Deleting the TGF-β Receptor Attenuates Acute Proximal Tubule Injury

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ABSTRACT

TGF-β is a profibrotic growth factor in CKD, but its role in modulating the kidney’s response to AKI is not well understood. The proximal tubule epithelial cell, which is the main cellular target of AKI, expresses high levels of both TGF-β and its receptors. To determine how TGF-β signaling in this tubular segment affects the response to AKI, we selectively deleted the TGF-β type II receptor in the proximal tubules of mice. This deletion attenuated renal impairment and reduced tubular apoptosis in mercuric chloride–induced injury. In vitro, deficiency of the TGF-β type II receptor protected proximal tubule epithelial cells from hydrogen peroxide–induced apoptosis, which was mediated in part by Smad-dependent signaling. Taken together, these results suggest that TGF-β signaling in the proximal tubule has a detrimental effect on the response to AKI as a result of its proapoptotic effects.


AKI, which is most commonly caused by ischemia and nephrotoxins, dramatically increases morbidity and mortality in hospitalized patients. The proximal tubule epithelial cell is a key target of AKI because of its high metabolic demands and unique vascular supply. After injury, proximal tubules undergo a loss of polarity, redistribution of integrins and junctional proteins, impaired adhesion, and, if severe, apoptosis or necrosis. Surviving epithelial cells de-differentiate, migrate over the denuded basement membrane, proliferate, and re-differentiate as part of the repair process. Growth factors modulate the proximal tubule’s response to AKI and facilitate the repair process.1–4 TGF-β is a growth factor that affects many cellular processes involved in injury and repair, but its role in AKI remains unclear.

Three mammalian TGF-β ligands (TGF-β1, -β2, and -β3) bind to the TGF-β type II receptor (TβRⅡ), a serine/threonine kinase, leading to phosphorylation of the TGF-β type I receptor and recruitment of intracellular signaling proteins Smad2 and 3.5,6 In canonical TGF-β signaling, Smads2/3 complex with Smad4, accumulate in the nucleus, and modulate DNA transcription. However, many noncanonical signaling proteins, such as the mitogen-activated protein kinases (MAPK), can mediate TGF-β signaling by Smad-dependent or -independent mechanisms.7,8 The complexity of TGF-β signaling may account for its pleiotropic effects, which vary depending on the target cell type and microenvironment.

TGF-β signaling has been shown to alter numerous cellular processes in vitro that may be beneficial or detrimental to the tubular response to AKI. TGF-β stimulates epithelial de-differentiation; thus, it may facilitate proximal tubule repair by accelerating de-differentiation of surviving epithelial cells, an important initial step in repair.9,10 TGF-β may also promote repair by increasing epithelial cell migration11 and upregulating integrin β1 expression, which increases...
cell/matrix adhesion. However, TGF-β can also increase proximal tubule apoptosis, which might play an important pathologic role in ischemic, septic, and toxin-induced forms of AKI. Furthermore, TGF-β may impair tubular recovery as it inhibits proximal tubule proliferation and retards re-differentiation in vitro.

Similarly, in vivo studies have had conflicting results regarding TGF-β’s role in AKI. TGF-β was shown to be protective in AKI because TGF-β1–deficient mice had worsened tubular injury after ischemia/reperfusion and volatile anesthetics improved the response to ischemia/reperfusion by a TGF-β–dependent mechanism. In contrast to these studies, the use of a neutralizing pan–TGF-β antibody did not significantly alter the acute response to ischemia/reperfusion, and TGF-β signaling was shown to have detrimental effects on the response to AKI because a TGF-β type I receptor inhibitor (ALK5) accelerated recovery and Smad3−/− mice had greater preservation of renal function and less histologic injury after ischemia/reperfusion injury.16,21 With such varied conclusions, TGF-β’s role in AKI remains unclear. Furthermore, these in vivo studies used chemical inhibitors or genetic techniques to systemically inhibit TGF-β activity, an approach that does not elucidate the role of TGF-β signaling specifically in the proximal tubule.

To define how TGF-β signaling in the proximal tubule affects the response to AKI, we deleted TβRII in the proximal tubule of mice using γ-glutamyl transferase-Cre (γGT-Cre; Tgfbr2fl/fox) and induced injury using mercuric chloride (HgCl₂). The γGT-Cre; Tgfbr2fl/fox mice had attenuated injury that was associated with reduced tubular apoptosis compared with wild-type littermates. Furthermore, proximal tubule epithelial cells lacking TβRII in vitro were resistant to apoptosis from oxidative and nonoxidative stressors. Thus, our data suggest that attenuating TGF-β signaling in proximal tubules protects against AKI by decreasing tubular apoptosis.

RESULTS

HgCl₂ Increased TGF-β Signaling in Renal Cortices

We initially defined whether TGF-β signaling was increased in the HgCl₂ model of AKI, which is a well established injury model that targets the proximal tubule by augmenting oxidative stress, a common mechanism of injury in clinical AKI. Wild-type mice had a significant increase in TβRII expression in renal cortices 3 days after HgCl₂ administration (Figure 1A); at 7 days, expression declined to levels of untreated mice (data not shown). Consistent with this, Smad2 phosphorylation was increased in renal cortical lysates 1 day after HgCl₂ administration (Figure 1, B and C). These data suggest that TGF-β plays a role in mediating the renal response to injury.

Deleting TβRII in Proximal Tubules Attenuated HgCl₂-Induced Injury

To determine how TGF-β signaling in the proximal tubule affects this AKI model, we generated mice lacking TβRII specifically in the proximal tubule by crossing the Tgfb2fl/fox mouse with one containing γGT-Cre (expressed in the proximal tubules at P10). We defined the location of Cre activity by intercrossing the γGT-Cre; Tgfbr2fl/fox and mT/mG reporter mice, which have ubiquitous expression of the membrane-bound fluorescent red tdTomato (mT) that is turned off and replaced by enhanced GFP (mG) in cells where Cre is active. The mT/mG γGT-Cre; Tgfbr2fl/fox mice had green fluorescence restricted to cortical tubules, consistent with the distribution of proximal tubules (Figure 2, A–C). We confirmed reduced TβRII expression in conditional knockout mice by immunoblots on renal cortices (Figure 2D).

When renal injury was induced by HgCl₂ (30 μmol/kg), the γGT-Cre; Tgfbr2fl/fox mice had less functional impairment as measured by BUN and creatinine compared with Tgfbr2fl/fox littermates. Although both genotypes reached their peak BUN level 3 days after injury, levels were significantly lower in the γGT-Cre; Tgfbr2fl/fox mice than the Tgfbr2fl/fox mice (BUN, 60 versus 86 mg/dl; P = 0.025) (Figure 3A). The BUN levels declined in both genotypes between days 4 and 7, and similar levels were reached at day 7. A significant difference in plasma creatinine levels (measured by HPLC) was also observed 3 days after injury (Figure 3B).

Consistent with our functional data, γGT-Cre; Tgfbr2fl/fox mice sustained less morphologic injury after treatment with HgCl₂. Epithelial injury was evident 1 day after injury (Figure 3, C and G), but the difference in response to HgCl₂ between γGT-Cre; Tgfbr2fl/fox and Tgfbr2fl/fox mice was most pronounced 3 days after injury (Figure 3, D and H). The γGT-Cre; Tgfbr2fl/fox mice had less epithelial flattening, tubular necrosis, and cast formation (Figure 3, E and I). At 7 days after HgCl₂, both groups of mice had some resolution of epithelial injury, but γGT-Cre; Tgfbr2fl/fox mice had fewer residual tubular casts (Figure 3, F and J). Injury scoring at day 3 (see Concise Methods section) showed that the γGT-Cre; Tgfbr2fl/fox mice had significantly less necrosis 1.28 ± 0.29 SE versus 2.33 ± 0.33 SE, P = 0.037; cast formation 1 ± 0.31 SEM versus 2.5 ± 0.22 SEM, P < 0.01; and tubular injury 1 ± 0.31 SEM versus 2.5 ± 0.22 SEM, P < 0.01 than the Tgfb2fl/fox mice (Figure 3K).

Tubular Apoptosis is Decreased in γGT-Cre; Tgfbr2fl/fox Mice

To investigate why the γGT-Cre; Tgfbr2fl/fox mice had attenuated injury after HgCl₂, tubular apoptosis, which TGF-β signaling promotes in vitro, was examined. We found significantly fewer terminal deoxynucleotidyl transferase–mediated digoxigenin–deoxyuridine nick-end labeling (TUNEL)–positive tubular cells in the cortex of γGT-Cre; Tgfb2fl/fox mice at 18 hours after HgCl₂ (Figure 4, A–C), and no TUNEL–positive cells in uninjured mice (data not shown). Cortical tissue immunoblots also showed significantly decreased cleaved caspase 3 expression in the γGT-Cre; Tgfbr2fl/fox compared with Tgfbr2fl/fox mice (Figure 4, D and E). These findings suggest that deleting TβRII in proximal tubules protects against apoptosis after HgCl₂.
Because TGF-β signaling can inhibit proximal tubule cell proliferation, we assessed whether the attenuated injury in γGT-Cre;Tgfbr2\textsuperscript{fl/fox} mice was due to enhanced epithelial cell proliferation. Quantification of Ki-67-positive tubular cells at days 0, 3, 5, and 7 after injury revealed that γGT-Cre;Tgfbr2\textsuperscript{fl/fox} mice had less, not more, proliferation (Figure 4, F–H). Therefore, the improved response of γGT-Cre;Tgfbr2\textsuperscript{fl/fox} mice to HgCl\textsubscript{2}-induced injury is not due to augmented tubular proliferation.

Because inflammation, which can be modulated by TGF-β signaling, plays an important pathophysiologic role in AKI, we assessed whether the improved response to HgCl\textsubscript{2} by γGT-Cre;Tgfbr2\textsuperscript{fl/fox} mice was due to alterations in inflammatory cell infiltration. No differences were found in macrophage or T cell infiltration between the genotypes, as indicated by immunoblots of F4/80 and CD3, respectively (Figure 4, I and J). Quantification of F4/80+ cells at 0, 3, and 7 days revealed a dramatic increase in macrophage infiltration but no difference between genotypes at 3 days after injury (39±7 versus 36±3 cells/high-powered field [hpf]) and a trend toward increased macrophages in the Tgfbr2\textsuperscript{fl/fox} mice at 7 days (75±10 versus 56±5 cells/hpf) (Figure 4, K, L, and O). The number of CD3+ cells was not significantly elevated at 3 days in Tgfbr2\textsuperscript{fl/fox} mice compared with baseline (2.7±0.3/hpf versus 2.4±0.45/hpf), and no differences were noted between genotypes (2.7±0.3 versus 3.6±0.3) (Figure 4, M and N), quantification of data not shown. These results suggest that the reduction in HgCl\textsubscript{2}-induced injury observed in γGT-Cre;Tgfbr2\textsuperscript{fl/fox} mice was not due to changes in tubular proliferation or inflammation but was, in part, due to reduced tubular apoptosis.

**γGT-Cre;Tgfbr2\textsuperscript{fl/fox} Mice Have Decreased Baseline Smad Activation**

To define how deleting TβRII in the proximal tubule affects TGF-β activity, Smad2/3 phosphorylation in the renal cortices of wild-type and the γGT-Cre;Tgfbr2\textsuperscript{fl/fox} mice was assessed (Figure 5, A–C). Cortical Smad2/3 activation was suppressed in the γGT-Cre;Tgfbr2\textsuperscript{fl/fox} uninjured kidneys (trend was nonsignificant for pSmad2 but significantly for pSmad3 at \( P, 0.05 \)), but differences in Smad activation between the genotypes (2.7±0.3 versus 3.6±0.3) (Figure 4, M and N), quantification of data not shown). These results suggest that the reduction in HgCl\textsubscript{2}-induced injury observed in γGT-Cre;Tgfbr2\textsuperscript{fl/fox} mice was not due to changes in tubular proliferation or inflammation but was, in part, due to reduced tubular apoptosis.

**Figure 1.** HgCl\textsubscript{2} stimulated TGF-β signaling in renal cortices. (A) Renal cortices were dissected from wild-type FVB mice that were injected with HgCl\textsubscript{2} 3 days earlier or were untreated. The tissue lysates were immunoblotted for TβRII expression. (B) Tissue lysates of renal cortices isolated 1 day after HgCl\textsubscript{2} were immunoblotted for pSmad2 and total Smad2. (C) Bands from six mice (three treated, three untreated) were quantified by densitometry and reported as means ± SEMs. *\( P<0.05 \). GADPH, glyceraldehyde 3-phosphate dehydrogenase.

**Figure 2.** Deletion of TβRII in the γGT-Cre;Tgfbr2\textsuperscript{fl/fox} mice. The mT/mG reporter mouse was crossed with our γGT-Cre;Tgfbr2\textsuperscript{fl/fox} mice, demonstrating membrane-bound red fluorescence (mT) in all cells except those where Cre is active and green fluorescence (mG) replaces mT (A and B). (C) The red (580–610 nm) and green (510–540 nm) wavelengths are merged, showing that Cre is primarily expressed in tubules in the cortex and corticomedullary renal tissue. (D) Cortical lysates of adult Tgfbr2\textsuperscript{fl/fox} and γGT-Cre;Tgfbr2\textsuperscript{fl/fox} mice were immunoblotted for TβRII expression with focal adhesion kinase (FAK) used as loading control.
two genotypes was lost after injury (Figure 5, D–F). Nuclear accumulation of pSmad2/3, a pattern consistent with activation, was predominantly localized to cortical tubules in both uninjured and injured kidneys (Figure 5I). Thus, deleting TβRII decreases basal Smad signaling, but no significant differences in Smad signaling were noted between the genotypes after HgCl₂-induced injury.

**TβRII⁻/⁻ Proximal Tubule Cells Were Protected against Apoptosis In Vitro**

To better understand how TGF-β signaling in proximal tubule cells affects apoptosis, we generated proximal tubule epithelial cells (PTCs) in vitro from Tgfrb2fl/fl mice (see Concise Methods section). Consistent with proximal tubule cells, TβRIIfl/fl PTCs expressed more ZO-1, E.cadherin, and claudin 2, a tight junction protein localized to the proximal tubule and thin descending loop of Henle, but less α-smooth muscle actin compared with interstitial cells (Figure 6A). The PTCs expressed more γGT, an enzyme produced by proximal tubules, than did collecting duct epithelial cells (Figure 6B). TβRII⁻/⁻ PTCs were produced by infecting the TβRIIfl/fl PTC with adenovirus-Cre, and deletion of TβRII was confirmed by immunoblots (Figure 6C). TβRII⁻/⁻ PTCs were unable to respond to TGF-β, as evidenced by a lack of Smad2 phosphorylation, after adding exogenous TGF-β1 (Figure 6D).

These PTCs were used to assess whether the protective effect of inhibiting TGF-β signaling on apoptosis in vivo could be recapitulated in vitro. Because HgCl₂-induced nephrotoxicity is mediated partially by oxidative stress⁵ and H₂O₂ is an important reactive oxygen species implicated in toxin-mediated and ischemic AKI, we examined the response of TβRIIfl/fl and TβRII⁻/⁻ PTCs to H₂O₂. Consistent with our in vivo results, TβRII⁻/⁻ PTCs had less cleaved caspase 3 than TβRIIfl/fl PTCs after incubation with varying doses of H₂O₂ for 12 hours (Figure 7A). Also, adding exogenous TGF-β1 was sufficient to induce apoptosis in TβRIIfl/fl PTCs and greatly potentiated its apoptotic response to H₂O₂, but apoptosis of TβRII⁻/⁻ PTCs was unaffected by TGF-β1 (Figure 7B). TβRII⁻/⁻ PTCs were also protected against anoikis, apoptosis due to disruption in cell/matrix interactions, induced by plating on PolyHEMA-coated plates (Sigma) (Figure 7C). The reduction in TβRII⁻/⁻ PTC apoptosis in response to

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**Figure 3.** γGT-Cre;Tgfrb2fl/fl mice were protected from HgCl₂-induced injury. (A) Plasma BUN levels (mg/dl) were measured at the time of injury and daily thereafter until euthanasia at day 7. Means are shown ± SEMs from 14 Tgfrb2fl/fl and 12 γGT-Cre; Tgfrb2fl/fl mice. *P<0.05. (B) Plasma creatinine 3 days after HgCl₂ injection was measured by HPLC on six Tgfrb2fl/fl and five γGT-Cre;Tgfrb2fl/fl mice. Means are shown ± SEMs. *P<0.05. (C–J) Tissue from mice at 1, 3, and 7 days after treatment with HgCl₂ was stained with hematoxylin and eosin. There was minimal epithelial injury 1 day after HgCl₂, but at 3 days, there was significantly more cast formation (black arrow in D) and tubular necrosis in the Tgfrb2fl/fl than in the γGT-Cre;Tgfrb2fl/fl mice. Both genotypes had resolving epithelial injury at 7 days, but fewer residual tubular casts were present in the γGT-Cre;Tgfrb2fl/fl mice. (K) Injury was scored at 3 days, as discussed in the Concise Methods section. The values represent the means of six Tgfrb2fl/fl and seven γGT-Cre;Tgfrb2fl/fl mice. Means are shown ± SEMs. *P<0.05.
Figure 4. γGT-Cre;Tgfrbr2\textsuperscript{fl/\textsuperscript{flo}} mice had less tubular apoptosis after HgCl\textsubscript{2}. (A and B) TUNEL staining was performed at 18 hours after injury with apoptotic nuclei staining brown (arrows). (C) TUNEL-positive tubular cells from 10 hpf per mouse were counted in a blinded fashion and reported as means using five mice per genotype ± SEMs. *P<0.05. (D) Cortical kidney lysates 18 hours after HgCl\textsubscript{2} injections were immunoblotted for cleaved caspase 3 expression using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control. Three representative mice per genotype are shown. (E) Bands were quantified by densitometry expressed as cleaved caspase 3:GAPDH means from five Tgfrbr2\textsuperscript{fl/\textsuperscript{flo}} and six γGT-Cre;Tgfrbr2\textsuperscript{flo/\textsuperscript{flo}} mice ± SEMs. *P<0.05. (F-H) Ki-67 staining was performed on five mice per genotype at 0, 3, 5, and 7 days after HgCl\textsubscript{2} treatment. (F and G) Representative staining at 3 days is shown. (H) Ki-67+ tubular cells were counted in 10 hpf per mouse at the different time points and expressed as means ± SEMs. (I and J) Cortical tissue lysates from three wild-type and four γGT-Cre;Tgfrbr2\textsuperscript{flo/\textsuperscript{flo}} mice at 3 days after HgCl\textsubscript{2} injection were immunoblotted for F4/80 and CD3 to detect macrophages and T cells, respectively. Representative staining of F4/80 (K and L) and CD3 (M and N) at 3 days is shown with positively staining cells in brown (arrows). F4/80 staining was performed on four mice per genotype at days 0, 3, and 7, and F4/80+ cells were counted in 10 hpf per mouse and shown as means ± SEMs (O).
Because the MAPK proteins p38 and extracellular receptor kinase (ERK) have both been shown to mediate TGF-β–dependent apoptosis in proximal tubule cells in vitro,13,14 we defined their role in TGF-β–dependent apoptosis after H2O2. Incubation with H2O2 increased p38 but not ERK phosphorylation in both TβRII flox/flox and TβRII −/− PTCs (Figure 8, H and I). Well established chemical inhibitors to p38 and ERK both increased the amount of apoptosis in H2O2-treated TβRII flox/flox and TβRII −/− PTCs to the same extent, suggesting that neither of these pathways mediated TβRII-dependent apoptosis (Figure 8J). These results suggest that the effects of TGF-β signaling on H2O2-mediated apoptosis were modulated by Smad2/3 but not by p38 or ERK pathways.

**DISCUSSION**

TGF-β is well established as a strong profibrotic growth factor in CKD, but its role in AKI is not as well studied and reports of how TGF-β modulates the response to AKI are inconsistent. Unlike previous studies that used systemic inhibition of TGF-β activity, we abrogated TGF-β signaling specifically in the renal proximal tubule and showed that it attenuated the decrease in renal function after HgCl2–induced AKI. This protective effect was associated with reduced tubular apoptosis in vivo. Proximal tubule cells lacking TβRII in vitro were also more resistant to apoptosis induced by H2O2, TGF-β1, and anoikis. Taken together, these findings suggest that TGF-β signaling in the proximal tubule has a detrimental effect on the response to AKI that is partly due to its proapoptotic effects.

The conflicting reports on how TGF-β modulates AKI may be attributed to the different methods used to inhibit TGF-β signaling. Our finding that deleting TβRII is renoprotective is consistent with the attenuated increase in serum creatinine and BUN observed in injured Smad3 −/− mice.21 In contrast to these genetic approaches, blocking TGF-β activity with chemical inhibitors did not show a significant effect on renal function after AKI.16,20 Genetic techniques may allow more thorough inhibition of TGF-β activity that is sustained over time, while an inhibitor is generally given at the time of injury and thus lacks the effects of chronic inhibition.

Our studies add to the field of TGF-β and AKI by defining the role of TGF-β signaling specifically in the proximal tubule, the main epithelial cell targeted by AKI. This cell-specific approach is important for understanding the role of TGF-β in...
Figure 6. PTCs were generated and TβRII deleted in vitro. (A and B) Cell lysates of PTCs, interstitial cells, and collecting duct (CD) cells were immunoblotted for expression of zona occludens-1 (ZO-1), E-cadherin, α-smooth muscle actin (α-SMA), claudin 2, and γ-glutamyltransferase (γGT) with focal adhesion kinase (FAK), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and α tubulin as loading controls. Vertical white lines separate samples rearranged from the same immunoblot. (C) Deletion of TβRII was confirmed by immunoblots of TβRII^{flkox/flkox} and TβRII^{+/−} PTCs. (D) Cell lysates of TβRII^{flkox/flkox} and TβRII^{−/−} PTCs treated with TGF-β1 (2.5 ng/ml) for various time points were immunoblotted for cleaved caspase 3 and GAPDH. (D) The Roche Cell Death ELISA was used to quantify apoptosis in PTCs both treated with H2O2 and plated on PolyHEMA. Experiments were repeated three times, and ratios of cell death to protein concentration were normalized to 1 for TβRII^{+/−} PTCs. The values for untreated cells (negative controls) are expressed relative to TβRII^{−/−} H2O2-treated PTCs as means ± SEMs.

renal injury because this growth factor’s variable effects depend on the target cell type and injury. This was demonstrated when we showed previously that deleting TβRII in the collecting system unexpectedly led to increased renal fibrosis in a unilateral ureteral obstruction model. This contrasts directly with our present study, in which deleting TβRII in the proximal tubule ameliorated renal injury. The reason for these divergent effects probably relates to the different injury models used. The unilateral ureteral obstruction model generates direct mechanical stress to the epithelium. Because TβRII probably plays an important role in maintaining epithelial integrity, deleting TβRII renders these epithelial cells more susceptible to stretch-induced injury. In contrast, mercuric chloride does not induce mechanical stress but rather oxidative injury that leads to apoptosis. In this different type of injury, the absence of TβRII was beneficial.

Blocking TGF-β was shown to reduce tubular apoptosis in models of chronic kidney injury, but this effect in AKI has not been well described. Tubular apoptosis was significantly less in the γGT-Cre;Tgfb2^{flox/flox} than in the Tgfb2^{+/−} mice after HgCl2, suggesting that resistance to apoptosis is the mechanism protecting from toxin-induced AKI and that TGF-β adversely affects the injury response rather than the repair process.

Although TGF-β was shown to reduce epithelial proliferation and adversely affect inflammation, neither accounted for the improved response to injury in γGT-Cre;Tgfb2^{flox/flox} mice. In fact, the Tgfb2^{flox/flox} mice had greater proliferation after injury, which is most likely explained by the presence of greater tubular injury, although impaired proliferation in the γGT-Cre;Tgfb2^{flox/flox} mice cannot be ruled out. Reduced inflammatory cytokine expression was the putative mechanism whereby Smad3^{−/−} mice were protected from ischemic injury, but there was no quantitative difference in inflammation in our γGT-Cre;Tgfb2^{flox/flox} compared with Tgfb2^{−/−} mice after injury. We did not assess qualitative differences in macrophage infiltration or cytokine expression profiles. An important difference between our work and other AKI and TGF-β studies is that we used a mercuric chloride nephrotoxicity model, which has a smaller inflammatory component compared with ischemic models of injury.

As expected, the γGT-Cre;Tgfb2^{flox/flox} mice had suppressed Smad activation in cortical tubules at baseline, but surprisingly, this difference was not present after injury. Some possible explanations include the following: (1) Deleting TβRII can induce a compensatory upregulation of activins, which can signal through Smads; (2) other proteins, such as angiotensin II, can transiently signal through Smads independent of TGF-β; and (3) most of the Smad activation occurs in the tubules, which have undergone significant injury and apoptosis in the Tgfb2^{flox/flox} mice. Consistent with our in vitro data, TGF-β1 was sufficient to induce apoptosis in wild-type PTCs, and it potentiated the
apoptotic response to oxidative stress (H2O2). In contrast to the paucity of literature on TGF-β and tubular apoptosis in AKI, many reports have described TGF-β’s proapoptotic effect on renal tubular cells in vitro.13,14,35,36 The reduced Smad signaling in proximal tubules lacking TβRII in vivo and in vitro suggests that reduced Smad signaling protects against apoptosis, and this was confirmed with siRNA studies. Smad2/3 signaling may augment apoptosis by altering cell cycle repressor elements or
reducing the antiapoptotic protein Bcl-2. Smad pathways have been implicated in TGF-β-mediated apoptosis of other epithelial cells, but one study concluded that TGF-β1 augmented proximal tubule apoptosis independent of Smad signaling. This study used Smad7 overexpression to inhibit Smad2, which might not be ideal because Smad7 per se promotes apoptosis.

Unlike in previous reports, the signaling proteins p38 and ERK were protective in our studies, consistent with findings implicating these MAPK pathways in Nrf2-dependent antioxidant production.

A paradigm has emerged in AKI research in which many growth factors (e.g., epidermal growth factor and IGF) are upregulated after injury and facilitate renal repair. Our studies indicate that TGF-β does not fit this paradigm, probably because its proapoptotic effects on epithelial cells outweigh any beneficial effects in the context of AKI. Thus, pharmacologic strategies targeting TGF-β signaling in the proximal tubule may offer a new therapeutic approach to AKI.

CONCISE METHODS

Animal Model

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. Tgbr2−/− mice were crossbred with mice containing Cre under control of the γGT promoter (gift from Eric Neilson). The γGT-Cre;Tgbr2−/− mice were further crossed with the mT/mG reporter mouse (purchased from Jackson Laboratory).

Mercuric Chloride Injury Model

A single injection of HgCl₂ (30 μmol/kg diluted in normal saline) was given subcutaneously to γGT-Cre;Tgbr2−/− and Tgbr2−/− male mice that were 8–10 weeks of age and generation 10 on the FVB background. At least five mice per genotype were used and euthanized at 18 hours and 3, 5, and 7 days after HgCl₂ injection.

ImmunobLOTS

Cortices were dissected from kidneys of HgCl₂-treated mice, placed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 2% SDS, 1% Triton, phosphatase and protease inhibitors), sonicated, and clarified by centrifugation. Equal amounts of protein were run on SDS-PAGE gels under reduced conditions, transferred onto nitrocellulose membranes, blocked in 5% milk, and incubated with various primary antibodies. Appropriate secondary antibodies were used before development with enhanced chemiluminescence. Primary antibodies were as follows: phospho-Smad2, total Smad2, total Smad3, phospho-ERK, total ERK, phospho-p38, total p38, and cleaved caspase 3 (Cell Signaling); phospho-Smad3, Smad7 (R&D); TBR1I (Santa Cruz); F4/80, CD3 (Serotek); ZO-1, Smad1, claudin 2 (Invitrogen); γGT (ANAspec); α-SMA (Sigma); and E-cadherin (BD Biosciences).

Renal Function

Whole blood (<25 μl) was collected daily from HgCl₂-treated mice, placed in heparinized tubes, and centrifuged to produce plasma, which was used with the Thermo Infinity Urea Reagent to determine BUN levels. Creatinine levels were determined by HPLC using 30 μl of plasma from a transcardiac puncture at the time of sacrifice by a previously described method (43) with the following modifications: (1) column was Zorbax SCX 2.1 × 100 mm, 5 μm connected to a Zorbax SCX Analytical Guard Column 4.6 × 12.5 mm, 5 μm; (2) mobile phase was 20 mM ammonium formate pH 4.1 at a rate of 0.2 ml/min; (3) absorbance measured at 225 nm with a standard curve of creatinine 25–200 ng.

Immunohistochemistry and Injury Score

Kidneys were harvested and fixed in 4% paraformaldehyde, paraffin embedded, and stained with hematoxylin and cosin, or incubated with primary antibodies to Ki-67, F4/80 (Abcam), CD3 (Serotek), pSmad2 (Cell Signaling), pSmad3 (Rockland), or TUNEL staining as previously described.

Kidneys were stained with TUNEL, F4/80, and CD3 positive tubular cells were quantified in a blinded fashion by counting 10 hpf per mouse and four to six mice per genotype. Renal injury was quantified by a renal pathologist who scored the percentage of tubular necrosis, cellular casts, and tubular injury (epithelial flattening, dilation, loss of brush border) using the following system: 0; <10%; 1, 11%–25%; 2, 26%–50%; 3, >50%. Six mice per genotype were scored in a blinded fashion with 10 fields (200×) reviewed per mouse.

Generation of PTCs

We generated PTCs from our Tgbr2−/− mice crossed with mice containing the Immortomouse transgene (H-2Kb-tsA58) using a modified protocol as described by Vinay et al. Briefly, cortices were isolated from 6-week-old mice, digested with collagenase, passed through a 70-micron filter, and separated on a Percoll gradient by centrifugation into four bands. The bottom (F4) band was removed, washed, and plated with DMEM/F12 media containing 2.5% FBS, hydrocortisone 50 ng/ml, insulin/transferrin/selenium 3 μg/ml, and penicillin/streptomycin/amphotericin. PTCs were incubated in 33°C with interferon-γ 10 ng/ml because the large tumor antigen of the Immortomouse transgene is thermolabile and interferon-inducible. Two weeks before experiments, PTCs in passages two to eight were transferred to 37°C and interferon-γ was removed.

Induction of Apoptosis/Anoikis

PTCs were incubated with 1–2 mM H₂O₂ in serum-free media for 12 hours to induce apoptosis. Inhibitors of ERK/MEK (U0126) and p38 (SB203582) were added to cells 45 minutes before H₂O₂. Higher doses of H₂O₂ (2 mM) were used in the experiments with the inhibitors because the carrier DMSO (equal amounts added to controls) had a protective effect on apoptosis. After incubation with H₂O₂, PTCs were lysed with radioimmunoprecipitation assay buffer and loaded onto SDS-PAGE gels for immunoblotting or were treated with the lysis buffer in the Roche Cell Death ELISA; instructions for this kit were followed.

To induce anoikis, 10-cm plates were coated with PolyHEMA (12 mg/ml, Sigma) as previously described. After plates dried overnight, they were washed with PBS and plated with 1 million PTCs for 18 hours. The media (and unattached cells) were collected, centrifuged at 200 g for 8 minutes, resuspended in 0.5 ml PBS, and divided in half.
One part was centrifuged and resuspended in radioimmunoprecipita-
tion assay buffer for immunoblotting, and the other was centri-
fuged, resuspended in the Roche Cell Death Assay lysis buffer (400 
µl), and then further diluted 1:2 for ELISA.

**Smad Inhibition with siRNA**

TβRIIflox/fox and TβRIIflox/fox PTCs were transfected with Smad2 siRNA (Ambion, Silencer Select predesigned) or scramble negative control (Ambion, Silencer) at 25 nM using lipofectamine RNAiMAX (Invitrogen) overnight. To knock down Smad3, the siRNAs from the following companies were used: Santa Cruz, Ambion (Silencer Select predesigned), and Dharmacon Smart Pool. Four days after transfection, cells were treated with H2O2 (1 mM) for 12 hours, and lysates were made to con-
firm knockdown of Smad2 and to assess apoptosis by cleaved caspase 3.

**Statistical Analyses**

We used the t test with unequal variance in Excel to compare two sets of data, with P<0.05 representing a statistically significant difference. All experiments subject to analysis were performed at least three times.

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**DISCLOSURES**

None.

**REFERENCES**

1. Humes HD, Ciesinski DA, Coimbra TM, Messana JM, Galvao C: Epidermal growth factor enhances renal tubule cell regeneration and re-
2. Coimbra TM, Ciesinski DA, Humes HD: Epidermal growth factor ac-
17. Guan Q, Nguyen CY, Du C: Expression of transforming growth factor-
20. Spurgeon KR, Donohoe DL, Basile DP: Transforming growth factor-
23. Zhao RK: Molecular interactions with mercury in the kidney. *Phar-
macol Rev* 52: 113–143, 2000