Experimental inhibition of porcupine-mediated Wnt O-acylation attenuates kidney fibrosis

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Activated Wnt signaling is critical in the pathogenesis of renal fibrosis, a final common pathway for most forms of chronic kidney disease. Therapeutic intervention by inhibition of individual Wnts or downstream Wnt/β-catenin signaling has been proposed, but these approaches do not interrupt the functions of all Wnts nor block non-canonical Wnt signaling pathways. Alternatively, an orally bioavailable small molecule, Wnt-C59, blocks the catalytic activity of the Wnt-acyl transferase porcupine, and thereby prevents secretion of all Wnt isoforms. We found that inhibiting porcupine dramatically attenuates kidney fibrosis in the murine unilateral ureteral obstruction model. Wnt-C59 treatment similarly blunts collagen mRNA expression in the obstructed kidney. Consistent with its actions to broadly arrest Wnt signaling, porcupine inhibition reduces expression of Wnt target genes and bolsters nuclear exclusion of β-catenin in the kidney following ureteral obstruction. Importantly, prevention of Wnt secretion by Wnt-C59 blunts expression of inflammatory cytokines in the obstructed kidney that otherwise provoke a positive feedback loop of Wnt expression in collagen-producing fibroblasts and epithelial cells. Thus, therapeutic targeting of porcupine abrogates kidney fibrosis not only by overcoming the redundancy of individual Wnt isoforms but also by preventing upstream cytokine-induced Wnt generation. These findings reveal a novel therapeutic maneuver to protect the kidney from fibrosis by interrupting a pathogenic crosstalk loop between locally generated inflammatory cytokines and the Wnt/β-catenin signaling pathway.

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Chronic injury to the kidney results in progressive scarring known as renal fibrosis. Renal fibrosis is a final common pathway for both immune-mediated glomerulonephritides and kidney diseases initiated by hemodynamic insults such as hypertension. The extent of renal fibrosis predicts progression to end-stage kidney disease in most forms of chronic kidney disease. Despite an intense focus of research in this area, no specific therapies are currently available to abrogate or reverse fibrosis in human chronic kidney disease.

In recent years, the Wingless-related integration site (Wnt) family of signaling proteins have emerged as potential targets of inhibition to control fibrosis in several target organs.1–6 In the so-called “canonical” pathway, Wnt triggers activation of β-catenin signaling that drives progressive fibrosis in various models of kidney disease.1–3 As expected, β-catenin inhibitor molecules have shown some efficacy in slowing the progression of renal fibrosis.2,5 However, as there are 19 different Wnt ligands that may signal via the canonical pathways, the alternative “non-canonical” pathways, or both, the efficacy of these direct β-catenin inhibitors may be limited.

To overcome the limitations of the available Wnt pathway inhibitors and to block all Wnt-dependent pathways concomitantly by preventing the secretion of all Wnts, we have employed a small-molecule inhibitor of Porcupine named Wnt-C59 (C59).8 Porcupine (PORCN) is a membrane-bound O-acyl transferase that resides in the endoplasmic reticulum and catalyzes the acylation of all mammalian Wnts at a conserved serine residue. This acylation is essential for the secretion and binding of all Wnts to their receptors.9,10 C59 is orally bioavailable and by inhibiting Wnt acylation blocks the secretion and activity of all Wnts.8

Here we report that preventing Wnt secretion with C59 therapy downregulates Wnt signaling and as a consequence dramatically reduces kidney fibrosis in the mouse unilateral ureteral obstruction (UUO) model. Preventing the secretion of Wnts using the PORCN inhibitor C59 blunts the proliferation of fibroblasts and the expression of key proinflammatory cytokines that are in turn capable of eliciting Wnt generation. Our results provide a compelling rationale for the clinical evaluation of PORCN inhibitors for the treatment of kidney fibrosis and other progressive scarring disorders.
RESULTS
PORCN inhibitor C59 blocks renal interstitial fibrosis in obstructive nephropathy

Wnt signaling is known to potentiate kidney fibrosis. We therefore tested whether inhibition of Wnt signaling with a PORCN inhibitor could limit the extent of renal fibrosis following injury in mice. We evaluated the severity of fibrosis in kidneys from mice treated with C59 or vehicle for 7 days following UUO. As illustrated by polarized images of the kidney sections stained with picrosirius red (Figure 1a), the obstructed kidneys from the vehicle group had markedly increased fibrosis compared to the contralateral kidneys. However, treatment with C59 significantly attenuated the level of fibrosis in the obstructed kidneys. Blinded morphometric analysis of the kidney sections (Figure 1b) confirmed that C59 therapy reduced the deposition of collagen fibrils in the obstructed kidney by half compared to vehicle-treated controls. Thus, treatment with C59 substantially mitigates renal fibrosis in the mouse UUO model.

Downregulation of Wnt signaling in obstructed kidneys of mice treated with PORCN inhibitor

Wnts act via the canonical signaling pathway to induce nuclear translocation of β-catenin. To determine if the beneficial effects of PORCN inhibition to prevent tissue fibrosis were mediated by downregulation of Wnt signaling pathways, we analyzed the cellular localization of β-catenin. In the vehicle-treated group, ureteral obstruction induced robust nuclear localization of β-catenin within the interstitial and epithelial cells of the kidney (Figure 2a). By contrast, treatment with C59 nearly eliminated Wnt-driven nuclear localization of β-catenin in cells of the obstructed kidney.

Upon translocation to the nucleus, β-catenin forms a complex with transcription factors T cell–specific transcription factor (TCF)/lymphoid enhancer binding factor (LEF) to drive transcription of key Wnt target genes including Axin2 and Nkd1. Accordingly, obstructed kidneys from the vehicle group had markedly increased expression of Axin2 and Nkd1, whereas this was significantly attenuated in the obstructed kidneys from C59-treated mice (Figure 2b and c).

Axin2-LacZ reporter mice have an integrated genetic reporter so that cells and tissues with Axin2 induction express β-galactosidase, with activity that is readily visible with a specific histochemical stain. Following UUO, Axin2-LacZ mice showed marked reporter activity in the obstructed kidney (Figure 2d). However, treatment with C59 nearly abrogated the UUO-mediated induction of Axin2 as detected in the reporter model (Figure 2d). Thus, Wnt signaling is critical to the pathogenesis of kidney fibrosis, and comprehensive inhibition of Wnt secretion by the PORCN inhibitor prevents kidney scarring in a robust model of renal fibrogenesis.

Decreased proliferation and function of fibroblasts in obstructed kidneys from C59-treated mice

Fibroblasts are the key effector cells in collagen deposition, and they accumulate in areas of severe interstitial fibrosis. β-catenin/TCF-dependent transcription promotes fibrogenesis by enhancing fibroblast proliferation and function. As treatment with C59 prevents nuclear accumulation of β-catenin and fibrosis, we examined whether blocking Wnt secretion impacts the proliferation of collagen-producing fibroblasts in the obstructed kidneys. To identify the fibroblasts, kidney sections were stained with an antibody against fibroblast-specific protein (Fsp-1) that primarily stains fibroblasts. In the vehicle-treated group, UUO strongly increased the level of Fsp-1 staining in the kidney (Figure 3a and b). By contrast, C59 treatment markedly reduced Fsp-1 staining in the obstructed kidney, consistent with an attenuated number of fibroblasts. At the mRNA level, C59 similarly reduced Fsp1 expression in the kidney following UUO (Figure 3c). These data indicate that PORCN inhibition limits UUO-induced kidney fibrosis in part by blocking the local accumulation of fibroblasts.

Figure 1 | PORCN inhibitor C59 blocks renal interstitial fibrosis in obstructive nephropathy. (a) Representative polarized images show reduced fibrotic lesions in PORCN inhibitor–treated mice. (b) Quantification of the fibrotic lesions in obstructed and contralateral kidneys as determined by picrosirius red staining, followed by blinded computer-aided morphometric quantitation in 15 contiguous high-power fields from each section, n = 9 mice per group. C59, Wnt-C59; PORCN, porcupine; UUO, unilateral ureteral obstruction.
Fibrosis is characterized by excessive scarring due to production, deposition, and contraction of extracellular matrix by proliferating fibroblasts. To characterize how the inhibition of Wnt secretion provides benefit in the mouse model of kidney fibrosis, we analyzed global changes in gene expression programs within the obstructed kidneys from vehicle- and C59-treated animals at day 7 of UUO. The disruption in Wnt signaling by C59 therapy had a broad impact on the expression of genes linked to the pathogenesis of kidney fibrosis. Five hundred fifty-six genes were differentially expressed in the control versus C59-treated UUO kidneys using a cutoff of 1.5-fold and false discovery rate $P$ value $< 0.05$ (Figure 4a). Ingenuity pathway analysis of the differentially expressed genes showed that UUO led to marked upregulation of genes associated with fibroblast-mediated deposition of extracellular matrix such as matrix metalloproteases and collagens (Figure 4b). C59 treatment also resulted in a reduction in the expression of fibroblast-associated genes including various collagens, matrix metalloprotease 2, fibronectin 1 (FN1), and

**Figure 2** | Downregulation of Wnt signaling in obstructed kidneys of mice treated with PORCN inhibitor. (a) Kidney sections were stained for β-catenin. Arrows indicate positively stained tubular epithelial and interstitial cells in the obstructed kidneys. Marked nuclear exclusion of β-catenin from obstructed kidneys of C59-treated mice compared to vehicle-treated mice is observed. (b,c) Expression of Axin2 (b) or Nkd1 (c) was measured using qRT-PCR in the kidneys following 7 days of treatment, $n = 8$ per group. Data shown are mean ± SEM. (d) Axin2-LacZ reporter mice were treated with or without C59 for 3 days. The kidneys were isolated and stained for LacZ. A significant reduction in staining for LacZ was observed in the kidneys from C59-treated mice compared to the control mice. C59, Wnt-C59; qRT-PCR, quantitative polymerase chain reaction; UUO, unilateral ureteral obstruction.
intercellular adhesion molecule 1 (ICAM-1), consistent with its effects on fibroblast proliferation (Figure 4b). The changes in expression of \(\text{Col1a1} \) and \(\text{FN1} \) were validated using real-time reverse transcriptase quantitative polymerase chain reaction (qRT-PCR) (Figure 4c and d). PORCN inhibition also reduced protein levels of \(\text{FN1} \) as examined by immunohistochemistry (Figure 4e). Taken together, these data confirm that inhibition of Wnt secretion comprehensively interrupts profibrotic signaling pathways in the obstructed kidney by blunting the proliferation of fibroblasts.

**Treatment with C59 reduces tubular damage in obstructive uropathy**

Chronic kidney disease ultimately culminates in kidney fibrosis that in turn predicts end-stage kidney disease. To determine whether C59 therapy preserves the architecture of the kidney tubules, we evaluated the severity of pathologic changes in the renal tubules at 7 days following UUO. The tubules in the control kidneys from both the groups were normal with eosinophilic cytoplasm. In the obstructed kidneys from the vehicle-treated group, most of the tubules were atrophic, having smaller cells and nuclei with basophilic cytoplasm, or dilated and filled with proteinaceous casts (Figure 5a). The severity of nephropathy was scored on a scale from 0 to 5 based on the extent of tubular atrophy, dilation, necrosis, and cell loss, with 0 representing no damage, and 1 to 5 marking a spectrum from minimal up to severe tubular damage. Here again, treatment with C59 yielded a quantitative reduction in the level of tubular damage induced by UUO (Figure 5a and b). At the mRNA level, microarray analysis confirmed the preservation of tubular integrity by C59 following UUO (Figure 5c). Specifically, expression of solute carriers expressed in the proximal nephron (\(\text{Slc5a10/Sglt5} \)), the thick ascending limb (\(\text{Slc12a1/Nkcc2} \)), and the collecting tubule (\(\text{Slc4a1/AE1} \)) was downregulated in obstructed kidneys but was restored toward normal by C59 therapy. Thus blocking Wnt secretion with a PORCN inhibitor mitigates the renal tubular injury that triggers the progression of kidney fibrosis.

**Treatment with C59 alters the proinflammatory milieu in the obstructed kidneys**

Mitigation of fibrosis with PORCN inhibition was accompanied by differential expression not only of multiple collagens but also of immune-signaling genes (Figure 4b). As proinflammatory cytokines produced in the kidney by intrinsic and/or infiltrating immune cells are key instigators of fibrosis, we measured the expression of several profibrotic cytokines in the kidneys from both vehicle- and PORCN inhibitor–treated animals (Figure 6). Indeed, there was a marked upregulation of \(\text{Tnf} \), \(\text{Tgfb} \), and \(\text{IL1b} \) in the thick ascending limb (\(\text{Slc12a1/Nkcc2} \)), and the collecting tubule (\(\text{Slc4a1/AE1} \)) was downregulated in obstructed kidneys but was restored toward normal by C59 therapy. Thus blocking Wnt secretion with a PORCN inhibitor mitigates the renal tubular injury that triggers the progression of kidney fibrosis.

Circulating mononuclear cells, particularly macrophages that infiltrate the kidney, have been reported to be a key source of profibrotic cytokines. However, we found similar levels of \(\text{F4/80}^+ \) macrophage infiltration in the UUO
kidneys from our vehicle-treated (4.6% ± 0.6%) and C59-treated groups (4.7% ± 0.5%). Importantly, in situ hybridization showed that transforming growth factor-β (TGF-β) was predominantly expressed in the interstitial cells and that C59 suppressed the expression of TGF-β (Figure 6e and f), suggesting that Wnts may promote renal fibrosis through the induction of profibrotic cytokines directly in renal stromal cells.

Upregulated expression of plasminogen activator inhibitor-1 (PAI-1) is a convergent pathway that mediates the fibrogenic actions of the Wnt/β-catenin and TGF-β pathways.22 PAI-1 promotes extracellular matrix deposition, and
mice lacking PAI-1 are protected against fibrosis induced by various pathogenic insults. As inhibition of Wnt secretion using C59 prevents the activation of Wnt/β-catenin target genes by preventing nuclear accumulation of β-catenin and decreases the expression of TGF-β, we measured renal expression of PAI-1 in the experimental groups. PAI-1 expression was high in the obstructed kidneys and was significantly decreased after treatment with C59 (Figure 6g).
Thus inhibition of Wnt signaling by C59 interrupts PAI-1-mediated renal fibrogenesis.

**Wnt ligand induction in intrinsic kidney cells and infiltrating macrophages after UUO**

Given the magnitude of protection from renal fibrosis afforded by PORCN inhibition, we posited that profibrotic cytokines could conversely stimulate Wnt/\(\beta\)-catenin activity. In that case, comprehensive Wnt inhibition by C59 would interrupt a positive feedback loop in which Wnts trigger the expression of cytokines that in turn provoke generation of Wnts. We therefore assessed the capacity of exogenous TGF-\(\beta\) and TNF-\(\alpha\) to regulate the expression of all 19 Wnt isoforms in primary kidney fibroblasts and a kidney epithelial cell line *in vitro*. Treatment with TNF-\(\alpha\) upregulated *Wnt1* and *Wnt10a* in the kidney epithelial cells but had no measurable effect on the expression of Wnt isoforms in kidney fibroblasts (*Figure 7a* and b). By contrast, TGF-\(\beta\) increased the expression of *Wnt5a* and *Wnt5b* in the kidney epithelial cells and led to an upregulation of *Wnt1* and *Wnt10b* in the kidney fibroblasts. An increase in expression of *Wnt5a*, *Wnt4*, and *Wnt7a* in the epithelial stromal cells of the ligated kidneys was also observed *in vivo*, using *in situ* hybridization assay (*Figure 7c*).

In acute kidney injury models, macrophages infiltrating the kidney constitute another source of Wnt ligands.\(^{25}\) We therefore profiled the expression of Wnt ligands in the CD11b\(^+\) macrophages infiltrating the kidney in our UUO model and observed an upregulation of *Wnt1*, *Wnt7a*, and *Wnt10a* in these cells compared to macrophages in the contralateral kidney (*Figure 7d*). These data suggest that both intrinsic kidney cells and infiltrating mononuclear cells generate Wnts in the obstructed kidney.

We posited that by disrupting a positive feedback cycle in which Wnt signaling promotes the expression of profibrotic cytokines that in turn induce additional Wnt expression, PORCN inhibition with C59 yields profound protection from kidney fibrosis. Consistent with that hypothesis, expression of...
Figure 7 | Upregulated expression of Wnts in kidney epithelial cells, fibroblasts, and macrophages. (a) HK-2 cells and (b) primary kidney fibroblasts were treated with TNF-α or TGF-β for 24 hours. Total RNA was isolated and expression of all 19 Wnts was analyzed using qRT-PCR. Wnts whose expression changed about 4-fold or more are shown. (c) Representative images of in situ hybridization for Wnt4, Wnt5a, and Wnt7a in the control and ligated kidney sections. (d) CD11b+ macrophages were isolated from control and ligated kidneys, and the expression of Wnts was analyzed using qRT-PCR, n = 3–4 per group. qRT-PCR, quantitative polymerase chain reaction; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; UUO, unilateral ureteral obstruction.
multiple Wnts, including Wnt1, 2, 4, 7a, and 10a, that is increased in the obstructed kidneys was significantly reduced with C59 treatment (Figure 8a). By contrast, while Wnt5a levels were increased in the obstructed kidneys, treatment with C59 did not alter its expression. We also tested whether C59 treatment influences the surface abundance of Wnt receptors by immunohistochemistry, but the expression of Frizzleds was not altered with C59 treatment (data not shown).

Finally, to examine if cytokine-mediated Wnt induction in intrinsic kidney cells could drive expression of Wnt target genes in those cells, we quantitated Axin2 mRNA levels in primary kidney fibroblasts or HK-2 epithelial cells exposed to TGF-β in culture. As shown in Figure 8b, TGF-β induced Axin2 in the kidney fibroblasts. Moreover, Axin2 induction was abrogated by treatment with C59 (Figure 8b). By contrast, we did not observe a significant change in Axin2 expression in epithelial cells following TGF-β or C59 treatment. Taken together, these data indicate that PORCN inhibition with C59 treatment attenuates the induction of profibrotic cytokines, reducing Wnt expression and disrupting a pathogenic positive feedback cycle to provide protection from kidney fibrosis.

**DISCUSSION**

Wnt/β-catenin signaling plays a fundamental role in the developing organism through the regulation of diverse cell growth and proliferation pathways. When injury in an organ occurs, patterns of fetal gene expression may be reactivated. However, inappropriate activation of various signaling pathways can alter tissue healing and permit scar formation. Kidney damage culminating in renal fibrosis induces profound activation of the Wnt signaling pathway, and the extent of kidney fibrosis predicts the progression of most forms of chronic kidney disease to end-stage renal disease. Accordingly, disruption of the Wnt/β-catenin

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**Figure 8 | Decreased expression of Wnts and Wnt signaling in C59-treated kidneys.** (a) Total RNA was isolated from the kidneys; expression of indicated Wnts was measured using qRT-PCR and was normalized to Hprt, n = 8 per group. (b) Primary kidney fibroblasts were treated with TGF-β with or without C59 (10 nM), total RNA was isolated after 24 hours, and Axin2 expression was measured. Data are representative of 3 independent experiments. C59, Wnt-C59; qRT-PCR, quantitative polymerase chain reaction; TGF-β, transforming growth factor-β.
pathway by specific antagonists has yielded measurable protection from fibrogenesis, particularly in the kidney.\textsuperscript{2,5,31} Nevertheless, these antagonists are not capable of concomitantly interrupting the actions of all 19 Wnt isoforms signaling via canonical and non-canonical pathways. Consistent with an important role for canonical Wnt signaling in kidney fibrosis, our study shows that blocking all Wnt secretion with PORCN inhibition profoundly inhibits the progression of kidney fibrosis in the UUO model.

The striking efficacy of PORCN inhibition is likely mediated by its effect on a diverse cascade of profibrotic signaling pathways in multiple cell lineages. Inhibition of Wnt secretion minimized the susceptibility to scarring in the obstructed kidney both by limiting the renal accumulation of fibroblasts and by restoration of gene expression of solute transporters expressed throughout the nephron, thus preserving normal tubular structure and function. One of the most profound effects of inhibiting the Wnt secretion was the subduing of the proinflammatory milieu composed of key profibrotic cytokines such as TGF-β, interleukin-1β, and TNF-α, all of which have been implicated in the pathogenesis of kidney scarring.\textsuperscript{32,33}

TGF-β1 is known to contribute to the formation of myofibroblasts through the activation of resident fibroblasts and by inducing the transition to mesenchyme of epithelial and endothelial cells via epithelial–mesenchymal or endothelial–mesenchymal transition, respectively.\textsuperscript{34–36} Consistent with the well-established function of TGF-β to promote the proliferation of fibroblasts,\textsuperscript{18,37} the blunted renal expression of TGF-β in the C59-treated group reduced proliferation of fibroblasts in the obstructed kidneys. However, although we observed a mild decrease in the expression of Snail and Slug in the treated kidneys, there were no significant changes in the expression of other epithelial–mesenchymal transition markers including vimentin, α-smooth muscle actin, and E-cadherin (data not shown).

The circulating mononuclear cells that infiltrate the kidney are a key source of profibrotic cytokines and are integral to the pathogenesis of kidney fibrosis.\textsuperscript{18–21} Moreover, while we did not observe differences in macrophage infiltration in the UUO kidneys from vehicle- and C59-treated groups, our data would suggest that renal infiltrating macrophages represent a key source of Wnts that require PORCN-dependent O-acylation to act on intrinsic kidney cells. Kidney epithelial cells are another potent source of cytokines including TGF-β and TNF-α,\textsuperscript{38,39} and activation of cytokine receptors directly on kidney parenchymal cells greatly augments the severity of renal fibrosis.\textsuperscript{40} Our data would indicate that C59 suppressed Wnt-induced generation of TGF-β in interstitial cells of the kidney. Wnts may thus promote renal fibrosis through the induction of profibrotic cytokines directly in intrinsic kidney cells.

Conversely, our study also shows that cytokines can enhance Wnt generation by kidney epithelial cells and fibroblasts. Stimulation of fibroblasts and renal epithelial cells in vitro with TNF-α and TGF-β induced several Wnts, including Wnt1, Wnt5, and Wnt10. UUO triggered induction of these and other Wnt isoforms in vivo, whereas C59 treatment attenuated the induction of all of these Wnts except Wnt5a. The failure of TGF-β to trigger induction of the Wnt target gene Axin2 in epithelial cell cultures suggests that the Wnts induced by TGF-β in kidney epithelial cells may act via paracrine mechanisms on fibroblasts more than via autocrine mechanisms in epithelial cells to drive fibrogenesis, particularly as TGF-β does induce Axin2 in fibroblasts via a PORCN-dependent pathway in our studies (Figure 8b). Thus, blocking Wnt secretion with C59 profoundly limits renal fibrogenesis by suppressing the renal expression of inflammatory cytokines that, in turn, induce further Wnt generation in fibroblasts and renal tubular cells. By interrupting this pathogenic feedback loop, PORCN inhibition shuts down crosstalk between inflammatory cells, the renal epithelia, and scar-forming fibroblasts, culminating in maximal renoprotection.

This therapeutic interruption of a pathogenic fibrotic cycle is reminiscent of the benefit that accrues from disrupting pathogenic intrarenal angiotensin II “augmentation,” wherein activation of AT\textsubscript{1}, angiotensin receptors triggers generation of angiotensinogen, the precursor of angiotensin II.\textsuperscript{38,41,42} Interestingly, the angiotensin II augmentation loop invokes the nuclear factor-κB inflammatory signaling cascade, a pathway that, like the Wnt cascade, is triggered by and drives expression of a broad array of profibrotic cytokines.\textsuperscript{43} Thus, in addition to negative feedback loops that restore homeostasis, nature has also evolved a set of pathogenic positive feedback loops that culminate in severe injury if unchecked. Blocking these pathogenic loops therefore holds significant promise as a therapeutic paradigm. However, to provide the strongest protection from tissue fibrosis with a minimum of unfavorable off-target consequences, additional studies will be needed to further specify the Wnt-dependent contributions of fibroblasts, epithelial cells, and inflammatory cells to the progression of renal fibrosis.

In summary, the current studies identify a potent strategy to preclude renal fibrogenesis by abrogating Wnt O-acylation and therefore cellular secretion of all Wnt isoforms. This intervention interrupts β-catenin signaling in multiple cell lineages, particularly kidney fibroblasts, and further attenuates profibrotic signaling cascades triggered by proinflammatory cytokines. In addition to its role in kidney fibrosis, the Wnt signaling cascade contributes to multiple fibrotic disorders in other target organs including systemic sclerosis, lung fibrosis, diabetic retinopathy, and dermal scarring.\textsuperscript{5,14,44–46} Wnt secretion inhibitors represent a promising translational opportunity to treat these fibrotic disorders as well. Novel PORCN inhibitors are being developed, and some are currently in phase I clinical trials for the treatment of Wnt-driven cancers.\textsuperscript{7} Our data lay the groundwork for clinical studies in which small-molecule inhibitors of PORCN can be tested in broad populations of humans suffering the consequences of chronic kidney disease and other fibrotic disorders.
Materials and methods

Compounds

For in vitro experiments, stock solutions of C59 were prepared in dimethylsulfoxide, and the final dimethylsulfoxide concentration in all biological assays was 0.1%.[8] For administration to the mice, C59 was resuspended in 0.5% methylcellulose and 0.1% Tween-80 by sonication for 20 minutes. The in vivo dose used in the UUO model was 25 mg/kg by oral gavage for the duration of the experiment. We selected the dose of 25 mg/kg based on its pharmacokinetic properties and maximum tolerated dose. At this dose, the mean exposure of C59 as calculated by AUC0-infinity was 98,761 ng*h/ml.

Animal care

All the animal studies were approved by the Durham Veterans Affairs Medical Center and Duke–NUS (National University of Singapore) Institutional Animal Care and Use Committees for compliance with regulations. Animals were housed in standard cages and were maintained as recommended in keratinocyte serum-free media containing 5 ng/ml epidermal growth factor and 0.05 mg/ml bovine pituitary extract. Primary kidney fibroblasts were established from kidney explants as described.[47] Briefly, the kidneys were minced and digested with collagen followed by plating on gelatin-coated petri plates. The fibroblasts obtained were cultured in Dulbecco’s modified Eagle’s medium and Ham’s F12 (1:1) with 20% serum. For analysis of renal macrophage RNA, kidneys were harvested at day 7 after UUO and minced, followed by incubation with 0.1% collagenase type I (Gibco, Thermo Fisher, Waltham, MA, USA) for 40 minutes at 37°C. Single-cell suspensions were obtained by filtering through a 40-μm cell strainer.[50] Thereafter, cells were stained with fluorescently labeled anti-CD45 and anti-CD11b (BD Biosciences, San Jose, CA), and subjected to fluorescent cell sorting. The gating strategy was as follows: first gate on single cells, second gate on CD45+ cells, third gate on CD11b+ cells. RNA was isolated from cells meeting these criteria for real-time PCR analysis.

Histopathologic analysis

Kidneys were fixed in 10% neutral buffered formalin and processed for staining. Briefly, the tissue samples were dehydrated through a graded series of ethanol, infiltrated with and embedded in paraffin. The tissue sections were then deparaffinized in xylene and rehydrated in a series of ethanol gradients. For β-catenin and Fsp-1 staining, after antigen retrieval, the endogenous peroxidase activity was blocked by incubation with H2O2. The sections were then incubated for 1 hour with 1:150 diluted β-catenin (catalog no. 610154, BD Transduction Laboratories), or 1:1000 diluted anti–Fsp-1 antibody (catalog no. ab27957, Abcam, Cambridge, UK) or 1:50 dilute fibronectin antibody (catalog no. 610077, BD Transduction Laboratories) followed by incubation with a horseradish peroxidase–conjugated secondary antibody. Incubation with 3,3′-diaminobenzidine (DAKO, Glostrup, Denmark) chromogen substrate resulted in brown staining of β-catenin–positive and Fsp-1–positive fibroblasts. The nuclei were counterstained with Mayer’s hematoxylin before dehydration and mounting.

For detecting total collagen fibrils, paraffin sections were stained with picrosirius red. The dewaxed and hydrated sections were incubated with picrosirius red solution for 1 hour and then washed with acidified water. Following staining, the sections were dehydrated through a series of ethanol gradients and mounted with Cytoseal (Thermo Fisher). On each section representative images were photographed and areas of positive signals were quantified by computer-assisted morphometric analysis using ImageJ.[138] (National Institutes of Health, Bethesda, MD). Scores were averaged for each animal, and then for each group. Kidney sections stained with picrosirius red were also examined under polarized microscopy.

An American College of Veterinary Pathologists board–certified veterinary anatomic pathologist (RMB) examined the hematoxylin and eosin–stained kidney sections from both the treated and untreated groups for tubular damage. The severity of tubular injury was scored on a scale from 0 to 5, with 0 representing no, 1 representing minimal, and 5 representing severe tubular damage.

In situ hybridization

In situ hybridization for Tgfβ, Wnt4, Wnt5a, and Wnt7a was performed using RNAscope 2.0 FPPE Assay (Advanced Cell Diagnostics, Hayward, CA). Briefly, 5-μm sections of FFPE blocks were treated for 1 hour at 60°C and 15 minutes with protease before hybridization with Tgfβ, Wnt4, Wnt5a, Wnt7a, or control probes. A horseradish peroxidase–based signal amplification system was then applied to the probes followed by visualization with 3,3′-diaminobenzidine. Positive staining was observed as brown, punctate dots. Technical controls included the bacterial gene DapB as a negative control and the housekeeping gene PPIB as a positive control. An American College of Veterinary Pathologists board–certified veterinary anatomic pathologist (RMB) scored for the Tgfβ expression levels. All regions of the kidney including cortex, medulla, and papilla were scored individually on a scale of 0 to 5 for the intensity and percentage of positively stained cells (cumulative score 0–15).

Quantitative RT-PCR analysis

Kidneys were harvested 4 hours after the last dose of C59, and total RNA was isolated with the RNeasy kit (Qiagen, Hilden, Germany). RNA was reverse transcribed with iScript reverse transcriptase and real-time quantitative PCR (qPCR) was performed with Ssofast EvaGreen assay from Bio-Rad (Hercules, CA). The following primers were used: Axin2 F:CTTCCCCACCCATGTGAAGA, R:TGGCTGGT GCAAAGACATAG; Fsp1 F:CTGGGGGAAAAGGACAGATGA, R:TGGC AGGACAGGAAGCACA; Cellat1 F:GTCCATTGCGATGGCTGCTC, R:CAAGTGCAGAGGGTGAAT; IL1β F:GCTGCTTCCAAACCTTT TGAC, R:AGCTTCTCACAGCCACAAAT; Trf5 F:ATACTAGTCGCT
AGGGAGTGTG, R:TCAGCTTGCCAGGAGGTG; F:GAAAGTCGCAAGAAGCAAGC, R:GGTTGAGGTGAAAGGCAGGAGTAqMan primers and probes were used for Tnflk and Tgflβ.

Microarray expression analysis
To define the gene expression patterns, Illumina (San Diego, CA) Expression Mouse WG-6 v2 Arrays genechips were used. The concentration and purity of the RNA samples was determined by Nanodrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the integrity of RNA was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Microarray expression analysis was conducted at the Genome Biology facility at Duke–NUS. All data normalization and selection of genes with changes in expression were performed using Partek (St. Louis, MO) analysis software. The genes whose expression differed more than 1.5-fold between experimental groups were considered significant. P values were controlled for multiple testing (false discovery rate) with P ≤ 0.05.

Statistical analysis
Data were analyzed using Prism version 5.0 (GraphPad, La Jolla, CA, USA). Data were analyzed using 2-way analysis of variance or t-test, and significance for all tests was set at P ≤ 0.05. The statistical test applied for each experiment is detailed in the respective figure legend. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001 in all instances.

DISCLOSURE
DMV has a patent interest in ETC159, a PORCN inhibitor related to drugs used in this study. All the other authors declared no competing interests.

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