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Blocking TGF-β and β-Catenin Epithelial Crosstalk Exacerbates CKD

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ABSTRACT

The TGF-β and Wnt/β-catenin pathways have important roles in modulating CKD, but how these growth factors affect the epithelial response to CKD is not well studied. TGF-β has strong profibrotic effects, but this pleiotropic factor has many different cellular effects depending on the target cell type. To investigate how TGF-β signaling in the proximal tubule, a key target and mediator of CKD, alters the response to CKD, we injured mice lacking the TGF-β type 2 receptor specifically in this epithelial segment. Compared with littermate controls, mice lacking the proximal tubular TGF-β receptor had significantly increased tubular injury and tubulointerstitial fibrosis in two different models of CKD. RNA sequencing indicated that deleting the TGF-β receptor in proximal tubule cells modulated many growth factor pathways, but Wnt/β-catenin signaling was the pathway most affected. We validated that deleting the proximal tubular TGF-β receptor impaired β-catenin activity *in vitro* and *in vivo*. Genetically restoring β-catenin activity in proximal tubules lacking the TGF-β receptor dramatically improved the tubular response to CKD in mice. Deleting the TGF-β receptor alters many growth factors, and therefore, this ameliorated response may be a direct effect of β-catenin activity or an indirect effect of β-catenin interacting with other growth factors. In conclusion, blocking TGF-β and β-catenin crosstalk in proximal tubules exacerbates tubular injury in two models of CKD.

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Tubulointerstitial fibrosis (TIF) is the hallmark of CKD and characterized by tubular atrophy and accumulation of extracellular matrix proteins. Growth factors are important modulators of TIF, and TGF-β is a key growth factor in renal injury. There are three TGF-β mammalian isoforms (TGF-β1, -β2, and -β3), which all bind to the TGF-β type 2 serine/threonine kinase receptor (TβRII). TβRII then phosphorylates the TGF-β type 1 receptor, which leads to recruitment and phosphorylation of intracellular Smad2/3s that accumulate in the nucleus and alter DNA transcription.[1,2] Smad-independent or noncanonical signaling pathways also exist (*e.g.*, MAPK), but all of these pathways require a functionally intact TβRII.[3,4]

TGF-β signaling also affects other growth factor signaling pathways important in the renal response to injury. Previously, our group showed that deleting TβRII in proximal tubule (PT) epithelial cells impaired responsiveness to hepatocyte growth factor.[5] The Wnt/β-catenin pathway is another pathway reported to interact with TGF-β signaling. β-Catenin has dual functions acting as both part of the adherens junction complex and a transcription factor.[6,7] In the

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absence of Wnt ligands, cytosolic β-catenin is phosphorylated by the destruction complex consisting of GSK-3β, Axin, and APC, which targets the protein for degradation.[8] However, when one of 19 Wnt ligands binds to the frizzled receptor, this forms an activated receptor complex and prevents β-catenin degradation by the destruction complex. Stabilization of β-catenin allows it to accumulate in the nucleus and interact with other transcription factors, such as TCF/LEF, to affect DNA transcription.[9]

TGF-β and Wnt/β-catenin affect similar biologic processes, such as organ development, cancer biology, cell differentiation, and cell proliferation/survival. Interactions between TGF-β and Wnt/β-catenin are critical for proper renal development. Activation of β-catenin in the ureteric bud–derived epithelia increased TGF-β2, thereby causing renal dysplasia.[10] TGF-β and Wnt/β-catenin pathways synergize in mesenchymal-derived cells of various organs to promote fibroblast activation, proliferation, and ECM accumulation.[11–13] Both TGF-β and Wnt/β-catenin signaling are upregulated in injured renal epithelia, but how these key pathways interact in renal tubules to affect the response to chronic kidney injury is unknown.

TGF-β promotes TIF in preclinical studies, but these previous studies have primarily modulated signaling systemically, an approach that does not define the role of epithelial TGF-β.[14,15] TGF-β promotes epithelial dedifferentiation and apoptosis in short-term *in vitro* experiments, but the role of epithelial TGF-β signaling in chronic injury is unclear. The PT is a specialized epithelial segment that can be both a target and a mediator of chronic renal injury. To define the role of proximal tubular TGF-β signaling in chronic renal injury, we selectively deleted TβRII in this epithelial compartment. We injured the mice using two different models of CKD: aristolochic acid and angiotensin II with uninephrectomy. In both models of injury, the mice lacking TβRII in the PT had increased tubular injury and fibrosis compared with littermate controls. Deleting TβRII in the PT impaired β-catenin activity both *in vitro* and *in vivo*. To determine whether the reduced β-catenin activity was causing the impaired response to chronic injury, we genetically stabilized β-catenin specifically in the PTs lacking TβRII. Activating β-catenin significantly improved the tubular injury of TβRII conditional knockout mice after chronic injury. Thus, we show that deleting TβRII in the PT worsens the response to two models of chronic injury, and this is partly due to reduced epithelial β-catenin activity.

RESULTS

Deleting TβRII in the PTs Worsens Injury in Models of CKD

To define the role of proximal tubular TGF-β signaling in CKD, we used γGT-Cre;Tgfbr2$^{fl/fl}$ mice, in which we have previously confirmed PT-specific deletion of TβRII.[16] The uninjured γGT-Cre;Tgfbr2$^{fl/fl}$ mice had no differences in histology or BUN compared with floxed control mice (Supplemental Figure 1). We injured the mice using aristolochic acid, which targets the PT and leads to TIF in both murine models and humans.[17,18] Six weeks after aristolochic acid, all mice had cortical tubular injury with increased matrix expansion between tubules (Figure 1A). However, the tubulointerstitial injury was much more severe in the conditional knockout compared with the floxed littermates, with greater epithelial cell flattening and tubular dilation (Figure 1, A and B). Furthermore, the aristolochic acid–treated γGT-Cre;Tgfbr2$^{fl/fl}$ mice had significantly increased TIF assessed by Sirius Red staining and collagen I transcripts compared with their littermate controls (Figure 1, C–E).

Given the pronounced histologic tubular injury in γGT-Cre;Tgfbr2$^{fl/fl}$ mice, we further characterized this tubular damage. The conditional knockout mice had much higher levels of kidney injury molecule-1 (KIM-1), a marker of proximal tubular injury, in renal cortices 6 weeks after aristolochic acid treatment (Figure 1F). There was a nonsignificant trend toward increased injury markers NGAL and plasminogen activator inhibitor-1 in the injured γGT-Cre;Tgfbr2$^{fl/fl}$ mice (Supplemental Figure 2). Additionally, BUN was significantly higher in the conditional knockout mice, suggesting greater renal functional impairment (Figure 1G). The conditional knockout mice had consistently increased tubular terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining, indicating cell death, compared with the floxed mice (Figure 1, H and I). These data show that γGT-Cre;Tgfbr2$^{fl/fl}$ mice have increased chronic tubular injury, functional impairment, and susceptibility to cell death in response to aristolochic acid.

To confirm whether abrogating TGF-β signaling in the PT worsens the response in other models of chronic renal injury, we also used the uninephrectomy plus angiotensin II (UniNx/AngII) model. This model produces TIF, and TGF-β signaling plays a significant role in the pathophysiology.[19,20] BP measurements (tail cuff plethysmography) confirmed that all angiotensin II–treated mice had significantly higher BPs than saline-treated animals (166±3 versus 129±4 mmHg), with no difference between angiotensin II–treated conditional knockout and floxed control mice (169±3 versus 170±4 mmHg). One month after injury, the floxed wild-type mice had mild tubular abnormalities, but the γGT-Cre;Tgfbr2$^{fl/fl}$ mice had severe tubular injury with interstitial matrix expansion (Figure 2, A and B). The injured Tgfbr2$^{fl/fl}$ mice had minimal fibrosis in contrast to significantly increased fibrosis in the conditional knockout mice (Figure 2, C and D). Consistent with the aristolochic acid injury model, the conditional knockout had increased BUN and KIM-1 expression after UniNx/AngII compared with in the floxed controls (Figure 2, E and F). In addition, albuminuria was greater in the injured γGT-Cre;Tgfbr2$^{fl/fl}$ mice (Figure 2G). Taken together, our data show that genetically inhibiting TGF-β signaling in the PT worsens tubular injury and TIF in two distinct models of CKD.

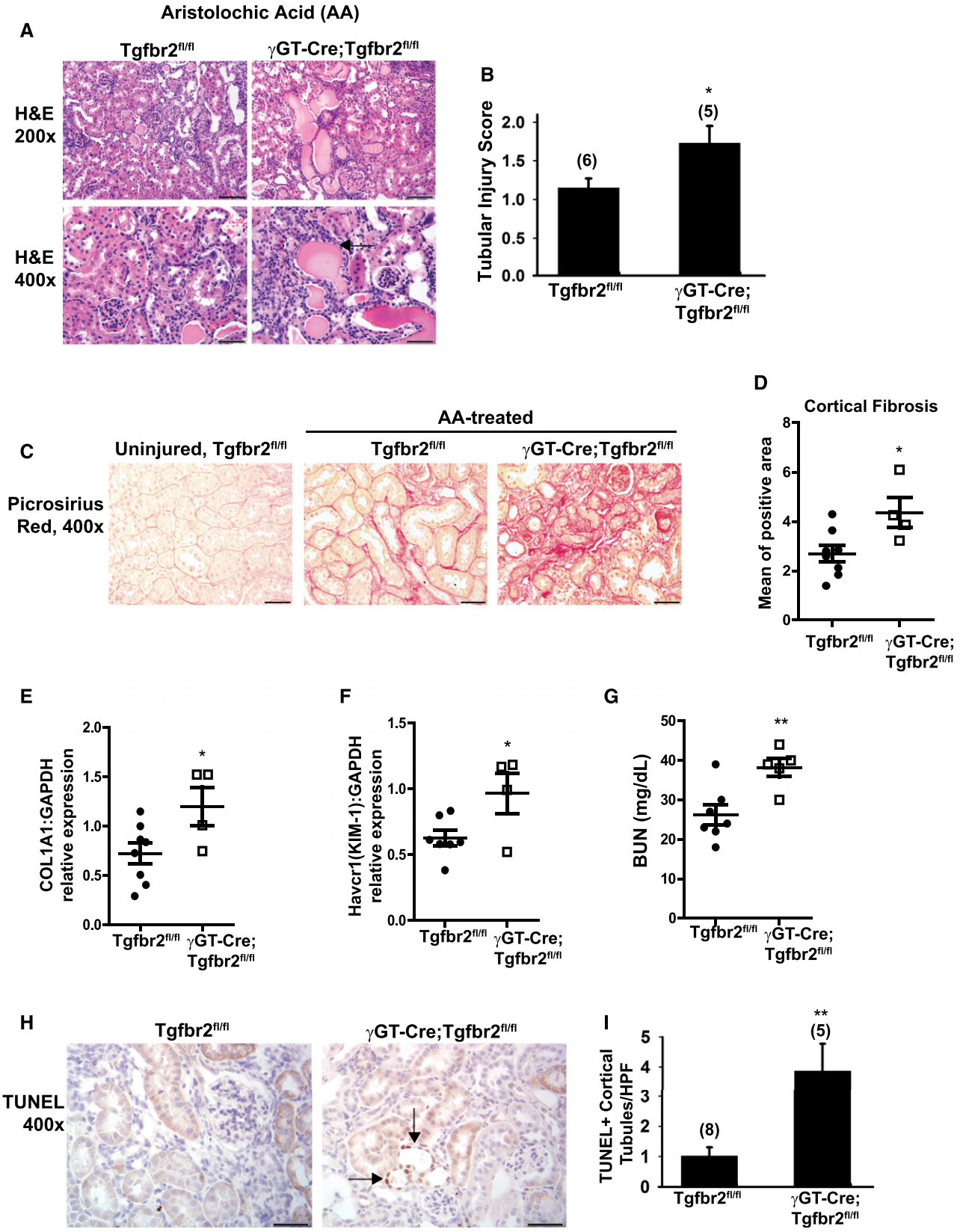

Figure 1. γGT-Cre;Tgfbr2$^{fl/fl}$ mice had increased tubular injury and TIF at 6 weeks after aristolochic acid injections. (A) Hematoxylin and eosin (H&E) staining was performed on kidneys from injured mice with tubular dilation, epithelial flattening, and cast formation (black arrow). (B) Quantification of tubular injury (Concise Methods). (C and D) To detect collagens, Picrosirius Red staining (Concise Methods)

Deleting TβRII in PTs Impairs β-Catenin Activity *In Vitro* and *In Vivo*

To explore the mechanism whereby deleting TβRII impairs tubular response to chronic injury, we performed RNAseq on renal epithelial cells with and without TβRII (Concise Methods). Wnt/β-catenin was the pathway most affected by deleting TβRII (Figure 3A, Supplemental Table 1). We validated these results by measuring expression of Axin2, an established downstream target of Wnt/β-catenin signaling and reliable reporter of activity, in cultured epithelial cells with and without TβRII.[8,21] Both PT cells (Figure 2B) and inner medullary collecting duct cells (data not shown) lacking TβRII had significantly lower levels of Axin2, reflecting reduced β-catenin activity, compared with cells with TβRII intact. To confirm this TβRII-dependent effect on β-catenin *in vivo*, we measured nuclear β-catenin (*i.e.*, active β-catenin) from injured renal cortices. Nuclear β-catenin was significantly reduced in aristolochic acid–treated γGT-Cre;Tgfbr2$^{fl/fl}$ mice compared with floxed littermates (Figure 3D), indicating that deleting TβRII impairs β-catenin activity *in vivo* and *in vitro*.

We next used TβRII$^{flox/flox}$ and TβRII$^{-/-}$ PT cells to further define how TGF-β signaling alters the Wnt/β-catenin pathway. PT cells were treated with exogenous TGF-β1 to assess for alterations in β-catenin protein expression and localization. In whole-cell lysates, TβRII$^{flox/flox}$ PT cells had slightly higher levels of β-catenin protein expression compared with TβRII$^{-/-}$ PT cells, but exogenous TGF-β1 did not significantly alter β-catenin levels (Figure 4, A and B). TGF-β1 did not alter nuclear β-catenin levels in TβRII$^{-/-}$ PT cells but did significantly increase nuclear β-catenin in TβRII$^{flox/flox}$ PT cells (Figure 4, C and D). These data indicate that TGF-β signaling promotes β-catenin activity through nuclear localization rather than protein expression.

To determine whether TGF-β signaling alters responsiveness to Wnt ligands, we stimulated TβRII$^{flox/flox}$ and TβRII$^{-/-}$ PT cells with Wnt3a and measured Axin2 by quantitative PCR (qPCR). TβRII$^{flox/flox}$ cells had much higher absolute levels of Axin2 after Wnt3a treatment, but the increase from baseline was comparable between TβRII$^{flox/flox}$ and TβRII$^{-/-}$ PT cells (Figure 4E). Similar results were found with Wnt5 and Wnt1 stimulation (Supplemental Figure 3). Other studies have shown that TGF-β signaling augments Wnt/β-catenin activity by phosphorylating GSK-3β at the inhibitory Ser-9 site.[22] To assess whether TGF-β signaling alters destruction complex activity, we used a GSK-3 inhibitor (BIO). Although BIO substantially increased β-catenin activity in TβRII$^{flox/flox}$ and TβRII$^{-/-}$ PT cells, the difference in Axin2 expression between the cells persisted, despite BIO treatment (Figure 4F). Failure of the GSK-3 inhibitor to rescue the suppressed level of β-catenin activity in TβRII$^{-/-}$ PT cells suggests that TGF-β–dependent effects on β-catenin activity are unlikely to be mediated through GSK-3β. These data indicate that TGF-β signaling augments β-catenin nuclear localization and increases β-catenin activity in a GSK-3–independent manner.

Augmenting β-Catenin Activity in PTs Lacking TβRII Improves Response to Injury

To determine how deleting TβRII in PT cells increases tubular injury and fibrosis after injury, we investigated whether TβRII$^{-/-}$ PT cells had increased TGF-β1 production or impaired redifferentiation after aristolochic acid treatment *in vitro*, but neither was observed (Figure 5A, Supplemental Figure 4). Because aristolochic acid can induce G2/M arrest in PT cells, resulting in a profibrotic epithelial phenotype, we then studied how TβRII alters the cell cycle's response to aristolochic acid.[23] Treatment with aristolochic acid substantially increased the number of G2/M-arrested TβRII$^{-/-}$ PT cells compared with TβRII$^{flox/flox}$ cells, and this was associated with a dramatic increase in CTGF production (Figure 5, B–D). We augmented β-catenin activity with BIO and found a statistically significant reduction in G2/M arrest and a nonsignificant trend toward reduction in CTGF transcripts in aristolochic acid–treated TβRII$^{-/-}$ PT cells (Figure 5, E and F). In addition, TβRII$^{-/-}$ PT cells had increased apoptosis, measured by cleaved caspase 3, after treatment with aristolochic acid compared with TβRII$^{flox/flox}$ cells, but this was significantly lowered by BIO (Figure 5, G–J). Thus, these data suggest that deleting TβRII may promote TIF and tubular atrophy in response to aristolochic acid by increasing G2/M arrest and epithelial apoptosis, effects that were ameliorated by the GSK-3 inhibitor BIO.

However, GSK-3β has targets distinct from β-catenin, and *in vitro* results do not always predict *in vivo* behavior. Therefore, we augmented β-catenin activity in our γGT-Cre;Tgfbr2$^{fl/fl}$ mice by crossing with Ctnnb1$^{(ex3)fl/fl}$ mice, in which β-catenin is conditionally stabilized.[24] Thus, the resulting γGT-Cre;Tgfbr2$^{fl/fl}$;Ctnnb1$^{(ex3)fl/fl}$ mice should have PT cells lacking TβRII but with augmented β-catenin activity. We validated that γGT-Cre;Tgfbr2$^{fl/fl}$;Ctnnb1$^{(ex3)fl/fl}$ mice had TβRII deleted by measuring TβRII expression in cortical lysates (Figure 6A) and showing that primary PTs from these mice had an impaired response to exogenous TGF-β1 (Figure 6B). Axin2 expression in renal cortices was increased 50-fold by qPCR in conditional KO mice compared with floxed controls (Figure 6C). The uninjured adult γGT-Cre;Tgfbr2$^{fl/fl}$;Ctnnb1$^{(ex3)fl/fl}$ mice had no histologic abnormalities or baseline

was quantified. (E) Collagen I production was assessed by qPCR of COL1A1 in injured renal cortices. (F) KIM-1 transcript levels were also quantified from renal cortices using qPCR. (G) Plasma BUN was measured from mice 6 weeks after injury. (H) TUNEL staining showed apoptotic nuclei (black arrows) in cortical tubules, which were (I) quantified with the number of mice in parentheses. Data are shown as means±SEM, with the number of mice in parentheses. Scale bars, 100 μM in A, upper panel; 50 μM in A, lower panel, C, and H. *$P<0.05$; **$P<0.01$. AA, aristolochic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPF, high-powered field.

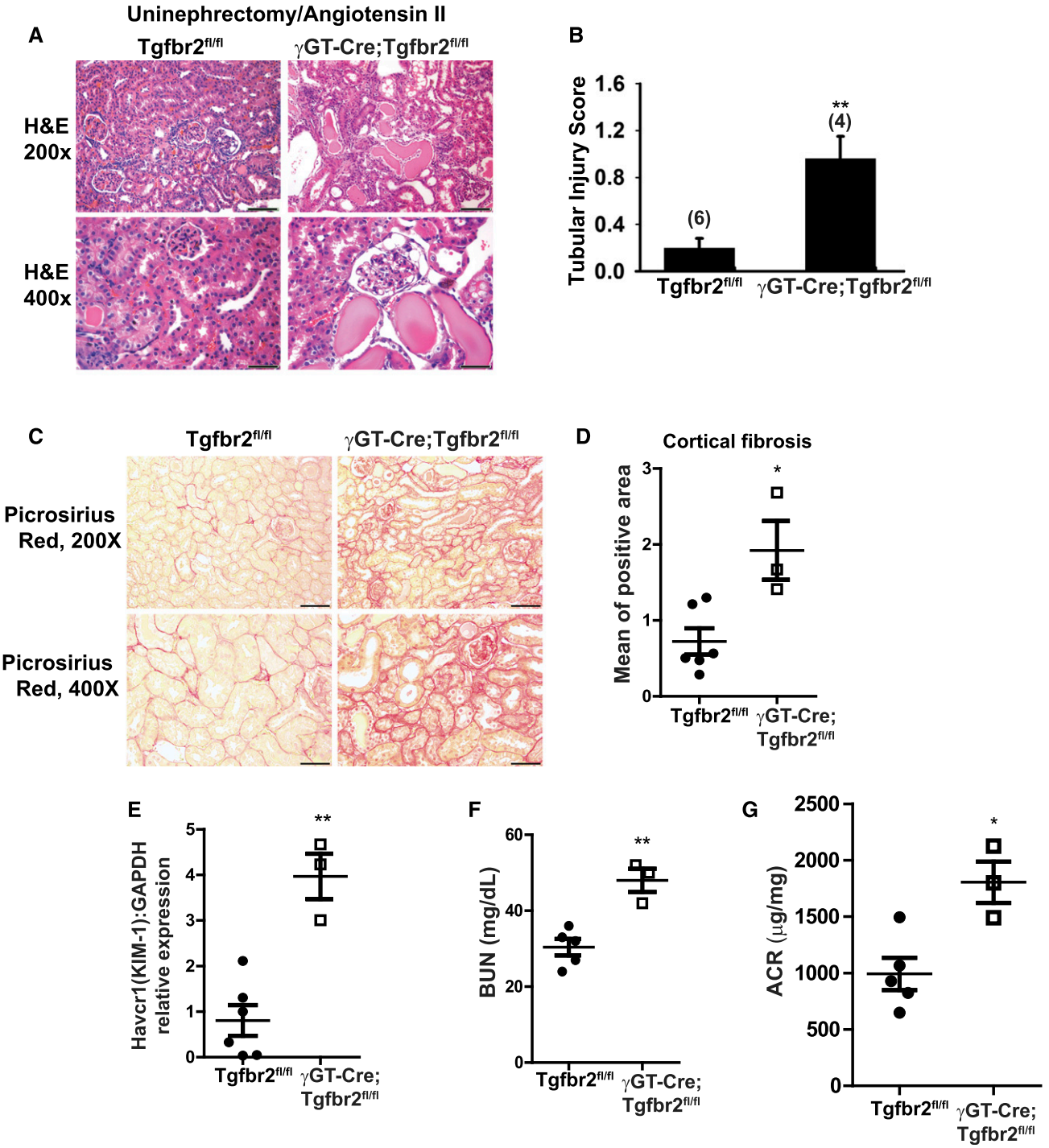

Figure 2. The conditional knockout mice also sustained greater tubular injury and TIF after injury by uninephrectomy/angiotensin II. (A) Renal cortices 4 weeks after UniNx/AngII infusions show increased tubular damage in the γGT-Cre;Tgfbr2$^{fl/fl}$ mice compared with floxed controls, which was (B) scored and quantified. (C and D) Picrosirius Red staining is shown, with quantification of the positive area. (E) KIM-1 expression was quantified in injured renal cortices using qPCR. (F) BUN was measured in plasma from mice 4 weeks after uninephrectomy and placement of angiotensin II minipumps. (G) The albumin-to-creatinine ratio (ACR) was quantified from urine during the fourth week of injury (Concise Methods). Data are shown as means±SEM, with the number of mice in parentheses. Scale bars, 100 μM in A, upper panel and C, upper panel; 50 μM in A, lower panel and C, lower panel. *$P<0.05$; **$P<0.01$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H&E, hematoxylin and eosin.

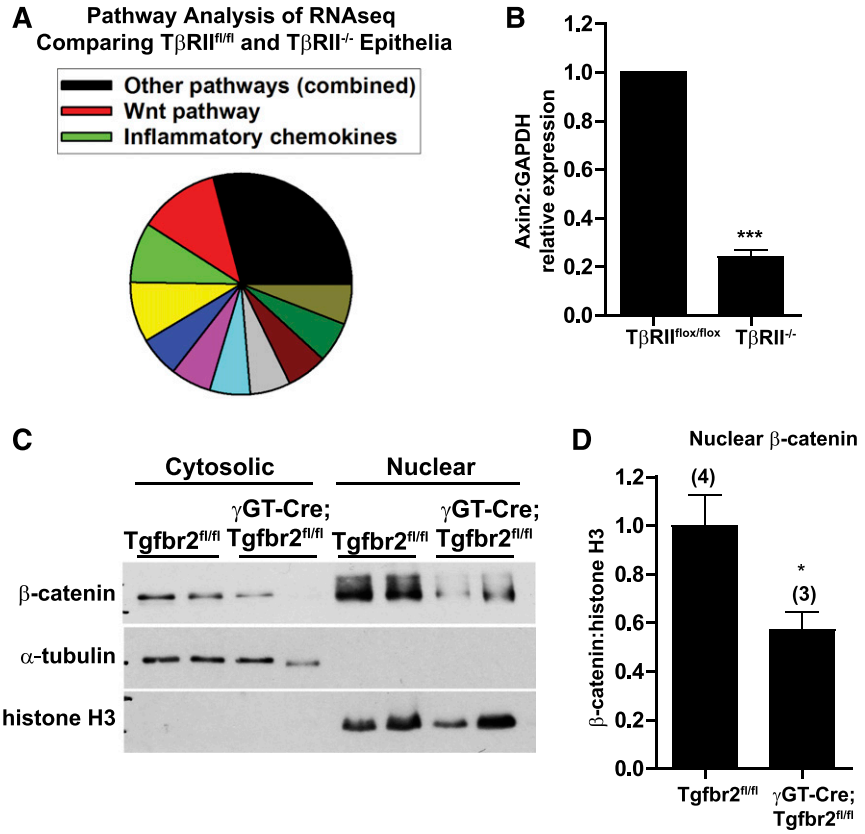

Figure 3. β-Catenin activity was reduced in TβRII$^{-/-}$ PT cells and renal cortices of γGT-Cre;Tgfbr2$^{fl/fl}$ mice compared with floxed controls. (A) RNAseq was performed on TβRII$^{fl/fl}$ and TβRII$^{-/-}$ inner medullary collecting duct cells (Concise Methods), and Wnt/β-catenin was the pathway most affected by deleting TβRII as assessed by PANTHER pathway analysis of statistically significant changes in gene expression. Supplemental Table 1 has a complete listing of pathways. (B) *Axin2*, a target gene of Wnt/β-catenin signaling, was measured in PT cells with or without TβRII grown in complete PT media using qPCR. Data are presented as means of three separate experiments ±SEM. (C) γGT-Cre;Tgfbr2$^{fl/fl}$ and floxed control mice were injured by aristolochic acid as described in Concise Methods, except that they were euthanized 3 weeks after the last injection. Nuclear and cytosolic fractions of renal cortices were isolated using ultracentrifugation (Concise Methods) and immunoblotted for β-catenin using α-tubulin and histone H3 as loading controls for the cytosolic and nuclear compartments, respectively. (D) Nuclear β-catenin from the injured renal cortices was quantified, with the number of mice in parentheses and shown as means±SEM. *$P<0.05$; ***$P<0.005$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

differences in BUN (Supplemental Figure 5). To assess whether activating β-catenin improves injury in the PTs lacking TβRII, we compared the response of γGT-Cre;Tgfbr2$^{fl/fl}$ and γGT-Cre;Tgfbr2$^{fl/fl}$;Ctnnb1$^{(ex3)fl/fl}$ mice to aristolochic acid–induced chronic injury. The injured γGT-Cre;Tgfbr2$^{fl/fl}$;Ctnnb1$^{(ex3)l/fl}$ mice had significantly less tubular damage with reduced tubular dilation, epithelial flattening, and tubular atrophy (Figure 6, D and E). TIF, measured by Sirius Red staining and collagen I expression, was also reduced in the injured γGT-Cre;Tgfbr2$^{fl/fl}$;Ctnnb1$^{(ex3)l/fl}$ mice (Figure 6, F–H). Both KIM-1 levels and BUN (Figure 6, I and J) at 6 weeks after injury were significantly reduced in the γGT-Cre;Tgfbr2$^{fl/fl}$;Ctnnb1$^{(ex3)fl/fl}$ mice compared with the aristolochic acid–injured γGT-Cre;Tgfbr2$^{fl/fl}$ mice. Thus, stabilizing β-catenin dramatically improved the response to chronic injury by PTs lacking TβRII.

DISCUSSION

TGF-β is widely considered a potent promoter of TIF during chronic kidney injury. However, our data show that deleting TβRII in the PT worsened the response to two different murine models of CKD. How do we reconcile our surprising finding with the literature? One possibility is that TGF-β–dependent effects on renal injury vary on the basis of location, because we blocked TβRII specifically in the PT in contrast to many approaches, which have systemically modulated TGF-β signaling.[14,15,25–28] However, other studies have shown that augmenting TGF-β1 adversely affects tubular epithelia by promoting cell death, autophagy, or dedifferentiation.[26,29–31] Thus, a more likely explanation is that the concentration of bioactive TGF-β is critically important for determining the response, with both too little and too much being detrimental. Others have shown that TGF-β's actions vary on the basis of the degree of stimulation or inhibition.[32,33] Excessive epithelial TGF-β signaling has deleterious effects,[29] but we show that abrogating TGF-β signaling also impairs the response to injury. Our finding is consistent with previous findings by our group and others, in which genetic blockade of TGF-β signaling, more potent than pharmacologic inhibition, impairs epithelial function or injury response.[34–37]

Our data suggest that deleting TβRII in PTs worsens the response to chronic injury due, in part, to compromised β-catenin signaling. Crosstalk between these two critical growth factor pathways has been shown in renal injury but primarily localized to the interstitial compartment, where it promotes TIF.[12] Others have shown that TGF-β and β-catenin signaling interact in the interstitial mesenchymal cells to promote fibroblast/pericyte proliferation, migration, and matrix production.[11,38,39] However, until our studies, there was not much known about how TGF-β and β-catenin signaling interact in injured epithelia to modulate the response to chronic renal injury. Wnt/β-catenin signaling can also promote many different responses depending on the microenvironment. The protective effect of stabilizing β-catenin in the PTs is unexpected, because β-catenin signaling augments epithelial

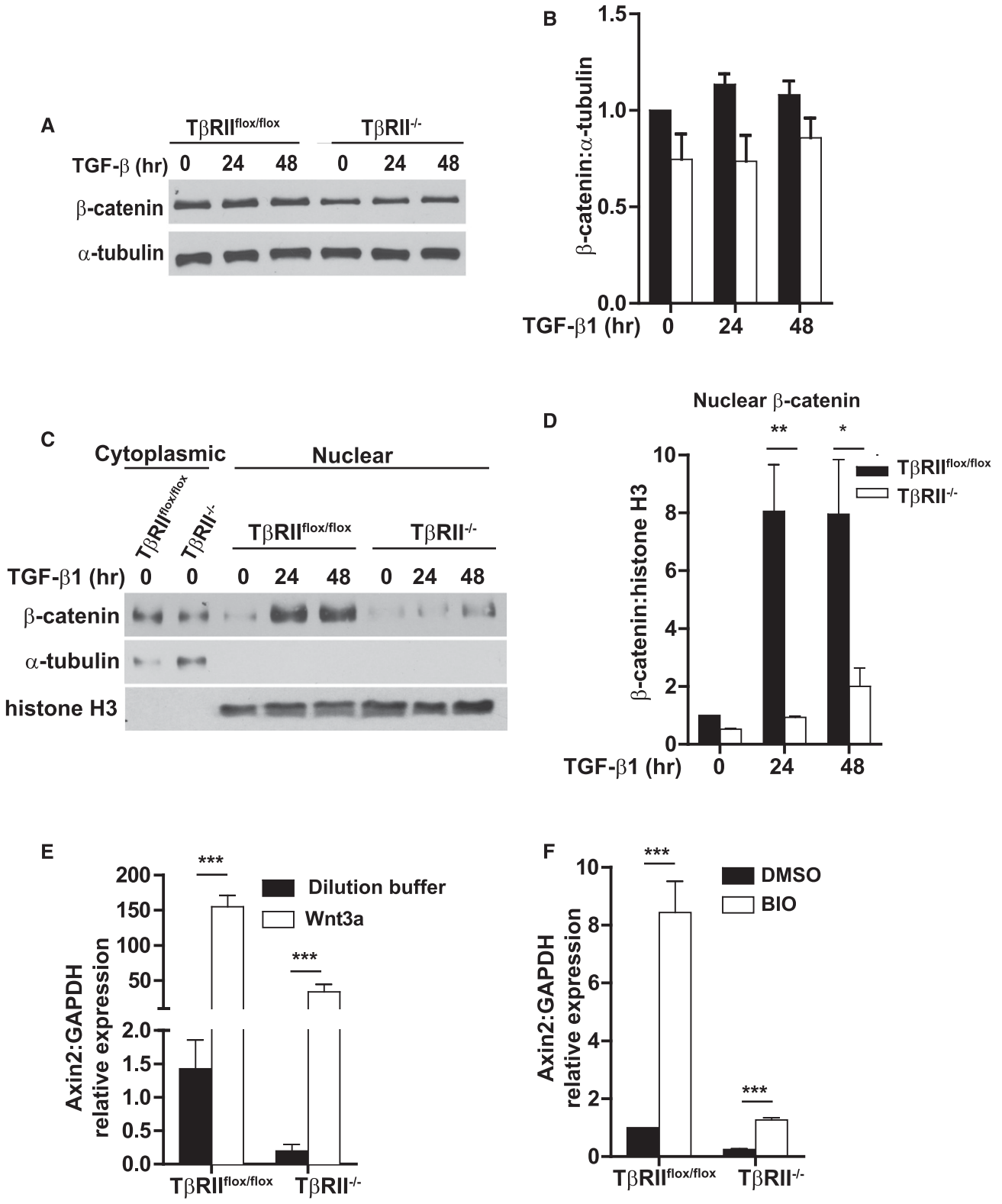

Figure 4. TGF-β signaling increases β-catenin nuclear localization and responsiveness to Wnt ligands. (A) PT cells were stimulated with TGF-β1 (2 ng/ml) for varying time points, and whole-cell lysates were immunoblotted for β-catenin and α-tubulin (loading control) and (B) quantified. (B and D) Quantification for β-catenin was normalized to that of untreated TβRII$^{flox/flox}$ PT cells. (C) PT cells were stimulated with TGF-β1 (2 ng/ml) for varying amounts of time; then, cytoplasmic and nuclear fractions were isolated and immunoblotted for β-catenin, with α-tubulin and histone H3 as loading controls for the cytoplasmic and nuclear fractions, respectively.

dedifferentiation, which is associated with TIF progression.[40,41] However, Wnt/β-catenin also mediates many protective effects in acutely injured epithelia, such as prevention of apoptosis through effects on mitochondria and survivin.[42,43]

Our *in vitro* data suggest that deleting TβRII in renal epithelia may augment fibrosis and tubular injury through increased G2/M arrest and apoptosis, respectively, and that these responses were ameliorated by increasing β-catenin activity using a GSK inhibitor. TGF-β signaling is known to induce G0/G1 arrest in epithelial cells, which may reduce the number of cells progressing to G2/M.[44–46] TGF-β1 has also been shown to induce G2/M arrest,[47] but this result was observed at a much higher dose (10 ng/ml) than used to induce the more commonly described G0/G1 arrest, thus showing divergent TGF-β–dependent effects depending on the concentration. G2/M renal epithelial arrest is associated with increased production of cytokines, such as CTGF, which may promote fibrosis through autocrine or paracrine effects.[23]

Deleting TβRII in PTs increases tubular cell death both *in vivo* and *in vitro*, which contrasts with our previous data, in which PTs lacking the receptor were protected from apoptosis in mercuric chloride–induced AKI.[16] TGF-β has well described opposing effects on apoptosis, even within the same cell type, which may be determined by the severity and chronicity of the insult.[48,49] During a strong insult, such as mercuric chloride, TGF-β signaling likely potentiates oxidative stress and promotes epithelial apoptosis. However, for a weaker and more progressive insult, such as aristolochic acid, TGF-β signaling may be advantageous. Thus, the effects of abrogating TGF-β signaling likely vary depending on the injury model (acute tubular injury versus progressive fibrosis) and microenvironment. The mechanisms whereby TGF-β signaling protects against PT apoptosis are unclear, although potential pathways include induction of autophagy and alteration of other growth factor signaling pathways.[5,50] Others have shown *in vitro* that prolonged exposure to TGF-β, such as may occur in chronic injury, protects against epithelial apoptosis.[49,51,52]

Our results differ from a study showing that deleting TβRII in renal tubules reduced TIF but enhanced inflammation after unilateral ureteral obstruction.[53] These discrepancies may be explained by differences in either the injury models, underscoring the importance of the microenvironment in TGF-β–dependent responses, or the genetic approaches to delete TβRII. Deleting TβRII in PTs alters many growth factor pathways,[5] and therefore, it is possible that our observed effects are not due to direct β-catenin–mediated actions but interactions between β-catenin and other pathways also affected by TβRII deletion. Augmenting β-catenin signaling in TβRII null PT cells was protective in our studies, but it remains to be seen if augmenting β-catenin activity in cells with TGF-β signaling intact also improves the response to injury. The genetic approach used to increase β-catenin activity likely produces supraphysiologic levels of β-catenin activity, a limitation of the Cre/lox approach.

Another unanswered question is whether other epithelial segments would benefit from stabilized β-catenin. Although deleting β-catenin in podocytes increased apoptosis in previous studies, augmenting β-catenin activity in podocytes resulted in an adhesion defect, suggesting that either too little or too much β-catenin activity in the podocyte is deleterious.[54] Because collecting duct epithelia have higher basal levels of β-catenin activity compared with PTs,[38] further increasing β-catenin activity may or may not be beneficial for the collecting system's response to injury.

There are many ways in which TGF-β signaling augments Wnt/β-catenin signaling. Studies have shown that TGF-β increases β-catenin activity by inhibiting GSK-3β activity,[22] but when we added a GSK-3 inhibitor to PT cells with or without TβRII, the difference in Axin2 expression persisted, suggesting that this was not the mechanism whereby TGF-β signaling enhanced β-catenin activity in our system. Transcriptional interactions between Smad2/3 and β-catenin or LEF/TCF may augment β-catenin signaling,[55–57] and this would be consistent with our findings. In addition, TGF-β1 was shown to phosphorylate β-catenin in alveolar epithelial cells at Y654, leading to increased β-catenin nuclear accumulation.[58] However, TGF-β can also increase Wnt/β-catenin signaling through effects on the ligands, the inhibitor dickkopff-1, and the receptor complex, and our data do not rule out these interactions.[38,59,60] TGF-β signaling clearly promotes β-catenin nuclear accumulation in our PT cells, but the exact mechanism likely involves multiple interactions and is beyond the scope of this study.

In summary, we show that deleting TβRII in the PT worsened tubular injury and TIF in two CKD models. The mechanism is partly due to impaired β-catenin signaling that results from deleting epithelial TβRII. Although TGF-β and β-catenin signaling are conventionally associated with TIF through effects on the fibroblast/pericyte compartment, our data suggest that some signaling from these pathways may improve the epithelial response to chronic injury. This is particularly relevant in light of the recently published clinical trial, in which blocking TGF-β failed to protect against progression of diabetic nephropathy.[61]

(D) Quantification of nuclear β-catenin (normalized to histone H3) is shown. (E) PT cells were incubated with Wnt3a (20 ng/ml) for 24 hours or dilution buffer, and then, levels of Axin2 transcripts were measured by qPCR. (F) PT cells were treated with either the GSK-3 inhibitor BIO (500 nM) or equivalent volumes of DMSO for 24 hours before measuring Axin2 by qPCR. Data are the means of three experiments ±SEM. *$P<0.05$; **$P<0.01$; ***$P<0.005$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

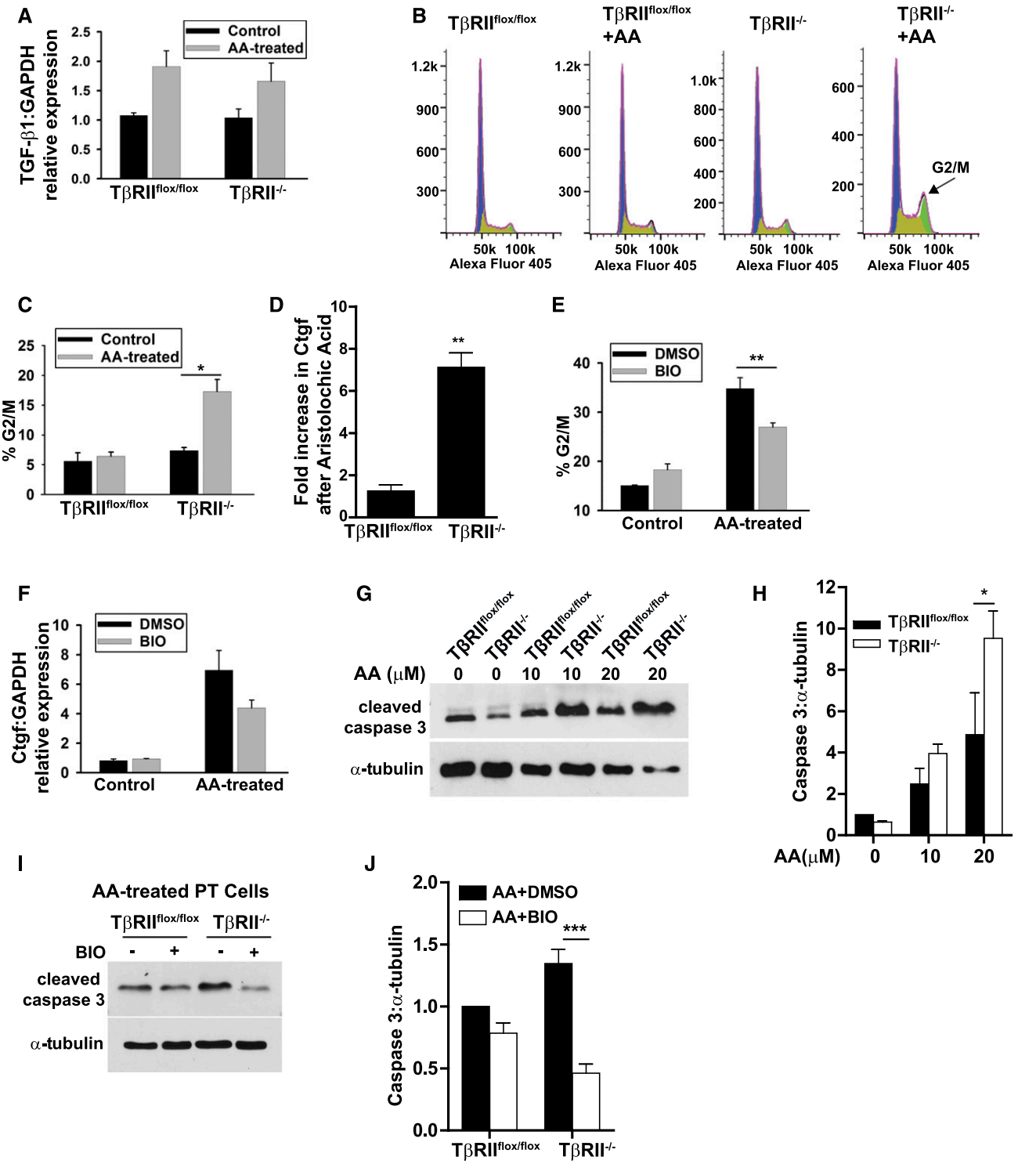

Figure 5. Increasing β-catenin activity in TβRII$^{-/-}$ PT cells reduces aristolochic acid–induced G2/M arrest and apoptosis. (A) PT cells were incubated for 7 days with either 20 μM aristolochic acid (Concise Methods) or PT complete media (control), and then, TGF-β1 mRNA expression was measured by qPCR. (B) PT cells with or without 20 μM aristolochic acid for 2 days underwent cell cycle modeling with DAPI by flow cytometry, and (C) the percentage of cells in G2/M is quantified. (D) PT cells treated with aristolochic acid (20 μM) for 7 days had CTGF mRNA measured by qPCR and expressed as a fold increase from baseline (no aristolochic acid treatment). (E) TβRII$^{-/-}$ PT cells were treated with the GSK inhibitor BIO (500 ng/ml) or equivalent amounts of DMSO with or without aristolochic acid (20 μM) (Concise Methods) for cell cycle analysis using flow cytometry, and G2/M arrest was quantified. (F) TβRII$^{-/-}$ PT cells were also treated with or without BIO and aristolochic acid for 7 days, and CTGF mRNA was quantified with qPCR. (G) PT cells were treated with varying amounts of

CONCISE METHODS

Animal Models

All procedures were approved by the Institutional Animal Care and Use Committee of Vanderbilt University and conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. To generate mice lacking TβRII in the PT, we crossed Tgfbr2$^{fl/fl}$ mice with those containing γGT-Cre as described and validated previously.[16] To conditionally stabilize β-catenin in the PT, γGT-Cre;Tgfbr2$^{fl/fl}$ mice were crossed with mice in which exon3 of β-catenin is floxed [Ctnnb1$^{(ex3)fl/fl}$] as previously described.[24] We used male mice 8–12 weeks old for all injury models.

Injury Models

For the aristolochic acid model, we injected γGT-Cre;Tgfbr2$^{fl/fl}$ mice and littermate floxed controls (N10 FVB background) with 4 mg/kg intraperitoneal aristolochic acid (Sigma-Aldrich) a total of six times over 2 weeks. The mice were euthanized 6 weeks after the last injection unless stated otherwise. The γGT-Cre;Tgfbr2$^{fl/fl}$; Ctnnb1$^{(ex3)fl/fl}$ and γGT-Cre;Tgfbr2$^{fl/fl}$ mice with which they were compared were N10 on Balb/c background, and they were injured using the same protocol, except that the dose used was 4.5 mg/kg aristolochic acid. For the UniNx/AngII model, we removed the right kidney and concurrently placed subcutaneous osmotic minipumps to deliver angiotensin II at 1.4 mg/kg per day as previously described.[19] The mice were euthanized 4 weeks later, and BP was measured using tail cuff plethysmography during the week before euthanasia.

Tissue Staining and Injury Score

Kidneys were harvested, fixed in 4% paraformaldehyde, and paraffin embedded, and sections were stained with hematoxylin and eosin, TUNEL staining as previously described,[34] or Picrosirius Red staining. Images were taken with a Nikon Eclipse E600 microscope. For quantification of TUNEL staining, 15 high-power fields ($\times$400) were taken per sample, and the number of cortical tubules with TUNEL$^+$ nuclei was counted while unaware of animal genotype. For Picrosirius Red, ten high-power fields of kidney cortex were taken per sample, and quantification of the positive area was done using ImageJ after subtracting out glomeruli and vessels. A pathologist unaware of the genotype scored renal injury from all nonoverlapping fields in the cortex ($\times$200 magnification) using the following system for tubular injury: 0= no injury, 1=1%–20% of area, 2=21%–50%, 3=51%–75%, and 4$\geq$75%. Tubular injury in the aristolochic acid model was defined as tubular sloughing, cast formation, dilation, degeneration, atrophy, or tubulitis. Tubular injury in the UniNx/AngII model was defined as protein cast formation, tubular dilation, cell swelling, vacuolization, or degeneration.

BUN Measurement and Proteinuria

At the time of euthanasia, whole blood was collected and placed in heparinized tubes, and plasma was used with the Thermo Infinity Urea Reagent to determine BUN levels. Proteinuria was quantified using the Exocell Albuwell M Test Kit and Creatinine Companion.

Subcellular Fractionation Using Renal Cortices

We isolated nuclear and cytosolic proteins from kidneys 3 weeks after the last aristolochic acid injection, such as in the previously published protocol.[62] Briefly, renal cortices were grossly dissected and minced using cold PBS with protease inhibitors (Sigma-Aldrich), resuspended in ice-cold buffer, homogenized, and centrifuged to yield supernatants, the cytosolic fractions, and the pellets (nuclear fractions). The pellets were solubilized as previously described, filtered, and ultracentrifuged. The nuclear pellets were resuspended in NE buffer and centrifuged; the supernatants were saved as soluble nuclear proteins, whereas the pellets, composed of DNA tightly bound proteins, were resuspended in RIPA buffer. The whole cytosolic fractions were centrifuged to pellet mitochondria, and the supernatants were ultracentrifuged to yield supernatants and pure cytosolic fractions.

For membrane preparations, renal cortices were minced in cold PBS and centrifuged, and the pellets were resuspended in a buffer containing 250 mM sucrose, 1 mM EDTA, 50 mM Tris·HCl, 5 mM $MgCl_2$, and protease inhibitors and mechanically homogenized followed by sonication. The homogenates were centrifuged for 10 minutes at 500$\times g$ at 4°C, and the supernatants were centrifuged for 1 hour at 75,000$\times g$ at 4°C to remove soluble proteins. Membranes were resuspended in RIPA buffer containing 1% NP40, and after centrifugation, the supernatants containing membrane protein fractions were saved.

Cell Populations

PT cells were generated from the Immorto-mouse crossed with the Tgfbr2$^{fl/fl}$ mice as previously described and characterized.[16] PT cells were grown at 33°C in DMEM/F12 supplemented with 2.5% FBS, hydrocortisone, insulin, transferrin, selenium, triiodothyronine, and penicillin/streptomycin (complete PT media) with IFN-γ.[16] Before experiments, PT cells were moved to 37°C, and IFN-γ was removed to induce differentiation. Deletion of TβRII in PT was achieved by adeno-Cre treatment *in vitro* and verified by immunoblots.[16]

For primary cultures, uninjured renal cortices were dissected, minced, and incubated in a shaker for 45 minutes in complete PT media with collagenase I (1 mg/ml), dispase (1 mg/ml), and DNAse at

aristolochic acid (0, 10, and 20 μM) for 7 days, and apoptosis was detected in cell lysates by immunoblotting for cleaved caspase 3, with α-tubulin as a loading control. (H) Expression of cleaved caspase 3 was quantified and normalized to that of uninjured TβRII$^{flox/flox}$ PT cells. (I) PT cells were treated with aristolochic acid plus either BIO (500 ng/ml) or DMSO for 7 days. (I and J) Cell lysates were immunoblotted for cleaved caspase 3 and α-tubulin and quantified. Data are the means of three experiments $\pm$SEM. *P<0.05; **P<0.01; ***P<0.005. AA, aristolochic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CTGF, connective tissue growth factor; DAPI, 4′,6-diamidino-2-phenylindole.

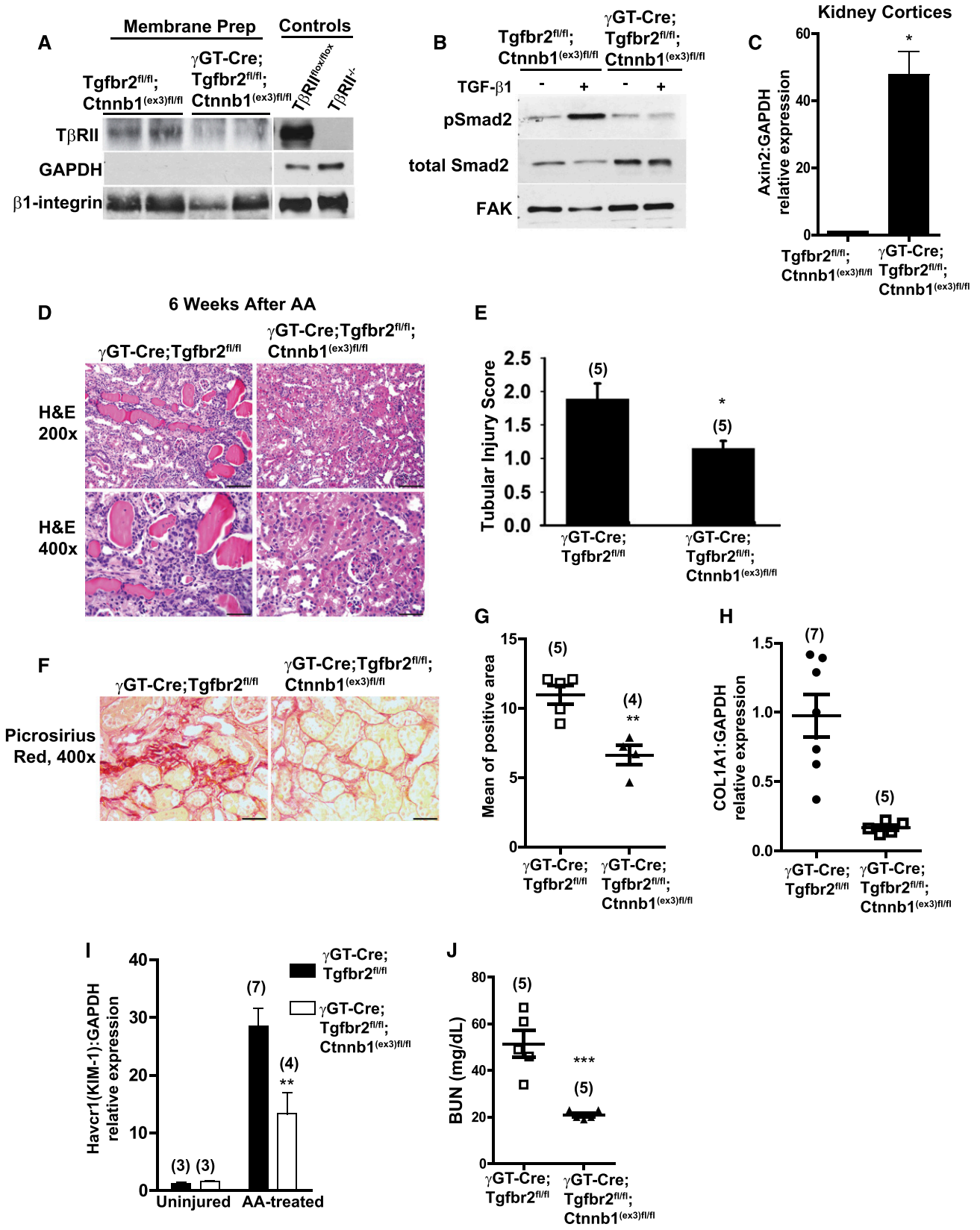

Figure 6. γGT-Cre;Tgfbr2$^{fl/fl}$;Ctnnb1$^{(ex3)fl/fl}$ mice have reduced tubular injury and preserved renal function compared with γGT-Cre; Tgfbr2$^{fl/fl}$ mice after aristolochic acid–induced chronic injury. (A) Membrane preparations of uninjured renal cortices were immunoblotted with TβRII to verify recombination in γGT-Cre;Tgfbr2$^{fl/fl}$;Ctnnb1$^{(ex3)fl/fl}$ mice, with β1-integrin as a loading control for membrane

37°C. The tissue was passed through a 70-μM filter to remove glomeruli, washed two times with complete PT media, plated, and used for experiments at passage 2.

Cell Experiments and Reagents

PT cells were plated at 30% confluency in complete PT media containing 20 μM aristolochic acid (unless otherwise specified) for 7 days. For some experiments, the GSK-3 inhibitor BIO (Sigma-Aldrich) or an equal concentration of DMSO (diluent for BIO) was added while being treated with aristolochic acid.

PT cells were serum starved for 24 hours before stimulation with TGF-β1 (R&D System), Wnt3a (Time Biosciences), Wnt5a (R&D Systems), or Wnt1 (Abcam) for an additional 24 hours. Nuclear and cytoplasmic cell fractionation was performed as previously described.[63] Briefly, PT cells were lysed with a hypotonic lysis buffer and centrifuged for 2 minutes at 16,000×*g* to separate the cytoplasmic (supernatant) from the nuclear compartment (pellet). The pellet was then resuspended in a hypertonic buffer and centrifuged for 30 seconds at 3000×*g* twice to extract pure nuclei (pellets), which were resuspended in RIPA buffer.

PCR

For RNA extraction of PT cells, direct lysis with RLT buffer (Qiagen) was performed; renal cortices were mechanically disrupted in Lysis Matrix Tubes (MP Biomedicals) containing RLT lysis buffer supplemented with 1% β-mercaptoethanol before clarification using RNeasy spin columns. RNA from both tissue and cells was extracted using the Qiagen RNeasy Kit, and Bio-Rad's iScript cDNA Synthesis Kit generated cDNA. Real-time qPCR was performed with 100 ng cDNA and SYBER Green Supermix using the BioRad CFX96 Thermal Cycler. Relative mRNA expressions were determined by $\Delta\Delta$CT equation, and after validation with a panel of housekeeping genes, Gapdh was used as a reference gene. Primer sequences are as follows (forward and reverse): Gapdh: 5′-AGGTCGGTGTGAACGGATTTG-3′ and 5′-TGTAGACCATGTAGTTGAGGTCA-3′; Havcr1 (KIM-1): 5′-AAACCAGAGATTCCCACACG-3′ and 5′-GTCGTGGGTCTTCCTGTAGC-3′; Col1a1: 5′-GGGTCTAGACATGTTCAGCTTTGTG-3′ and 5′-ACCCTTAGGCCATTGTGTATGC-3′; Axin2: 5′-TGAGCTGGTTGTCACCTACT-3′ and 5′-CACTGTCTCGTCGTCCCA-3′; Lcn2 (NGAL): 5′-CACCACGGACTACAACCAGTTCGC-3′ and 5′-TCAGTTGTCAATGCATTGGTCGGTG-3′; Ctgf: 5′-TTCTGCGATTTCGGCTCC-3′ and 5′-ACCATCTTTGGCAGTGCACA-3′; Serpine1 (plasminogen activator inhibitor-1): 5′-CCGATGGGCTCGAGTATG-3′ and 5′-TTGTCTGATGAGTTCAGCATC-3′; and Tgfb1: 5′-GCCACTGCCCATCGTCTACT-3′ and 5′-CACTTGCAGGAGCGCACAAT-3′.

RNAseq

Inner medullary collecting duct cells with and without TβRII as previously described and characterized[34] were used for RNA isolation with the Qiagen RNeasy Kit including a DNAse step. The Vanderbilt VANTAGE Core performed quality control on the RNA as well as RNAseq, with 30 million reads per sample. Benjamini–Hochberg correction for multiple testing was performed to determine significance. PANTHER was then used for pathway analysis of genes that were altered in a statistically significant manner.

Assessment of Cell Cycle

PT cells were serum starved for 24 hours to synchronize cell cycle and then treated with aristolochic acid (20 μM) for 48 hours, and BIO (500 nM) or equivalent amounts of DMSO were added for the last 24 hours in some experiments. The cells were trypsinized and fixed in ice-cold 70% ethanol for ≥2 hours. After washing with PBS, the cells were resuspended in PBS/0.5%FBS containing 1 μg/ml DAPI per 1×10^6 cells. Samples were analyzed using a BD LSRFortessa Analyzer (BD Biosciences) and FlowJo analysis software at the Vanderbilt University Medical Center Flow Cytometry Shared Resource.

Immunoblots and Reagents

Cells were lysed using Mammalian Lysis Buffer (Sigma-Aldrich) plus protease and phosphatase inhibitors (Sigma-Aldrich), sheared using an insulin syringe, clarified by centrifugation, and quantified using the BCA protein assay. Tissue lysates were generated as described above in Subcellular Fractionation Using Renal Cortices. Both cell and tissue proteins were separated by SDS-PAGE and incubated with the following primary antibodies: β-catenin, cleaved caspase 3, p-Smad2, total Smad2, α-tubulin, GAPDH, and histone H3 (Cell Signaling); TGF-β type 2 receptor and focal adhesion kinase (Santa Cruz); and E.cadherin and β1-integrin (BD Biosciences). Bands on autoradiography film were quantified using ImageJ software.

Statistical Analyses

We used the paired *t* test with unequal variance to compare two sets of data, with $P<0.05$ considered statistically significant. All experiments subject to analysis were performed at least three times.

fractions. TβRII$^{flox/flox}$ and TβRII$^{-/-}$ PT whole-cell lysates were used as controls, and a white line separates lanes that were moved within the same immunoblot. (B) Primary PTs (Concise Methods) were made from γGT-Cre;Tgfbr2$^{fl/fl}$;Ctnnb1$^{(ex3)fl/fl}$ mice and floxed controls and stimulated with TGF-β1 (2 ng/ml) for 20 minutes, and cell lysates were immunoblotted for pSmad2 to indicate lack of responsiveness to TGF-β in γGT-Cre;Tgfbr2$^{fl/fl}$;Ctnnb1$^{(ex3)fl/fl}$ mice. (C) To assess recombination at the Ctnnb1 locus, RNA was isolated from uninjured renal cortices, and Axin2 was measured by qPCR, with the means of three animals per genotype shown ±SEM. (D) Hematoxylin and eosin (H&E) sections from γGT-Cre;Tgfbr2$^{fl/fl}$;Ctnnb1$^{(ex3)fl/fl}$ and γGT-Cre;Tgfbr2$^{fl/fl}$ mice 6 weeks after aristolochic acid injections are shown. (E) Tubular injury after aristolochic acid was quantified and expressed as means±SEM with five mice per genotype. (F and G) Picrosirius Red staining is shown with quantification of the positive area. (H) Collagen I mRNA was measured by qPCR from renal cortices. (I) To detect KIM-1 in injured kidneys, qPCR was performed for Havcr1 (*KIM-1* gene) in renal cortices and shown as means±SEM, with number of mice in parentheses. (J) Plasma BUN is also shown as mean±SEM, with number of mice in parentheses. Scale bars, 100 μM in D, upper panel; 50 μM in D, lower panel and F. *$P<0.05$; **$P<0.01$; ***$P<0.005$. AA, aristolochic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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DISCLOSURES

E.L. is a founder of a biotechnology company, StemSynergy Therapeutics, that seeks to develop drugs against the Wnt pathway. None of the other authors have potential conflicts of interest.

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