Capture Microscopy and qRT-PCR

RFP⁺CoRL were isolated by LCM, as described (Tretiakova and Hart, 2011), with the Leica Laser Microdissection Systems LMD6500 and LMD7000 (Leica Microsystems Inc., Buffalo Grove, IL).

Immunoperoxidase and Immunofluorescent Staining

Formalin-fixed paraffin-embedded mouse, rat, and human kidney sections or frozen tissue sections (4–20 μm thick) were used. Following standard antigen retrieval steps, Avidin-biotin based, polymer-based, and fluorochrome-based staining was performed as previously described (Kimura et al., 1995; Wagner et al., 2014). Further details are described in *Supplemental Experimental Procedures*.

Quantitative Analysis

Absolute numbers of renin⁺, RFP⁺, WT1⁺, and RFP⁺/WT1⁺ expressing cells were counted in the JGC of fluorescence-stained sections. Results were expressed as a percentage of co-localized RFP⁺/WT1⁺ cells per number of renin⁺ cells. The percentage of proliferating (BrdU⁺) CoRL co-expressing WT1 in the JGC was measured by dividing the number of RFP⁺/WT1⁺/BrdU⁺-stained cells by the total number of RFP⁺ cells in the JGC. The total number of glomeruli and glomeruli with RFP⁺ cells were counted to determine the percentage of glomeruli with RFP⁺ cells. Absolute numbers of RFP⁺, MYH11⁺, CK18⁺, MYH11⁺/RFP⁺, and CK18⁺/RFP⁺-expressing cells were counted in the JGC. Results were expressed as a percentage of MYH11⁺/RFP⁺ and CK18⁺/RFP⁺ cells per number of RFP⁺ cells in the JGC. Podocyte number was measured on 150 ± 20 glomeruli per animal on p57/periodic acid-Schiff-stained sections (Zhang et al., 2015; Lichtnekert et al., 2016).

Statistical Analysis

Groups were compared using a one-way or two-way ANOVA for multiple comparisons with Bonferroni post hoc analysis with significant set at p < 0.05. Data are presented as means ± SEM. All data were analyzed in GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2017.08.020>.

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