Activation of podocyte Notch mediates early *Wt1* glomerulopathy

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The Wilms' tumor suppressor gene, *WT1*, encodes a zinc finger protein that regulates podocyte development and is highly expressed in mature podocytes. Mutations in the *WT1* gene are associated with the development of renal failure due to the formation of scar tissue within glomeruli, the mechanisms of which are poorly understood. Here, we used a tamoxifen-based CRE-LoxP system to induce deletion of *Wt1* in adult mice to investigate the mechanisms underlying evolution of glomerulosclerosis. Podocyte apoptosis was evident as early as the fourth day post-induction and increased during disease progression, supporting a role for *Wt1* in mature podocyte survival. Podocyte Notch activation was evident at disease onset with upregulation of *Notch1* and its transcriptional targets, including *Nrarp*. There was repression of podocyte *FoxC2* and upregulation of *Hey2* supporting a role for a Wt1/FoxC2/Notch transcriptional network in mature podocyte injury. The expression of cleaved Notch1 and HES1 proteins in podocytes of mutant mice was confirmed in early disease. Furthermore, induction of podocyte HES1 expression was associated with upregulation of genes implicated in epithelial mesenchymal transition, thereby suggesting that HES1 mediates podocyte EMT. Lastly, early pharmacological inhibition of Notch signaling ameliorated glomerular scarring and albuminuria. Thus, loss of *Wt1* in mature podocytes modulates podocyte Notch activation, which could mediate early events in *WT1*-related glomerulosclerosis.

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Glomerulosclerosis accounts for 5% to 10% of pediatric and adult end-stage kidney disease and recurs in 15% to 30% of patients following kidney transplantation.[1] Mutations in genes encoding transcription factors which regulate podocyte differentiation have been implicated in human glomerulosclerosis and include Wilms' tumor 1 (WT1).[2–7] WT1 encodes a nuclear protein containing 4 zinc fingers that bind DNA and RNA and is highly expressed in mature podocytes.[8–11] Mutations in the regions involved in DNA binding or zinc finger formation have been reported in patients with Denys-Drash syndrome, a disorder associated with infantile diffuse mesangial sclerosis, gonadal dysgenesis, and Wilms' tumor.[12–14] Mutations disrupting an alternative splice donor site in intron 9, results in Frasier syndrome, a disorder associated with focal segmental glomerulosclerosis (FSGS), predisposition to gonadoblastoma, and male pseudohermaphroditism.[15,16] Furthermore, mutations in *WT1* have been reported in isolated primary steroid-resistant nephrotic syndrome, supporting a role for aberrant Wt1 function in the pathogenesis of glomerulosclerosis.[5,7]

Mechanisms underlying the development of *WT1*-related glomerulosclerosis are poorly understood with no effective treatments available. Recent studies employing chromatin immunoprecipitation sequencing, RNA sequencing, exon array, and bioinformatic analyses have shown a specific role for WT1 in regulating the podocyte-specific transcriptome. WT1 can bind to both promoters and enhancers of 18 known podocytopathy genes.[17,18] Mutations in *WT1* could lead to altered transcriptional regulation of these genes, which is necessary for podocyte differentiation and function, and contribute to the pathogenesis of glomerulosclerosis.[17,18]

Genes regulating podocyte differentiation have also been implicated in the pathogenesis of *WT1*-related disease. *De novo* expression of PAX2 protein and mRNA, a paired box transcription factor repressed by WT1 during nephrogenesis, has been observed in podocytes of Denys-Drash syndrome patient biopsies.[19] Furthermore, re-expression of Pax2 in podocytes, cell cycle re-entry, and reduced expression of podocyte proteins such as nephrin and α-actinin-4 have been reported in heterozygous $Wt1^{tmT396/+}$ mice with glomerulosclerosis by 8

months of age.[20] These findings suggested that *Wt1*$^{tmT396/+}$ podocytes dedifferentiate and revert to an immature phenotype during disease progression.[20]

During glomerulogenesis, podocyte fate induction is regulated by Notch, a highly conserved and ubiquitous pathway that transduces short-range signals between neighboring cells.[21–24] Ligand binding initiates regulated intramembrane proteolysis with subsequent nuclear translocation of the Notch intracellular domain (NICD) where it associates with RBPJ-κ, a DNA-binding protein, and promotes transcription of target genes (e.g., hairy enhancer of split [Hes]), which regulates tissue-specific differentiation. *Notch1*, *Notch2,* and downstream transcriptional targets *Hes1* and *Hey1* are expressed in podocyte precursors in the S-shaped body and are down-regulated during terminal differentiation.[25,26] Ectopic podocyte Notch activation in differentiating and mature podocytes is associated with both diffuse mesangial sclerosis and FSGS, respectively, the latter being mediated by p53-induced podocyte apoptosis and the former associated with *de novo* Pax2 expression.[27,28] Given the interplay between WT1 and Notch during glomerulogenesis and the fact that both diffuse mesangial sclerosis and FSGS phenotypes occur with mutations in *WT1* as well as ectopic podocyte Notch activation, we hypothesized a role for podocyte Notch activation in the development of glomerulosclerosis related to loss of Wt1 function.

In a model where *Wt1* was deleted in mature podocytes, using a tamoxifen-based CRE-LoxP system, we observed increased podocyte loss during the development of glomerulosclerosis. At disease onset, we found upregulation of Notch pathway transcripts in mutant podocytes. At the same time point, we observed repression of *FoxC2* and upregulation of *Hey2* transcripts in primary mutant podocytes. Cleaved Notch1 and HES1 proteins were evident in mutant podocytes at disease manifestation. Induction of podocyte HES1 expression *in vitro* was associated with increased expression of *Slug* and *Snail* transcripts, genes implicated in epithelial to mesenchyme transition. Pharmacological inhibition of Notch with gamma-secretase inhibitors at disease onset rescued the severity of glomerulosclerosis and albuminuria. These data support a role for early Notch activation in the manifestation of *Wt1* glomerulopathy, which may be mediated via repression of podocyte *FoxC2*.

RESULTS

Early glomerulosclerosis is evident at 5 days post tamoxifen induction in *CAGG-CreER*$^{TM+/-}$;*Wt1*$^{f/f}$ transgenic mice

Wt1 deletion in mature podocytes results in glomerulosclerosis with compromised renal function by day 7 post tamoxifen induction in adult *CAGG-CreER*$^{TM+/-}$;*Wt1*$^{f/f}$ transgenic mice.[29] To investigate events leading to the induction of disease in these mice, we first determined the earliest point at which we could detect glomerulosclerosis after *Wt1* deletion. Tamoxifen was administered for 3 consecutive days by i.p. injection to 5-week-old *CAGG-CreER*$^{TM+/-}$;*Wt1*$^{f/f}$ transgenic mice and mice were nephrectomized at 4, 5, 6, and 12 days following injection. Successful *Wt1* deletion was demonstrated by recombination polymerase chain reaction (PCR) and the reduction of Wt1 expression in glomeruli (Supplementary Figure S1). Following light microscopy analysis of periodic acid–Schiff (PAS)-stained kidney sections, we determined the severity of glomerulosclerosis by a semiquantitative analysis at each time point in *CAGG-CreER*$^{TM+/-}$;*Wt1*$^{f/f}$ mutants, *CAGG-CreER*$^{TM-/-}$;*Wt1*$^{f/f}$ controls, and heterozygous *CAGG-CreER*$^{TM+/-}$;*Wt1*$^{f/+}$ mice (Figure 1a–d).

Heterozygous mice did not develop glomerulosclerosis by day (D) 12 postinduction (PI) (Supplementary Figure S2). At D4 PI, mutants exhibited early segmental glomerulosclerosis with focal foot process effacement and a trend toward higher levels of albuminuria ($P = 0.21$) (Figure 1a and a′; Supplementary Figures S3A and S4). By D5 PI, glomerular scarring was more extensive (≥ score 2) in mutants compared with control and heterozygous mice ($^*P < 0.05$) (Figure 1b, Supplementary Figure S3B). Progression of disease was further supported by an increase in urine albumin-creatinine ratio in mutants compared with controls ($^*P = 0.01$) (Figure 1b′). By D6 PI, extensive glomerulosclerosis with tubules containing protein casts were observed in mutants relative to controls with increased albuminuria (Figure 1c and c′, Supplementary Figure S3C). Late stage disease with global glomerulosclerosis was observed at D12 PI (Figure 1d and e, Supplementary Figure S3D), with peritubular cells expressing vascular smooth muscle actin, consistent with progression of tubulointerstitial disease (Supplementary Figure S5). We conclude that podocyte function appears compromised within 6 days of *Wt1* deletion in mature podocytes. Therefore, investigations into the mechanisms underlying manifestation of disease should be undertaken within this time frame.

Cleaved-Caspase-3 protein expression increases in glomeruli and podocytes of mutant mice at D5, D6, and D12 PI

Podocyte apoptosis has previously been implicated in the pathogenesis of glomerulosclerosis.[30] Therefore, we next investigated whether apoptosis is evident during disease induction in *CAGG-CreER*$^{TM+/-}$;*Wt1*$^{f/f}$ transgenic mice. Expression of Cleaved Caspase-3 protein was observed within mutant glomeruli as early as D4 PI but quantitatively, was not statistically significantly different compared with control glomeruli (Figure 2a) ($P = 0.06$, NS). By D5 PI, at the onset of early glomerulosclerosis, we observed an increased number of Cleaved Caspase-3/DAPI-positive cells in mutant glomeruli (Figure 2c, c′, and d) ($^{***}P < 0.0001$), consistent with a temporal increase in apoptosis. Terminal deoxynucleotidyltransferase–mediated 2′-deoxyuridine 5′-triphosphate nick end-labeling (TUNEL)-positive mutant glomerular cells were evident at D5 PI but were absent in controls (Supplementary Figure S6). TUNEL staining of D6 PI primary mutant podocytes was also higher than for controls (Figure 2e, e′, and f) ($^*P = 0.04$). At the same time point, we observed increased Caspase-3/7 positivity in primary mutant podocytes (Supplementary Figure S7) and an increased number of Annexin V/Sytox blue-positive primary mutant podocytes compared with controls (Supplementary