Paracrine Wnt1 Drives Interstitial Fibrosis without Inflammation by Tubulointerstitial Cross-Talk

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ABSTRACT

AKI with incomplete epithelial repair is a major contributor to CKD characterized by tubulointerstitial fibrosis. Injury–induced epithelial secretion of profibrotic factors is hypothesized to underlie this link, but the identity of these factors and whether epithelial injury is required remain undefined. We previously showed that activation of the canonical Wnt signaling pathway in interstitial pericytes cell autonomously drives myofibroblast activation *in vivo*. Here, we show that inhibition of canonical Wnt signaling also substantially prevented TGFβ–dependent myofibroblast activation *in vitro*. To investigate whether Wnt ligand derived from proximal tubule is sufficient for renal fibrogenesis, we generated a novel mouse strain with inducible proximal tubule Wnt1 secretion. Adult mice were treated with vehicle or tamoxifen and euthanized at 12 or 24 weeks postinjection. Compared with vehicle-treated controls, kidneys with tamoxifen–induced Wnt1 expression from proximal tubules displayed interstitial myofibroblast activation and proliferation and increased matrix protein production. PDGF receptor β–positive myofibroblasts isolated from these kidneys exhibited increased canonical Wnt target gene expression compared with controls. Notably, fibrotic kidneys had no evidence of inflammatory cytokine expression, leukocyte infiltration, or epithelial injury, despite the close histologic correlation of each with CKD. These results provide the first example of noninflammatory renal fibrosis. The fact that epithelial–derived Wnt ligand is sufficient to drive interstitial fibrosis provides strong support for the maladaptive repair hypothesis in the AKI to CKD transition.

CKD represents a substantial unmet medical need. Despite decades of research, therapeutic advances are lacking, in part because the underlying mechanisms are poorly understood. An important recent model for CKD progression suggests that, rather than heal completely, tubular injury may lead to incomplete repair in a process that is maladaptive.[1] For example, injured proximal tubule cells that fail to redifferentiate after injury may undergo cell cycle arrest, and in this state, they are proposed to promote profibrotic responses through the secretion of soluble factors that are hypothesized to subsequently act on myofibroblast progenitors in the interstitium.[2] Importantly, all experimental models supporting this theory are themselves characterized by both epithelial injury and inflammation, states that strongly correlate with fibrosis itself.[3–5]

Whether epithelial–derived paracrine factors are sufficient to induce tubulointerstitial fibrosis in the absence of injury and inflammation remains unknown.

A large body of work implicates the Wnt pathway in the pathogenesis of CKD.[6] The 19 mammalian Wnt ligands are expressed in spatially regulated and

dynamic patterns during development and in disease.[7] Wnt ligands are secreted by a lipid-modified glycoprotein that activates either a canonical or noncanonical pathway on receptor binding.[6] Wnt1 ligand is a prototypic activator of canonical Wnt signaling, acting through β-catenin with Axin2 and LEF1 target gene activation.[6,8–10] Wnt pathway activation occurs during kidney fibrosis,[11] and we have shown that genetic stabilization of β-catenin in pericytes and perivascular fibroblasts is sufficient to drive myofibroblast differentiation.[12] A number of canonical Wnt ligands is known to be induced after kidney injury, including Wnt1, and cultured proximal tubule cells express Wnt1.[13,14]

Here, we have investigated the role of paracrine epithelial to interstitial Wnt signaling in the pathogenesis of kidney fibrosis. We generated a novel mouse model characterized by inducible expression of Wnt1 exclusively in proximal tubule epithelial cells. We asked whether induction of proximal tubule Wnt1 (PT-Wnt1) expression in an adult mouse is sufficient to drive tubulointerstitial fibrosis by paracrine signaling. We show that epithelial secretion induces canonical Wnt target gene expression in platelet–derived growth factor receptor–β (PDGFRβ) –positive interstitial cells and that these cells gain expression of the myofibroblast marker α–smooth muscle actin (αSMA). Mice develop renal fibrosis with enhanced matrix accumulation. Remarkably, interstitial fibrosis occurs in the complete absence of leukocyte recruitment, inflammation, or epithelial injury, providing strong evidence that epithelial Wnt ligand acts in a paracrine fashion on interstitial myofibroblast progenitors to induce renal fibrosis.

RESULTS

Canonical Wnt Signaling Is Necessary for TGFβ– Mediated Myofibroblast Activation

To investigate the role of the Wnt pathway in myofibroblast transition, we first assessed the effect of inhibiting the Wnt pathway in an established *in vitro* model of myofibroblast activation. As expected, the rat kidney fibroblast line NRK-49F responded to TGFβ with upregulation of αSMA and fibronectin mRNA and protein expression (Figure 1, A and B). Because some TGFβ-dependent responses require canonical Wnt signaling,[15–17] we asked whether inhibition of Wnt signaling might abrogate induction of myofibroblast markers. We exposed NRK-49F cells to XAV939, a tankyrase inhibitor that inhibits the canonical Wnt pathway and stabilizes Axin2.[18] Indeed, XAV939 substantially reduced expression of αSMA and fibronectin in response to TGFβ (Figure 1, A and B). We next tested whether Wnt pathway inhibition reduced TGFβ-smad signaling using an smad binding element luciferase (SBE-luc) reporter.[19] The SBE-luc reporter was introduced into NRK-49F cells by lentiviral transduction, and TGFβ alone induced SBE activity as expected. XAV939 reduced SBE activity, consistent with its ability to inhibit αSMA and fibronectin expression in response to TGFβ (Figure 1C). In support of this result, XAV939 also

reduced nuclear translocation of phospho-smad3 (Figure 1D). Thus, inhibiting the canonical Wnt pathway inhibits TGFβ– induced myofibroblast activation in an smad-dependent fashion.

Wnt Pathway Potentiates Fibrinogenic Targets of TGFβ

Because inhibition of the canonical Wnt pathway reduced TGFβ–dependent myofibroblast activation, we next investigated whether Wnt pathway activation might potentiate myofibroblast activation. We chose Wnt1 because it is a prototypic activator of canonical Wnt signaling.[6,8,10] Wnt1 had no effect on αSMA expression but did modestly increase basal fibronectin expression in NRK-49F cells (Figure 2, A and B). Longer incubation times or higher doses of Wnt1 also failed to upregulate αSMA (data not shown). We next exposed NRK-49F cells to Wnt1 and TGFβ for either 16 (mRNA) or 48 hours (protein) and examined myofibroblast marker expression. In each case, Wnt1 stimulated additive αSMA, collagen I, and fibronectin expression compared with TGFβ alone (Figure 2, A–C).

β-Catenin has been reported to directly interact with smad proteins.[20–24] Our results were consistent with a direct interaction of the two pathways, and therefore, we tested whether β-catenin and smad3 directly interact in NRK-49F cells in response to TGFβ. We treated NRK-49F cells with vehicle or TGFβ for 48 hours. Coimmunoprecipitation with anti–β-catenin antibody of cellular lysates from NRK-49F cells treated with TGFβ or vehicle revealed an interaction between endogenous β-catenin and psmad3 only after TGFβ stimulation (Figure 2D). These results are consistent with a model, whereby β-catenin activation potentiates TGFβ-smad signaling by direct binding and nuclear translocation of phospho-smad3.

Wnt Pathway Induces Kidney Fibroblast Proliferation

The Wnt pathway has been shown to promote cell proliferation in lung and dermal stem cells[25,26] and fibroblasts.[27–29] This prompted us to investigate whether the Wnt pathway has a similar effect in kidney fibroblasts. We observed that treatment with Wnt1 increased numbers of Ki67+ NRK-49F cells *in vitro* (Figure 2E). To quantify this proliferative response, we performed flow cytometry for Ki67. Our analysis revealed that treatment with Wnt1 dramatically increased the number of cycling (Ki67+) NRK-49F cells (Figure 2, F and G). Thus, activation of the canonical Wnt pathway by Wnt1 promotes proliferation of kidney fibroblasts *in vitro*.

SLC34a1$^{GCE/+}$;R26$^{Wnt1-GFP/+}$ Mouse for Inducible PT-Wnt1 Expression and TGFβ Activation

To test whether the profibrogenic activities of canonical Wnt pathway activation were sufficient to drive renal fibrosis *in vivo*, we generated a novel mouse model by crossing an inducible proximal tubule CreER^{t2} line (SLC34a1$^{GCE/+}$)[30] with a line that allows conditional expression of Wnt1 after Cre activity (R26$^{Wnt1-GFP/+}$).[8] Bigenic SLC34a1$^{GCE/+}$; R26$^{Wnt1-GFP/+}$ mice
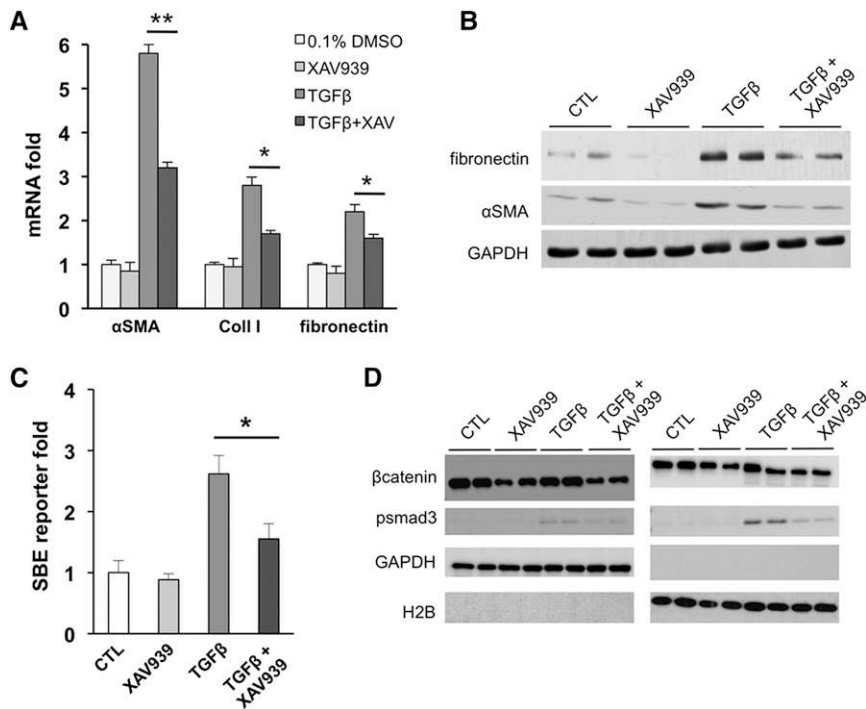
Figure 1. Inhibition of the Wnt pathway prevents TGFβ-induced fibrosis in NRK-49F cells. (A) TGFβ-induced increase in fibronectin, collagen I, and αSMA gene transcription was significantly inhibited by XAV939. (B) Protein lysates from NRK-49F cells reveal inhibition of fibronectin and αSMA protein expression by XAV939. (C) NRK-49F cells stably transduced with SBE-luc construct show that XAV939 inhibits TGFβ–induced SBE transcription. (D) Cytosolic and nuclear fractions of NRK-49F protein extracts reveal that XAV939 markedly reduces the nuclear translocation of psmad3. *P<0.05; **P<0.01.

Proximal Tubule–Derived Wnt1 Induces Interstitial Fibrosis by Activating Perivascular Fibroblasts/Pericytes

Next, we investigated αSMA and fibronectin protein expression in PT-Wnt1 versus control kidneys. Both of these fibrotic markers were significantly upregulated in the cortex at both time points (Figure 3, B, D, and E). To further characterize the model, we examined sections of cortex stained with αSMA and Lotus tetragonolobus lectin (LTL; proximal tubule marker). There was a clear and substantial increase in interstitial αSMA+ myofibroblasts in PT-Wnt1 kidneys compared with control (Figure 3F). PDGFRβ is expressed on all renal myofibroblasts,[31] and confocal imaging confirmed that interstitial myofibroblasts in PT-Wnt1 kidneys all express PDGFRβ (Figure 4A). Because activated myofibroblasts secrete matrix proteins, we examined collagen I and fibronectin expression. We observed local areas of increased matrix deposition in the cortex when stained for both collagen I and fibronectin (Figure 4B). Semi-quantitative analysis of fluorescence intensity confirmed a modest but significant increase in expression for both matrix proteins (Figure 4C). Taken together, these results indicate that sustained proximal tubule expression of Wnt1 is sufficient to induce interstitial myofibroblast activation.

Epithelial Wnt1 Drives Interstitial Myofibroblast Proliferation

We observed significantly increased interstitial myofibroblast number in PT-Wnt1 kidneys and that Wnt1 stimulated fibroblasts proliferation *in vitro* (Figure 3, A and B). Therefore, we asked whether myofibroblast proliferation might be contributing to interstitial fibrogenesis. Kidney sections from PT-Wnt1 or control kidneys were costained with the cell proliferation marker Ki67 and either LTL or αSMA. Ki67+ cells were quantified according to location in the tubular or interstitial compartment (Figure 5, A and B). We measured cell proliferation only in the interstitium and not in tubules (Figure 5, A and B), and this proliferation occurred in αSMA+ myofibroblasts (Figure 5, C and D).

Evidence that Proximal Tubule–Derived Wnt1 Acts Directly on Interstitial Myofibroblast Progenitors in the Absence of Epithelial Injury and Inflammation

Although the fibrotic phenotype of PT-Wnt1 kidneys was dramatic, it required 12 weeks to develop and therefore, could have reflected an indirect signaling cascade, whereby epithelial
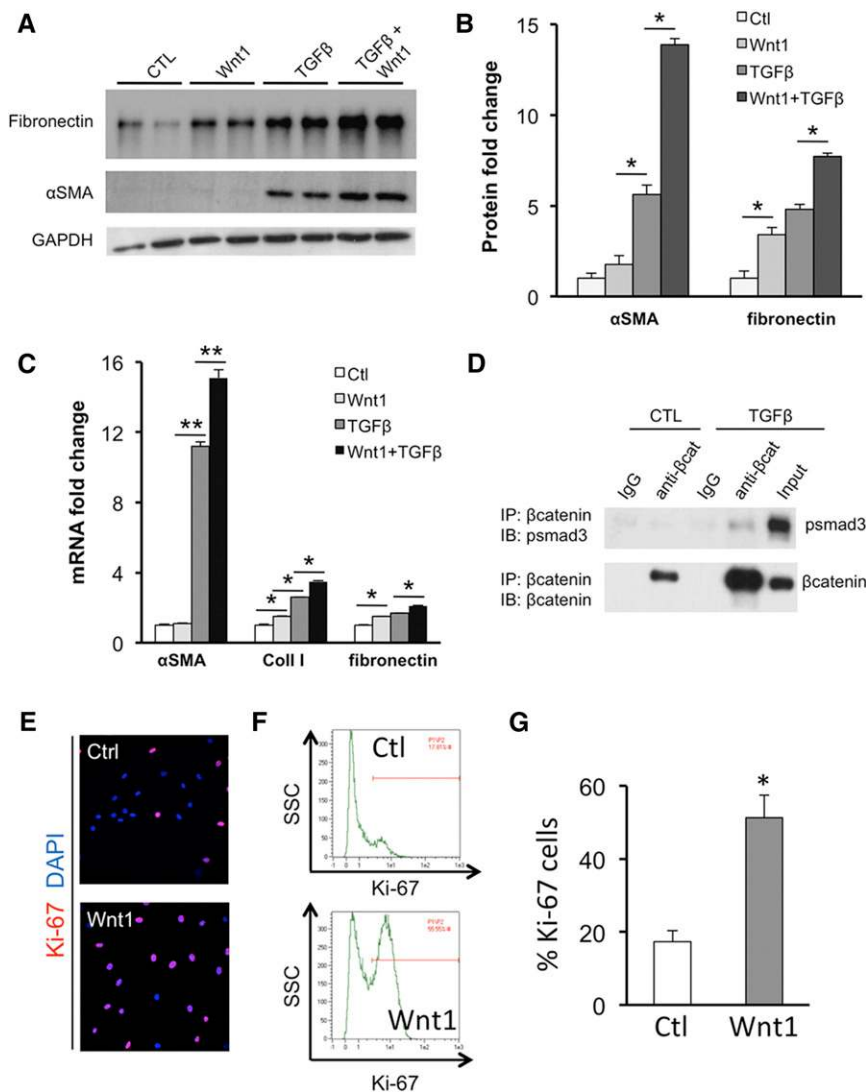
(Hereafter referred to as PT-Wnt1) express Wnt1 only in the proximal tubule and only after tamoxifen administration (Figure 3A). Groups of 8-week-old mice differing only in whether they contained an SLC34a1[GCE] allele were both given tamoxifen, and euthanized at two time points: 12 and 24 weeks postinjection (Figure 3A). Note that the SLC34a1-CreER[t2] line drives recombination only in cortex but not medulla. Consistent with this, we found that the Wnt1 gene and canonical pathway readouts Axin2 and LEF1 were significantly upregulated only in the cortex from PT-Wnt1 mice but not in medulla or control kidneys at both time points (Figure 3B, Supplemental Figure 1). The absolute increase in Wnt1 expression was low, consistent with the weak promoter activity of the Rosa26 locus in adult kidney (B.D. Humphreys, unpublished data). In addition, mRNA encoding fibrotic markers αSMA and fibronectin were significantly upregulated in PT-Wnt1 kidneys. We also observed a modest but significant increase in TGFβ gene transcription in the kidney cortex (Figure 3B). Consistent with the cortical–limited TGFβ gene upregulation, psmad3 protein was only increased in the PT-Wnt1 kidney cortex compared with its control (Figure 3C). This analysis suggests that proximal tubule–derived Wnt1 causes upregulation of canonical Wnt signaling, fibrotic markers, and TGFβ.

Figure 2. Wnt pathway potentiates fibrogenic targets of TGFβ and drives proliferation in NRK-49F cells. (A) Wnt1 (2 ng/ml) induces fibronectin protein and potentiates TGFβ–induced αSMA expression. (B) Quantification of protein expression (ImageJ). (C) Wnt1 augments TGFβ-induced transcription of fibronectin, collagen I, and αSMA. Wnt1 alone also significantly increases gene transcription of collagen I and fibronectin. (D) Coimmunoprecipitation reveals a direct interaction between β-catenin and psmad3 on TGFβ stimulation. (E) Wnt1 (2 ng/ml in 0.3% FBS) increased proliferation as assessed by Ki67 staining in NRK-49F cells after 16 hours of incubation. (F and G) Quantification of Ki67 staining by FACS (n=3 in each group). *P<0.05; **P<0.01.

Wnt1 induces the expression of other profibrotic factors in other cells or potentially, epithelial cells through autocrine signaling. Another possible indirect mechanism would be through induction of epithelial injury or kidney inflammation, because each is strongly associated with tubulointerstitial fibrosis.[32]

To address this, we compared macrophage infiltration in control versus PT-Wnt1 kidneys and observed no difference (Figure 6, A and B). We also performed quantitative PCR (qPCR) analysis of known inflammatory markers at 20 and 32 weeks. We found no increase in CD45 expression, a type I transmembrane protein expressed on all hematopoietic cells

except erythrocytes.[33] We also measured gene transcription of chemokines and their receptors involved in macrophage and neutrophil recruitment and activation, including CCL2, CCR2, CXCL1, CXCL2, CXCL10, CX3CL1, and CX3CR1, as well as the proinflammatory cytokines IL-6 and TNFα. There was no difference in expression for any of these inflammatory genes between PT-Wnt1 and control kidneys (Figure 6C, Supplemental Figure 1).

To examine epithelial injury, we stained for the proximal tubule injury marker kidney injury molecule-1 (Kim-1)[34] and the dedifferentiation marker vimentin.[35] We observed no Kim-1 or vimentin staining in PT-Wnt1 kidneys compared with controls, although we could easily detect epithelial expression of these markers in kidneys after ischemia-reperfusion injury (Figure 6, D and E). We also could not find any evidence of bush border damage when proximal tubule epithelia were examined by electron microscopy (Figure 6F). Finally, there was no evidence of glomerular injury, because podocyte foot processes were intact in PT-Wnt1 kidneys (Figure 6G).

Finally, we asked whether interstitial myofibroblasts showed evidence of canonical Wnt pathway activation, which would be expected if Wnt1 was binding to Wnt receptors directly on these cells. We sorted PDGFRβ+ cells[36] from single-cell suspensions of kidneys from PT-Wnt1 or controls, isolated RNA, and performed qPCR for canonical Wnt pathway targets or myofibroblast genes. This revealed increased expression of canonical Wnt pathway readouts Axin2 and LEF1 as well as increased αSMA and fibronectin mRNA in PDGFRβ+ cells from PT-Wnt1 kidneys compared with controls (Figure 7A). We also observed modest but significant increases in BUN and serum creatinine in PT-Wnt1 mice at the 32-week time point, sug-

gesting that the noninflammatory fibrosis induced in this model reduced parenchymal function (Figure 7B). Collectively, these results provide strong evidence that Wnt1 acts directly on interstitial myofibroblast progenitors to drive canonical Wnt signaling and fibrosis in the absence of inflammation and epithelial injury.

DISCUSSION

This study provides new data concerning the role of paracrine signaling in kidney fibrosis, an area of significant current
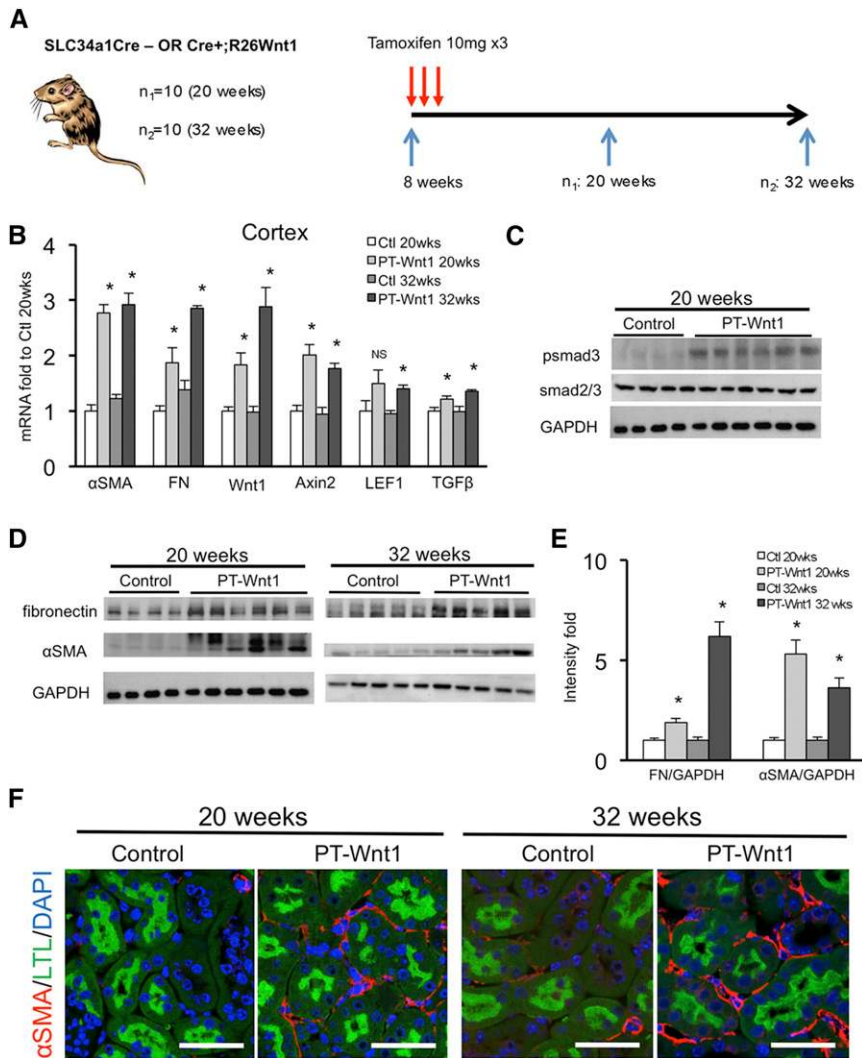
Figure 3. Epithelial–derived Wnt ligand drives kidney fibrosis. (A) Cartoon depicting the experimental approach. Control (SLC34A1$^{+/+}$;R26$^{Wnt1/+}$; Ctrl) or bigenic (SLC34A1$^{GCE/+}$;R26$^{Wnt1/+}$; PT-Wnt1) mice were administered tamoxifen at 8 weeks and euthanized at either 20 or 32 weeks of age. (B) At stated time points, mRNA from kidney cortex was subject to qPCR for fibrotic readouts (αSMA and fibronectin), Wnt1 itself, canonical pathway readouts (Axin2 and LEF1), or TGFβ. Results are expressed as fold increase compared with cortical kidney tissue from control mice at 20 weeks. All of these genes, except for LEF1 at 20 weeks, were significantly upregulated in PT-Wnt1 mice. (C) Kidney cortical tissue shows increased psmad3 protein in PT-Wnt1 compared with control kidneys, which is a sign of TGFβ activation. (D) Protein lysates from control or PT-Wnt1 kidneys at 20 or 32 weeks show increased fibronectin and αSMA expression in PT-Wnt1 kidneys (E) Quantification of the Western blot signal (ImageJ). (F) Confocal images of the renal cortex comparing control and PT-Wnt1 kidneys at both time points. There is a diffuse increase in αSMA staining (red) directly adjacent to the LTL–positive proximal tubule (green) in all PT-Wnt1 kidneys compared with control. Scale bar, 50 μm. *$P<0.05$.

interest. Myofibroblasts arise primarily from kidney–resident mesenchymal stem cell–like progenitors.[37] Their activation represents a final common pathway for all CKD and subsequent matrix deposition, and scar formation is the common pathologic pathway.[38–40] Beyond the master fibrotic regulator TGFβ,

however, the signals that drive myofibroblast proliferation and activation from pericyte and perivascular fibroblast progenitors *in vivo* remain poorly understood. Although strong data implicate epithelial injury and cell cycle arrest in interstitial fibrosis, there is little direct evidence implicating specific soluble mediators that might communicate from the tubule to the interstitium.[32,41]

There are three main findings in this study. First, we provide unequivocal evidence that epithelial-derived Wnt1 is sufficient to drive interstitial fibrosis in the absence of any injury. This observation strongly supports a model of renal fibrogenesis involving epithelial to interstitial cross-talk. Second, we show that epithelial injury can be dissociated from renal fibrogenesis, despite its well characterized association with fibrosis in all preclinical models of which we are aware. Third, we have dissociated renal fibrosis from kidney inflammation. To varying degrees, inflammation accompanies every form of disease-associated CKD in humans. Although inflammation may influence fibrosis (either positively[42,43] or negatively[42,44]), this is the first study to show that inflammation is not itself required to drive tubulointerstitial fibrosis.

In two prior studies, a model for inducible overexpression of TGFβ in renal epithelia was generated and shown to drive fibrosis.[45,46] In this model, TGFβ also induced epithelial autophagy and ultimately, tubular decomposition beginning several days after the appearance of interstitial fibrosis. Whether this tubular injury might also have contributed to the interstitial phenotype through release of other downstream factors could not be determined. In this work, the fibrotic reaction was milder, taking weeks, which allowed for clear and complete dissociation of epithelial injury and interstitial fibrosis. The fact that myofibroblasts in our model exhibited canonical Wnt pathway activation further supports the notion that Wnt1 is directly binding to myofibroblasts and their precursors in the interstitium.

Although Wnt1 clearly showed additive profibrotic effects with TGFβ *in vitro*, it had modest effects when administered alone. This contrasts with the robust profibrotic phenotype of Wnt1 expression alone *in vivo*. Several factors may account for this discrepancy. First, the time course for development of fibrosis *in vivo* required weeks to develop, and therefore, comparing the *in vitro* (1–2 days) with the *in vivo* (months)
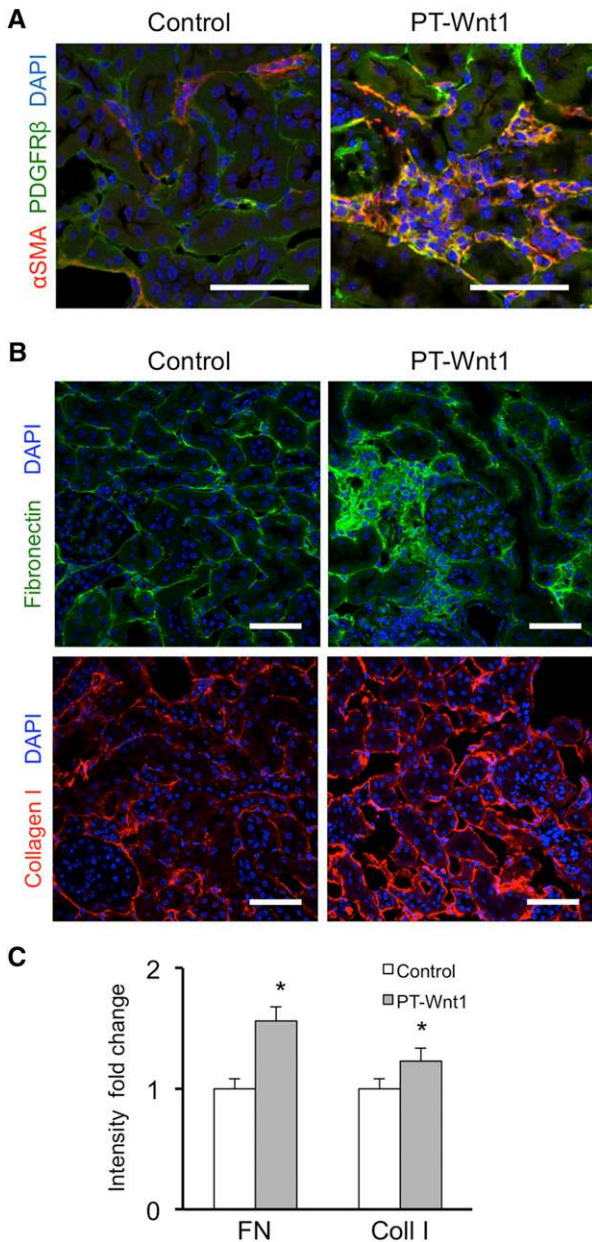
Figure 4. Epithelial Wnt1 induces myofibroblast activation and increased matrix deposition. (A) Confocal images of cortical kidney sections showing colocalization of αSMA (myofibroblast; red) with PDGFRβ (stroma; green) in PT-Wnt1 mice. There are no myofibroblasts evident in control littermates. (B) Confocal images of PT-Wnt1 kidney cortex show increased deposition of matrix proteins fibronectin and collagen I. (C) Quantification of the immunofluorescence signal intensity in the confocal images. Scale bar, 50 μm. *P<0.05.

perivascular fibroblasts could be an incomplete model of the *in vivo* situation.

The precise signaling mechanism by which Wnt1 alone drives myofibroblast activation remains undefined, although our *in vitro* evidence suggests that direct physical interaction of β-catenin with smad proteins may be involved. Indeed, β-catenin and TGFβ-regulated smads cooperatively activate a number of target genes, including connective tissue growth factor, which itself has been implicated in renal fibrogenesis.[47] The modest upregulation of TGFβ and psmad3 in PT-Wnt1 kidneys is consistent with cooperation between the Wnt and TGFβ pathways. One potential mechanism for TGFβ upregulation is through increased stiffness induced by Wnt–driven matrix protein expression. Increased tissue stiffness is a known activator of latent TGFβ.[48] The extent to which these
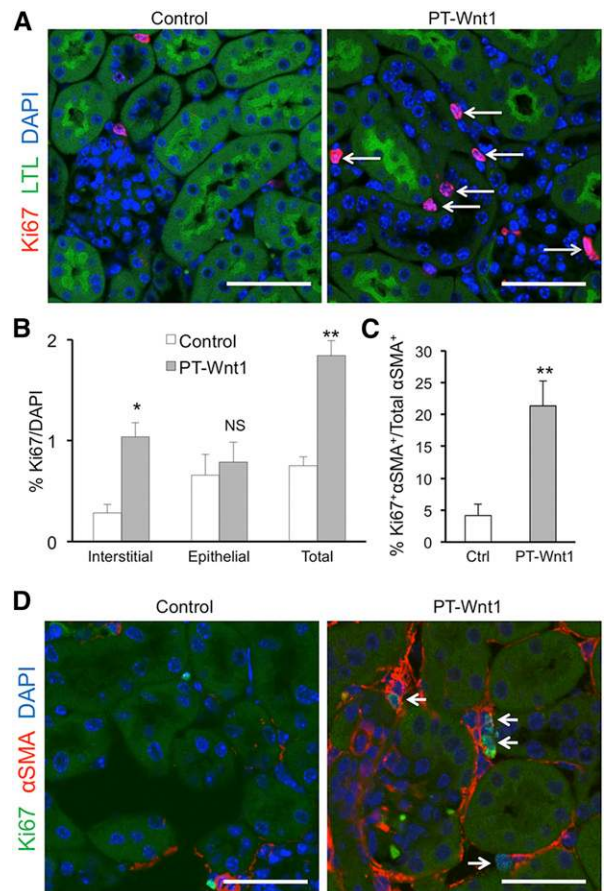

Figure 5. Epithelial–derived Wnt ligand increases myofibroblast proliferation. (A) Cortical kidney sections stained with anti-Ki67 antibody (red), anti-LTL antibody (green), and DNA marker DAPI (blue). There is a significant increase in Ki67+ cells (arrows) in the interstitium of the cortex in PT-Wnt1 mice. (B) Quantification of proliferative cells reveals that they are limited to the interstitium without any epithelial proliferation. (C) Percentage of Ki67+/αSMA double–positive cells to the total αSMA pool. There is a >4-fold increase in myofibroblast proliferation induced by Wnt1 secretion. (D) Increased proliferation (arrows) in myofibroblasts from PT-Wnt1 mice. Scale bar, 50 μm. *P<0.05; **P<0.01.

experiments may be inappropriate. Second, over this time period, it is possible that Wnt-dependent signaling might activate other profibrotic pathways, including increasing the availability of local TGFβ, which could then synergize with Wnt signaling. Indeed, we were able to measure modest induction of TGFβ mRNA in PT-Wnt1 kidneys. Third, the fidelity of the NRK-49F cell line to kidney pericytes and
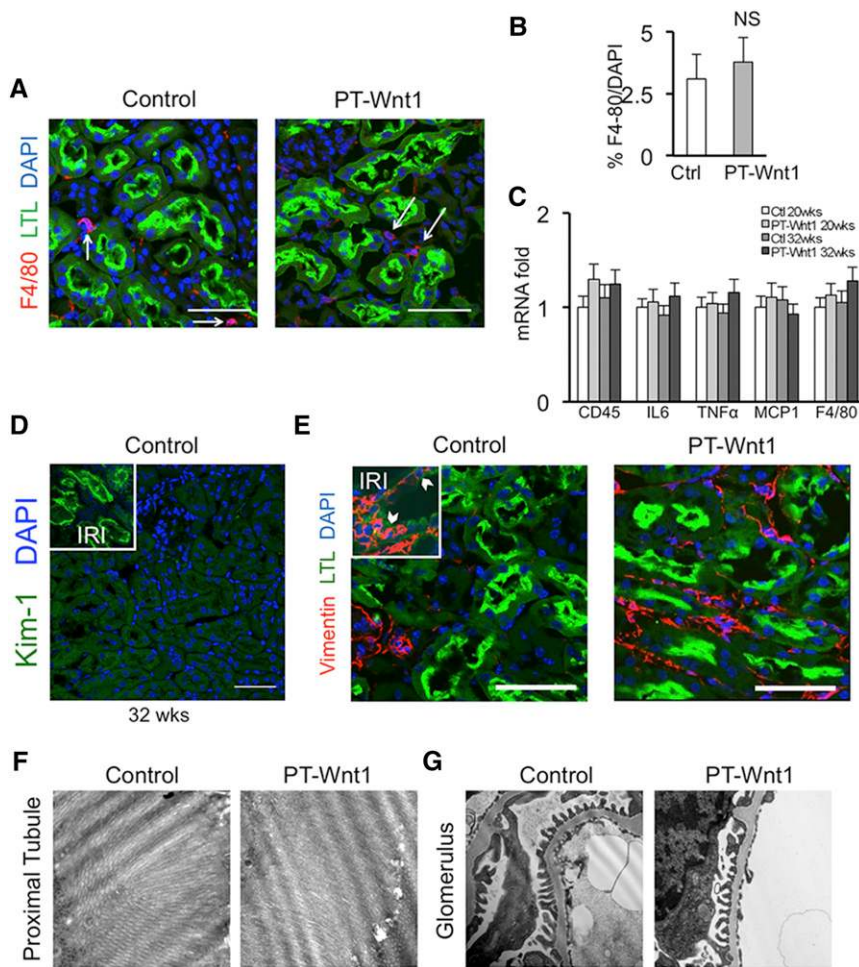
Figure 6. PT-Wnt1 kidneys are fibrotic without inflammation or epithelial injury. (A and B) There is no increase in macrophage number in PT-Wnt1 kidneys compared with control. Macrophages (arrows) identified by F4/80 staining. Quantification of macrophage abundance confirms equal numbers between groups. (C) qPCR of cortical kidney tissue reveals the absence of inflammatory marker expression in PT-Wnt1 kidneys compared with control. (D) There is no evidence of epithelial injury in PT-Wnt1 kidneys. Kim-1 protein (green) was measured by immunofluorescence using an IRI kidney as a positive control (Inset). Kim-1, a sensitive marker of epithelial injury, was undetectable in PT-Wnt1 kidneys. Scale bar, 50 μm. (E) Vimentin is normally expressed in kidney interstitium but induced in epithelial cells after injury (red; Inset). Epithelial vimentin was undetectable in PT-Wnt1 epithelium, although it was readily detected in epithelium from IRI kidneys. Scale bar, 50 μm. (F) Brush border from the proximal tubule was intact, which was reflected by electron microscopy, in PT-Wnt1 kidneys. (G) Podocyte foot processes were intact in PT-Wnt1 glomeruli as well.

pathways synergistically converge in our model will require additional delineation.

It is notable that the rise in BUN and serum creatinine in PT-Wnt1 mice was so modest, despite mild to moderate myofibroblast and matrix protein accumulation at 32 weeks. It is well known that creatinine is an insensitive marker of GFR loss, especially in mild disease. Thus, one possible explanation for this finding is that renal fibrosis was not advanced enough and

that GFR would fall more dramatically at later time points. However, combined with our failure to detect any evidence of tubular injury, an alternative explanation is that interstitial fibrosis itself does not promote parenchymal loss. Rather, ongoing epithelial injury may be required for loss of kidney function, which has been postulated for the AKI to CKD transition.

It is increasingly recognized that epithelium may repair incompletely after injury, and this failed repair triggers secretion by the epithelium of profibrotic factors that cause localized peritubular fibrosis (a recent review is in ref. 49). This central role of tubular epithelial responses to acute injury provides a cellular model for understanding the very strong epidemiologic link between episodes of AKI and increased future risk of CKD and ESRD. Our results provide support for this model, because we were able to dissociate epithelial injury and inflammation from interstitial fibrosis. The specificity of our inducible model allows us to conclude with strong conviction that paracrine signals from proximal tubule are sufficient to drive interstitial myofibroblast activation and proliferation, leading to increased matrix protein deposition—the histologic hallmark of fibrosis. Although we cannot rule out that Wnt1 protein levels were increased in the circulation, the most parsimonious explanation for our results is direct signaling from proximal tubule to adjacent pericytes, fibroblasts, and myofibroblasts. In human kidney injury syndromes, we hypothesize that epithelial injury and/or failed repair cause upregulation of Wnt ligands and other profibrotic soluble factors, which subsequently act on adjacent interstitium, driving a fibrotic response. Although the precise identity of myofibroblast progenitors responding to such paracrine signals has been the subject of considerable controversy, our recent evidence strongly implicates resident Gli1+ mesenchymal stem cell–like pericytes as the major myofibroblast progenitor population in kidney and other solid organs.[37]

In summary, we show that epithelial–derived Wnt ligand drives interstitial fibrosis by paracrine signaling. Our model emphasizes the role of cross-talk between tubular and interstitial compartments and highlights the therapeutic potential of targeting the Wnt signaling pathway for treatment of CKD.
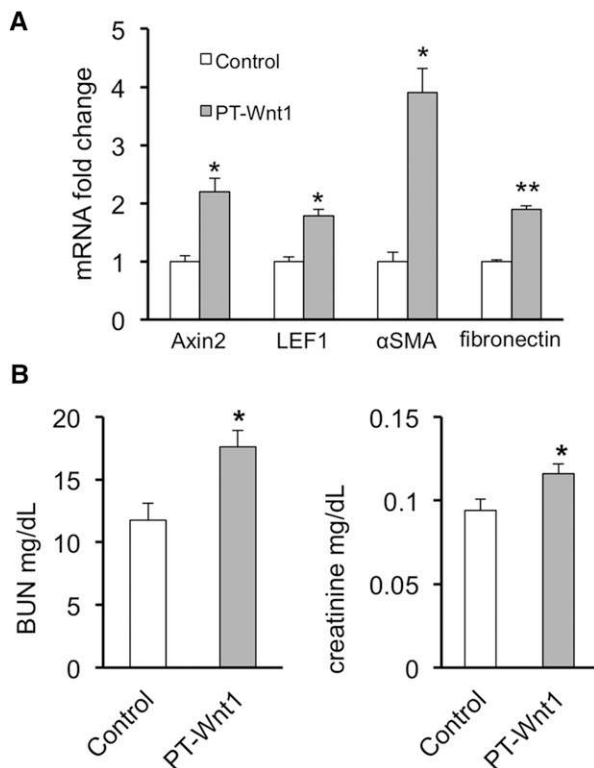
Figure 7. The canonical Wnt pathway is activated in interstitial myofibroblasts from PT-Wnt1 kidneys. (A) Stroma from cortex was isolated by FACS using PDGFRβ antibody in both PT-Wnt1 mouse and their control littermate kidneys. The sorted cells were collected and immediately processed for qPCR. There was a significant increase in the canonical Wnt readouts Axin2 and LEF1 in PT-Wnt1 stroma, suggesting that they are directly responding to epithelial-derived Wnt1. These sorted PDGFRβ cells also had significantly higher expression of αSMA and fibronectin. (B) Serum creatinine and BUN data at the 32-week time point reveal a modest but significant decrease in kidney function in PT-Wnt1 mice compared with controls. *$P<0.05$; **$P<0.01$.

CONCISE METHODS

Creation of the SLC34a1$^{GCE/+}$;R26R$^{Wnt1-GFP/+}$ Bigenic Mouse

We made use of the Cre-Lox system to generate a bigenic mouse model with sustained Wnt1 production in the proximal tubule epithelium. SLC34a1-GFPCreER^{T2} (SLC34a1^{GCE}) mice were generated by gene targeting as described.[30] These SLC34a1^{GCE} mice were crossed against mice containing a Wnt1-GFP fusion construct inserted into the Rosa-26 locus downstream of a floxed triple–polyadenylation STOP sequence.[8] In bigenic mice, administration of tamoxifen induces recombination of the STOP sequence, leading to Wnt1 expression exclusively in proximal tubule cells.

Animal Experiments

All experiments were performed according to the animal experimental guidelines issued by the Animal Care and Use Committee at Harvard University. Tamoxifen was dissolved in corn oil (Sigma-Aldrich, St. Louis, MO) containing 3% (vol/vol) ethanol at a concentration of 20 mg/ml. At 8 weeks of age, adult mice were given 0.5 ml (10 mg) tamoxifen by gavage every other day for three doses. After either a 12- or 20-week chase, mice were euthanized for analysis. Serum creatinine was assessed by HPLC at the O'Brien Core Center for Acute Kidney Injury Research in Birmingham, AL.

Tissue Preparation

Mice were anesthetized, killed, and immediately perfused with PBS through the left ventricle, and kidneys were removed. For frozen sections, kidneys were fixed with 4% paraformaldehyde for 2 hours on ice, incubated in 30% (vol/vol) sucrose at 4°C overnight, and embedded in optimum cutting temperature compound (Sakura FineTek), and 6-μm sections were cut. For paraffin sections, kidneys were fixed with 10% (vol/vol) formalin and paraffin embedded, and 5-μm sections were cut.

Immunofluorescence Analyses and Antibodies

For bromodeoxyuridine staining, sections were rehydrated and permeablized with 0.1% Triton X-100 in PBS for 5 minutes. Antigen retrieval was performed using heated citrate (Antigen Unmasking Solution; VectorLabs). For immunostaining of NRK-49F cells, kidney tissues were permeablized with 0.1% Triton X-100 and fixed with 4% (vol/vol) paraformaldehyde for 5 minutes. Samples were blocked with 5% (vol/vol) normal goat serum in PBS and incubated with primary antibodies, including rat anti-PDGFRβ (catalog no. 14–1402; eBioscience, San Diego, CA), Cy3-conjugated αSMA (1:500; catalog no. C6198; Sigma-Aldrich), rabbit antifibronectin (1:500; catalog no. ab23750; Abcam, Inc., Cambridge, MA), rat anti-F4/80 (catalog no. ab6640; Abcam, Inc.), FITC–conjugated anti–LTL (FL-1321; 1:1000; Vector Laboratories), goat anti–Kim-1 (AF1817; 1:500; R&D Systems), and rabbit anti-Ki67 (VP-RM04; 1:500; Vector Laboratories). Secondary antibodies were FITC, Cy3, or Cy5 conjugated (Jackson ImmunoResearch Laboratories) and incubated for 1 hour. Nuclear counterstaining was performed using DAPI and followed by mounting in Prolong-Gold (Invitrogen). Images were obtained by confocal (Nikon C1 Eclipse; Nikon) or standard (Nikon Eclipse 90i; Nikon) microscopy.

RNA Extraction and Real-Time qPCR

Cortex and medulla tissue samples were subdissected out of mouse kidney and snap frozen in liquid nitrogen. Total RNA was extracted from tissue or 1×10^6 cells using RNeasy Kits (Qiagen) followed by cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time detection of PCR product was performed using SYBR Green Master Mix (Bio-Rad), and all of the reactions were done in duplicate.

Separation of PDGFRβ–Positive Interstitial Cells Using FACS

Kidney cortex was minced, and a single-cell suspension was generated through liberase digestion for 30 minutes at 37°C. Cells were washed two times with PBS, filtered through 40- and 35-μm cell strainers, resuspended in PBS and 2% FBS with 1:1000 anti-PDGFRβ, and subjected to FACS using FACSAria II (BD Bioscience). Dead cells (DAPI+) were excluded during FACS. PDGFRβ+ DAPI− cells were collected in DMEM and 10% FBS, washed with PBS, and RNA extracted. Data were analyzed by FlowJo software (Version 7.5; TreeStar, Inc.).

Western Blot

To determine the relative amount of αSMA and fibronectin protein in kidneys from PT-Wnt1 mice versus wild-type littermates, the cortex and medulla of kidneys were separated and homogenized in radioimmunoprecipitation assay buffer with protease inhibitors and phosphatase inhibitors using a handheld rotor; the total protein was quantified by Bradford Assay, and 20 μg were separated by 10% PAGE. Proteins were transferred to polyvinylidene difluoride membrane, blocked in 5% milk in PBS, probed overnight at 4°C with mouse anti-αSMA (catalog no. A2547; 1:2000; Sigma-Aldrich) or antifibronectin rabbit antifibronectin (catalog no. ab23750; 1:6000; Abcam, Inc.), and probed with anti–rabbit or –mouse horseradish peroxidase (1:5000; Dako, Carpinteria, CA) for 1 hour at room temperature, and the antigen antibody complex was visualized using the ECL Detection System (PerkinElmer, Waltham, MA).

SBE-Luc Assay

The SBE-luc plasmid[19] was purchased from Addgene (Cambridge, MA). A pLenti expression construct carrying the SBE-luc plasmid was created (Life Technologies, Waltham, MA). NRK-49F cells were transduced, and cells expressing the SBE-luc plasmid were selected using G418 (Sigma-Aldrich). SBE-luc–transduced NRK-49F cells were treated with TGFβ (2 ng/ml), XAV939 (10 μM), or a combination for 16 hours, and then, the cells were harvested and analyzed. The luciferase assay kit was purchased from Promega (Madison, WI).

Cell Culture Experiments

NRK-49F cells (ATCC) were grown in Basal Media Eagle (Gibco, Billings, MT) with 5% FBS supplemented with penicillin and streptomycin and 2 mmol/L glutamine. For cells' response to ligands, cells were grown on six-well plates, serum starved by incubating in 0.5% FBS for 12 hours, and then, stimulated for 16 hours for RT-PCR experiments. Cells were starved overnight by replacing media with 0.5% FBS and subsequently treated with recombinant Wnt1 at 2 μg/ml (category no. 120–17; PeproTech) or TGFβ at 2 ng/ml (category no. 100–21; PeproTech). RNA was subsequently extracted, and real-time qPCR was performed as above.

Statistical Analyses

Data are given as means±SEMs. Statistical analysis was performed using the unpaired *t* test to determine differences between two groups and ANOVA to compare data among groups. *P* values of <0.05 were considered statistically significant. Each experiment was repeated at least two times with similar results.

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