Sustained Activation of Wnt/β-Catenin Signaling Drives AKI to CKD Progression

Liangxiang Xiao,*† Dong Zhou,† Roderick J. Tan,‡ Haiyan Fu,† Lili Zhou,* Fan Fan Hou,* and Youhua Liu*†

*State Key Laboratory of Organ Failure Research, National Clinical Research Center of Kidney Disease, Nanfang Hospital, Southern Medical University, Guangzhou, China; and Departments of Pathology and Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

ABSTRACT
AKI is increasingly recognized as a major risk factor for progression to CKD. However, the factors governing AKI to CKD progression are poorly understood. In this study, we investigated this issue using moderate (20 minutes) and severe (30 minutes) ischemia/reperfusion injury (IRI) in mice. Moderate IRI led to acute kidney failure and transient Wnt/β-catenin activation, which was followed by the restoration of kidney morphology and function. However, severe IRI resulted in sustained and exaggerated Wnt/β-catenin activation, which was accompanied by development of renal fibrotic lesions characterized by interstitial myofibroblast activation and excessive extracellular matrix deposition. To assess the role of sustained Wnt/β-catenin signaling in mediating AKI to CKD progression, we manipulated this signaling by overexpression of Wnt ligand or pharmacologic inhibition of β-catenin. In vivo, overexpression of Wnt1 at 5 days after IRI induced β-catenin activation and accelerated AKI to CKD progression. Conversely, blockade of Wnt/β-catenin by small molecule inhibitor ICG-001 at this point hindered AKI to CKD progression. In vitro, Wnt ligands induced renal interstitial fibroblast activation and promoted fibronectin expression. However, activated fibroblasts readily reverted to a quiescent phenotype after Wnt ligands were removed, suggesting that fibroblast activation requires persistent Wnt signaling. These results indicate that sustained, but not transient, activation of Wnt/β-catenin signaling has a decisive role in driving AKI to CKD progression.

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Correspondence: Dr. Fan Fan Hou, Division of Nephrology, Nanfang Hospital, Southern Medical University, 1838 North Guangzhou Avenue, Guangzhou, China 510515, or Dr. Youhua Liu, Department of Pathology, University of Pittsburgh School of Medicine, S-405 Biomedical Science Tower, 200 Lothrop Street, Pittsburgh, PA 15261. Email: ffhouguangzhou@163.com or liuy@upmc.edu

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AKI is responsible for about 2 million deaths each year worldwide, and its incidence is rising.¹⁻³ AKI is increasingly common in critically ill patients, and those patients with severe AKI requiring dialysis have a mortality rate of >50%. In contrast to the traditional belief that survivors of AKI tend to fully recover renal function, there is growing evidence that patients who survive an episode of AKI will have a significant risk of developing progressive CKD and even ESRD.¹²⁻⁴ Evidence is also mounting that the severity and frequency of AKI seem to be closely correlated with poor patient long-term outcome,⁵⁻⁶ suggesting that AKI may be a predictive and causative factor for subsequent development of CKD. In this context, delineation of the underlying mechanisms governing the different courses of long-term outcome after AKI will not only shed new light on understanding the pathophysiology of AKI-CKD progression but also is instrumental in designing rational strategies for therapeutic intervention.

Wnt/β-catenin is a developmental signaling pathway that plays an essential role in regulating...
nephron formation during embryogenesis, injury repair, and pathogenesis of kidney diseases.\(^7,8\) Wnt proteins transmit their signal through interaction with the Frizzled family of receptors and their coreceptors, members of the LDL receptor–related protein-5 and -6. On binding to their receptors, Wnts trigger a cascade of downstream events, resulting in dephosphorylation of β-catenin. This leads to the stabilization of β-catenin and renders its translocation into the nucleus, where it binds to T cell factor/lymphoid enhancer–binding factor to stimulate its target genes.\(^9,10\) Although Wnt/β-catenin is relatively silent in normal adult kidneys, this signaling is robustly reactivated after a wide variety of kidney injuries.\(^11–16\)

Previous studies from our laboratory show that tubule-specific ablation of β-catenin in mice aggravates AKI after either ischemic or nephrotoxic insults, suggesting that Wnt/β-catenin activation is renoprotective, at least in the initial stage after insults.\(^17\) Along this line, an earlier study has also shown the beneficial effect of macrophage-derived Wnt7b in promoting tubular repair and kidney regeneration after AKI.\(^18,19\) However, chronic activation of this signaling seems to be detrimental, leading to development and progression of CKD. This notion is consistent with copious reports that Wnt/β-catenin controls a battery of fibrogenic genes, such as Snail,\(^15\) plasminogen activator inhibitor-1 (PAI-1),\(^20\) matrix metalloproteinase-7 (MMP-7),\(^21\) fibroblast-specific protein 1 (Fsp1),\(^22\) and multiple components of the renin-angiotensin system.\(^23,24\) On the basis of these observations, it is conceivable to speculate that an early and transient activation of Wnt/β-catenin after AKI may be renoprotective by facilitating tubular repair and regeneration, whereas sustained activation of the same signaling could promote AKI to CKD progression.

To test this hypothesis, we have studied the pattern and dynamics of Wnt/β-catenin expression in both moderate and severe AKI models induced by ischemia/reperfusion injury (IRI). We further manipulated this signaling in vivo by either overexpressing Wnt1 or inhibiting β-catenin–mediated gene transcription. Our results indicate that sustained but not transient activation of Wnt/β-catenin is a key determinant for driving AKI-CKD progression.

**RESULTS**

**Level of Wnt/β-Catenin Activation Correlates with AKI Severity**

We first examined the effect of different severity of AKI induced by IRI on the long-term outcomes of kidney function in mice. As shown in Figure 1A, ischemia/reperfusion after bilaterally clamping renal pedicles for 20 minutes triggered moderate AKI. Serum creatinine level was significantly elevated at 1 day after 20-minute IRI but returned to the baseline at 3 and 10 days (Figure 1A). However, 30-minute IRI caused more severe AKI, with a higher level of serum creatinine at 1 day. More importantly, serum creatinine did not decrease at 3 days after 30-minute IRI (Figure 1B), suggesting a persistent kidney failure. Even at 10 days after 30-minute IRI, serum creatinine level remained significantly higher than the baseline (Figure 1B). Consistent with these functional data, kidney morphology was restored at 10 days after 20-minute IRI, whereas morphologic lesions were evident in the kidneys at 10 days after 30-minute IRI (Figure 1C). These results suggest that the severity of the injury is a key determinant dictating the divergent outcomes of AKI, because 20-minute IRI leads to recovery, whereas 30-minute IRI triggers progression to CKD.

To delineate the mechanism governing the different outcomes of AKI, we investigated the expression of Wnt/β-catenin in the kidneys after IRI. As shown in Figure 1D, numerous Wnt ligands, such as Wnt1, Wnt2, Wnt3, Wnt3a, Wnt7a, Wnt8a, and Wnt8b, were significantly induced at 1 day after IRI. Compared with moderate AKI induced by 20-minute IRI, severe AKI by 30-minute IRI exhibited an exaggerated induction of many Wnt ligands, including Wnt1, Wnt3, Wnt7a, Wnt8a, and Wnt8b (Figure 1D). Similarly, renal β-catenin protein abundance was dramatically increased at 3 days after IRI, and the degree of β-catenin induction was closely correlated with the severity of AKI (Figure 1, E and F). Collectively, these results indicate that Wnt/β-catenin is activated in both moderate and severe AKI, but severe AKI is associated with an exaggerated activation of this signaling.

**Wnt/β-Catenin Activation Is Transient in Moderate AKI with Recovery**

We further examined the dynamics of Wnt/β-catenin activation in AKI at different time points after IRI. To that end, expression of Wnt ligands was assessed in moderate AKI induced by 20-minute IRI, in which kidney function and structure promptly recovered (Figure 1). As shown in Figure 2A, RT-PCR analyses displayed a transient activation of the majority of Wnt ligands at 1 day after 20-minute IRI followed by a return to the baseline level at 3 and 10 days after injury. On the basis of the characteristic features of Wnts regulation, we classified four dynamic patterns of Wnt expression, although the magnitude of induction for each individual Wnt after 20-minute IRI was different. As shown in Figure 2B, the majority of Wnt ligands, such as Wnt1, Wnt2, Wnt3, Wnt3a, Wnt7a, Wnt8a, Wnt8b, and Wnt16, displayed a similar expression pattern with a transient induction peaking at 1 day, but their mRNA levels quickly returned to the baseline at 3 and 10 days after IRI injury. A few Wnts exhibited different expression patterns, whereas the expression of Wnt5b and Wnt11 was not changed throughout the course of the experiments (Figure 2B). Notably, the induction of β-catenin protein predominantly took place at 3 days after 20-minute IRI, a time point lagging behind Wnt induction (Figure 2, C and D). However, β-catenin protein also returned to the baseline at 10 days after injury. Taken together, these results suggest that moderate AKI after 20-minute IRI, which is characterized by transient kidney failure and recovery (Figure 1A), is associated with a transient Wnt/β-catenin activation.
Sustained Activation of Wnt/β-Catenin Is Associated with AKI-CKD Progression

We next investigated the expression pattern and dynamics of Wnt/β-catenin in severe AKI induced by 30-minute IRI, which are characterized by developing progressive CKD (Figure 1B).25 As shown in Figure 3A, the expression of the majority of Wnt genes was induced at 1 day after 30-minute IRI and then, slightly reduced at 3 days but increased again at 10 days after injury. According to the dynamics of Wnts induction, four distinct patterns of Wnts expression could be identified. As shown in Figure 3B, most Wnt ligands, such as Wnt1, Wnt5b, Wnt7a, Wnt7b, Wnt9b, Wnt10a, and Wnt10b, were induced at 1 day and somewhat decreased at 3 days but increased again at 10 days after 30-minute IRI. Other patterns of Wnt induction were also observed, but they only represented the minority of the Wnt alterations in the kidneys after 30-minute IRI (Figure 3B). Correspondingly, Western blotting also revealed that renal β-catenin protein was induced at 1, 3, and 10 days after 30-minute IRI, with a peak at 3 days (Figure 3C and D). Therefore, in contrast to moderate AKI after 20-minute IRI, severe AKI after 30-minute IRI, characterized by progressing to CKD, is associated with a sustained activation of Wnt/β-catenin signaling.

In Vivo Expression of Wnt Accelerates AKI to CKD Progression

To establish a role of sustained Wnt/β-catenin activation in AKI-CKD progression, we sought to upregulate Wnt signaling in the kidneys in vivo after AKI. To this end, we investigated whether in vivo expression of Wnt1 gene in mice accelerates AKI to CKD progression. Wnt1 was chosen, because it is the...
prototype of Wnt that transmits its signal by canonical pathway and because its expression was upregulated in AKI (Figures 1–3). As shown in Figure 4A, hemagglutinin (HA)–tagged Wnt1 expression vector (pHA-Wnt1) or empty vector (pcDNA3) was administered intravenously at 5 days after unilateral ischemia/reperfusion injury (UIRI) by a hydrodynamic–based gene transfer technique, an approach that results in significant renal expression of the transgenes.26,27 As shown in Figure 4B, Wnt1 mRNA was induced in the kidneys at 6 days after a single injection of Wnt1-expressing plasmid (11 days after UIRI). Wnt1 protein was also induced as shown by Western blot analyses of whole-kidney lysates (Figure 4C). Notably, this increase in Wnt1 protein was primarily because of the transgene expression of exogenous Wnt1 as shown by Western blotting using anti-HA antibody (Figure 4C). Consistently, immunohistochemical staining revealed that Wnt1 and its downstream β-catenin protein were predominantly induced in kidney tubular epithelium after pHA-Wnt1 plasmid injection (Figure 4D). Of note, endogenous Wnt1 was also mainly expressed in renal tubular epithelium (Figure 4D, IRI +pcDNA3). Collectively, these data indicate that the hydrodynamic–based gene delivery of pHA-Wnt1 plasmid resulted in overexpression of Wnt1 in the same sites as endogenous gene in vivo. We found that overexpression of Wnt1 in vivo exacerbated kidney dysfunction and increased serum creatinine and BUN levels at 11 days after UIRI (6 days after plasmid injection) (Figure 4, E and F), suggesting that sustained activation of Wnt/β-catenin accelerates AKI to CKD progression.

**In Vivo Expression of Wnt Augments Renal Fibrosis after AKI**

We next examined the effect of sustained expression of Wnt1 on renal fibrosis, the final common outcome of progressive CKD.28–30 As shown in Figure 5, in vivo expression of Wnt1 induced its downstream mediator and target genes in the kidneys after ischemic AKI. Western blot analyses illustrated that delivery of the Wnt1-expressing vector upregulated renal expression of β-catenin and PAI-1 protein after IRI compared with pcDNA3 controls (Figure 5, A–C). Similarly, exogenous Wnt1 induced renal mRNA expression of MMP-7 and Fsp1 as determined by quantitative real-time RT-PCR (qRT-PCR) analyses (Figure 5, D and E). Immunohistochemical staining also revealed that overexpression of Wnt1 promoted the expression of β-catenin target genes, such as MMP-7 and renin (Figure 5F). Notably, weak renin staining was only observed in the juxtaglomerular apparatus of normal kidneys (Figure 5F, open arrow), whereas renin was predominantly induced in renal tubular epithelial cells after kidney injury (Figure 5F, solid arrows), consistent with previous observations.31–33

We further investigated the effects of exogenous Wnt1 on matrix gene expression and renal fibrotic lesions after AKI. As shown in Figure 6, A–D, in vivo expression of exogenous Wnt1 significantly induced the mRNA expression of α-smooth muscle.
actin (α-SMA), fibronectin, and types I and III collagens in the kidneys after IRI as shown by qRT-PCR analyses. Accordingly, Western blot analyses of whole-kidney lysates also revealed that exogenous Wnt1 promoted renal expression of α-SMA and fibronectin proteins (Figure 6, E–G). Similar results were obtained when kidney sections were immunostained with antibodies against α-SMA and fibronectin (Figure 6H). Furthermore, Masson Trichrome staining (MTS) also showed that exogenous Wnt1 in vivo aggravates renal fibrotic lesions after IRI (Figure 6, I and J). These data indicate that sustained activation of Wnt/β-catenin after severe AKI is a major driver promoting kidney function decline and fibrogenesis.

**Figure 3.** Severe AKI after 30-minute IRI is associated with sustained activation of Wnt/β-catenin. (A) Representative RT-PCR analysis reveals that most Wnt genes were induced at 1 day after severe AKI induced by 30-minute IRI compared with sham controls and that Wnt induction was sustained at 3 and 10 days. Numbers (1–3) indicate each individual animal in a given group. (B) Graphic presentation shows the distinct pattern of Wnt expression in severe AKI after 30-minute IRI. Different Wnts with similar dynamic pattern were grouped. D, day. (C) Representative Western blot and (D) graphic presentation show that β-catenin protein was induced at 1, 3, and 10 days after 30-minute IRI, with a peak expression at 3 days. Data are presented as fold induction over sham controls. *P<0.05 versus sham controls (n=4). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

### Blockade of Wnt/β-Catenin Signaling Hinders AKI-CKD Progression

To further confirm a role of the sustained Wnt/β-catenin signaling in driving AKI-CKD progression, we examined whether blockade of Wnt/β-catenin signaling can protect kidneys against developing CKD after AKI. To this end, we assessed the effect of ICG-001, a small molecule inhibitor that blocks β-catenin–mediated gene transcription,34–36 in an established IRI–induced kidney injury. As shown in Figure 7A, ICG-001 was administered through daily intraperitoneal injections starting at 5 days after UIRI. We found that inhibition of Wnt/β-catenin signaling by ICG-001 ameliorated both serum creatinine and BUN levels at 11 days after IRI compared with vehicle controls (Figure 7, B and C). Similarly, qRT-PCR analyses showed that ICG-001 reduced the mRNA expression of fibronectin, types I and III collagens, and α-SMA in the kidneys (Figure 7, D–G). MTS also revealed that ICG-001 ameliorated renal collagen deposition and fibrotic lesions at 11 days after IRI (Figure 7H). Quantitative data on renal fibrotic lesions as determined by using computer–aided morphometric analyses are presented in Figure 7I. These results indicate that blockade of Wnt/β-catenin by ICG-001 starting at 5 days after UIRI efficiently protects the kidneys against developing CKD after AKI.

ICG-001 Represses Wnt/β-Catenin Downstream Target Genes

We then investigated the effect of blocking Wnt/β-catenin by ICG-001 by examining the expression of its target genes. As shown in Figure 8, A–E, Western blot analyses showed that β-catenin, fibronectin, α-SMA, and PAI-1 proteins were dramatically induced in the kidneys after AKI induced by 30-minute IRI compared with sham controls, indicating an active AKI-CKD progression in this model. However, administration of ICG-001 starting at 5 days after initial injury almost completely abolished the expressions of β-catenin (Figure 8B), fibronectin (Figure 8C), α-SMA (Figure 8D), and PAI-1 protein (Figure 8E). Immunohistochemical staining also showed that β-catenin, fibronectin, and α-SMA proteins were markedly induced after UIRI, whereas ICG-001 largely abolished the induction of these proteins (Figure 8F). Consistently, qRT-PCR results also revealed that ICG-001 repressed the mRNA expression of MMP-7 and


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Snail1 (Figure 8, G and H), two direct downstream targets of Wnt/β-catenin signaling.\(^1^{15,21}\)

Fibroblast Activation Requires Persistent Wnt/β-Catenin Signaling In Vitro

To elucidate the mechanism underlying sustained Wnt/β-catenin activation driving AKI to CKD progression, we studied fibroblast activation, a key event in renal fibrogenesis,\(^2^{8,30}\) by using an in vitro model system. To this end, conditioned media were collected from human kidney proximal tubular epithelial cell (HKC-8) transfected with various Wnt expression vectors, including Wnt1, Wnt2, Wnt3a, Wnt4, and Wnt16. This Wnt–rich conditioned medium (Wnt-CM) contained a cocktail of the Wnts that were induced after IRI. As shown in Figure 9A, normal rat kidney interstitial fibroblast (NRK-49F) cells were treated with Wnt-CM using different protocols that imitate in vivo conditions. Protocol c in Figure 9A mimicked moderate AKI after 20-minute IRI, with a low-level, transient Wnt exposure, whereas protocol d modeled severe AKI after 30-minute IRI, with high-level, persistent Wnt exposure (Figure 9A). As shown in Figure 9B, transient incubation with a low level of Wnt-CM (10%) for 24 hours was sufficient to induce fibroblast activation as assessed by fibronectin expression. However, after Wnt-CM was removed, fibronectin induction in fibroblasts was eradicated, indicating its reversion to a quiescent phenotype. These data suggest that fibroblast activation in vitro requires persistent Wnt/β-catenin activation.

Consistent with this notion, we found that continuous incubation of NRK-49F cells with Wnt-CM for 48 hours resulted in massive expression of fibronectin (Figure 9B). Notably, the fibrogenic effect of Wnt-CM was abolished by ICG-001 (Figure 9, C and D), indicating an indispensable role for Wnt/β-catenin signaling in mediating fibroblast activation. Analyses of the mRNA expression by qRT-PCR also revealed that persistent Wnt signaling was obligatory for induction of fibronectin, collagens I and III, and Fsp1 in cultured fibroblasts (Figure 9, E–H). Similarly, prolonged incubation with a high level of Wnts resulted in maximal induction of fibronectin, collagen III, and vimentin proteins in NRK-49F cells. These results indicate that persistent but not transient Wnt signaling is required for sustaining fibroblast activation and matrix production in vitro.
DISCUSSION

After AKI, the kidney sometimes fully recovers its structure and function by an adaptive repair and regeneration or undergoes maladaptive responses in other circumstances, leading to the development of progressive and fibrotic CKD.37 Exactly what factors dictate such divergent outcomes of AKI, however, remain a mystery. In this study, we have investigated the molecular mechanisms underlying the distinct fates of the kidneys after AKI. Our results show that sustained activation of Wnt/β-catenin signaling plays a decisive role in driving CKD progression after AKI. This conclusion is supported by several lines of evidence, which include (1) sustained but not transient activation of Wnt/β-catenin is associated with developing CKD after AKI, (2) prolonged activation of Wnt/β-catenin through in vivo expression of Wnt1 after AKI accelerates CKD progression, (3) blockade of Wnt/β-catenin after AKI with small molecule inhibitor prevents AKI-CKD progression and ameliorates renal fibrosis, and (4) fibroblast activation in vitro is a reversible process that requires persistent Wnt signaling. Collectively, these findings establish a crucial role for a sustained and exaggerated Wnt signaling in driving AKI to CKD progression. Our studies also provide novel insights into the mechanisms that control the different long-term outcomes of AKIs of varying severity.

It is widely recognized that the long-term outcome of patients who survive an episode of AKI is divergent. Although some patients with AKI may have better prognosis with full recovery of kidney function, others unfortunately will progress to CKD with declining renal function. Although numerous host factors, such as preexisting conditions and genetic makeup, play a role, the severity of AKI seems to be the most important and robust predictor of poorer outcome.3,6 For instance, in patients who underwent cardiac surgery, the magnitude of serum creatinine concentration during the postoperative hospital course is found to be directly linked to CKD progression and mortality.38,39 Consistent with these data, we found that, in mice with identical age, sex, and genetic backgrounds, the severity of ischemia, per se, is

Figure 5. In vivo expression of exogenous Wnt1 promotes the expression of β-catenin target genes. (A–C) Western blot analyses show the expression of β-catenin and PAI-1 in different groups. Numbers (1–3) indicate each individual animal in a given group. (A) Representative Western blot and quantitative data for (B) β-catenin and (C) PAI-1 are shown. Data are presented as fold induction over sham controls. Ctrl, control. *P<0.05 versus sham controls; †P<0.05 versus pcDNA3 alone (n=5). (D and E) qRT-PCR analyses show that expression of Wnt1 in vivo promoted renal expression of (D) MMP-7 and (E) Fsp1 after IRI. *P<0.05 versus sham controls; †P<0.05 versus pcDNA3 alone (n=5). (F) Representative micrographs show renal induction of MMP-7 and renin at 6 days after plasmid injection. Arrows indicate positive staining. Open arrow shows renin expression in the juxtaglomerular apparatus. Scale bar, 50 μm.
the single most crucial factor for determining the ultimate outcome of AKI, with 20-minute IRI triggering transient AKI followed by recovery of renal function and 30-minute IRI causing severe AKI followed by progression to CKD (Figure 1). Therefore, by merely altering the duration of ischemia, one can establish both models of moderate AKI with full recovery and severe AKI destined to progressive CKD in the same setting (Figure 1). Such IRI models with different outcomes provide an unparalleled system to interrogate the mechanisms dictating the different long-term outcomes of AKI.

One novel finding in this study is that severe AKI is coupled to a sustained Wnt/β-catenin activation. Although reactivation of Wnt/β-catenin is a common event in both moderate and severe AKI, the duration and magnitude of activation are quite different between these two IRI models with divergent outcomes. Transient activation of Wnt/β-catenin is associated with moderate AKI after 20-minute IRI (Figure 2). Given the fact that Wnt signaling promotes tubular cell survival by inhibiting apoptosis and augmenting cell proliferation,40–42 such a transient activation of Wnt/β-catenin in the early stage of AKI seems beneficial by minimizing the injury and promoting repair and regeneration. Consistent with this view, genetic knockout of the β-catenin gene in tubule-specific cells show aggravate kidney injury after both ischemic and nephrotoxic insults.17 It should be pointed out that the biologic action elicited by transient Wnt/β-catenin signaling, such as fibroblast activation and matrix production, is controllable and reversible (Figure 9). Therefore, transient Wnt/β-catenin activation after moderate AKI would lead to predominantly adaptive repair, with complete resolution of the injury and full recovery of kidney function. Taken together, it is reasonable to conclude that the differences in duration of

Figure 6. Exogenous Wnt1 in vivo drives CKD progression after AKI. (A–D) qRT-PCR shows the relative mRNA levels of (A) α-SMA, (B) fibronectin, (C) collagen I, and (D) collagen III in different groups. Relative mRNA levels were determined after normalization with β-actin and expressed as fold induction over controls. Ctrl, control. *P<0.05 versus sham controls; †P<0.05 versus pcDNA3 (n=5). (E) Western blot analyses of renal expression of fibronectin and α-SMA. Kidney lysates were immunoblotted with specific antibodies against fibronectin (FN), α-SMA, and β-actin. Numbers (1–3) indicate each individual animal in a given group. (F and G) Graphic presentation of renal protein levels of fibronectin and α-SMA. *P<0.05 versus sham controls; †P<0.05 versus pcDNA3 (n=5). (H) Immunofluorescence and immunohistochemical staining show fibronectin and α-SMA expression in the kidneys at 6 days after Wnt1 plasmid injection. Kidney sections were stained with specific antifibronectin and α-SMA antibody. Scale bar, 50 μm. (I) Kidney sections were stained with MTS. Representative micrographs from different groups as indicated are shown. Scale bar, 50 μm. (J) Quantitative determination of renal fibrotic lesions in different groups. *P<0.05 versus sham controls; †P<0.05 versus pcDNA3 alone (n=5).
Wnt/β-catenin activation after AKI determine the divergent paths to adaptive repair or maladaptive responses, thereby leading to disparate outcomes.

There are several reasons that could account for why a sustained activation of Wnt/β-catenin leads to CKD. As illustrated in vitro (Figure 9), persistent Wnt signaling, a scenario similar to severe AKI, leads to sustained fibroblast activation and matrix production, whereas activated fibroblasts easily revert back to quiescent phenotype when Wnt signaling is transient, a condition closely resembling moderate AKI. As such, sustained activation of Wnt/β-catenin would result in irreversible fibroblast activation, which contributes to the development of fibrotic lesions. Furthermore, sustained activation of Wnt/β-catenin in severe AKI will cause an exaggerated induction of several key target genes, such as Snail1, PAI-1, MMP-7, Fsp1, and renin-angiotensin system genes (Figures 5 and 6), all of which are relevant to the progression of CKD.15,20,21,23 The importance of sustained Wnt signaling in driving AKI to CKD progression is also substantiated by in vivo expression of Wnt1, the prototype of Wnt ligands that is highly induced after IRI (Figures 2 and 3), or inhibiting Wnt/β-catenin signaling by using a small molecule inhibitor ICG-001.34,43,44 Notably, we selected to manipulate Wnt/β-catenin at 5 days after IRI (Figures 4 and 7), a time point that extends beyond the initial injury and early repair/regeneration phases after AKI, to assess the potential role of Wnt signaling in modulating AKI to CKD progression. These data clearly suggest that a sustained activation of Wnt/β-catenin beyond the injury and repair phases after AKI would result in maladaptive responses characterized by persistent fibroblast activation, excessive matrix accumulation, and renal fibrosis.

Figure 7. Blockade of Wnt/β-catenin signaling by small molecule inhibitor ICG-001 hinders AKI-CKD progression. (A) Diagram shows the experimental design. Arrows indicate the time point of UIRI and unilateral nephrectomy (UNx). The green arrowhead indicates the time point when ICG-001 or vehicle was injected. (B and C) Graphic presentation shows that ICG-001 reduced the levels of serum creatinine (Scr) and BUN at 11 days after UIRI compared with vehicle controls. Ctrl, control. *P<0.05 versus sham controls; †P<0.05 versus vehicle alone (n=6). (D–G) qRT-PCR shows that ICG-001 reduced the mRNA expression of (D) fibronectin, (E) collagen I, (F) collagen III, and (G) α-SMA in the kidneys after IRI compared with vehicle controls. *P<0.05 versus sham controls; †P<0.05 versus vehicle alone (n=6). (H) Kidney sections were stained with MTS. Representative micrographs from different groups as indicated are shown. The arrow indicates positive staining. Scale bar, 50 μm. (I) Quantitative determination of renal fibrotic lesions in different groups. *P<0.05 versus sham controls; †P<0.05 versus vehicle alone (n=6).
Given the significant therapeutic potential of blocking AKI to CKD progression, it is important to determine whether a later intervention (such as 10 days after AKI) by targeting Wnt/β-catenin is still therapeutically effective for longer follow-up periods. Our experiments were terminated at 11 days after severe IRI, because kidney function decline and renal fibrosis already developed at that time point (Figure 7). We envision that the kidney at 5 days after severe IRI or beyond is destined to CKD. Because late administration of ICG-001 has been shown to reduce kidney injury in other CKD models, such as unilateral ureteral obstruction and adriamycin nephropathy, it is reasonable to speculate that later interventions (starting at 10 or 14 days) will also be beneficial. Additional studies are warranted to test this hypothesis.

Emerging evidence suggests that AKI and CKD should be viewed as an intertwined clinical entity rather than two disparate diseases as previously perceived. In this study, we propose that the differences in the dynamics and duration of renal Wnt/β-catenin activation play a decisive role in dictating whether kidneys recover or progress to CKD after acute injury. Although more studies are needed, this study provides the

Figure 8. ICG-001 represses Wnt/β-catenin target genes and reduces fibrotic lesions. (A) Western blot analyses show that ICG-001 inhibited renal expression of β-catenin, fibronectin, α-SMA, and PAI-1 after IRI. Numbers (1–3) indicate each individual animal. Ctrl, control. (B–E) Quantitative data for (B) β-catenin, (C) fibronectin, (D) α-SMA, and (E) PAI-1 are shown in different groups. *P<0.05 versus sham controls; †P<0.05 versus vehicle alone (n=6). (F) Immunostaining shows that ICG-001 reduced the expression of β-catenin, fibronectin, and α-SMA after IRI; kidney sections from different groups were stained with specific antibodies against β-catenin, fibronectin, and α-SMA, respectively. Scale bar, 50 μm. (G and H) qRT-PCR analyses show that ICG-001 inhibited MMP-7 and Snail1 mRNA expression in vivo. *P<0.05 versus sham controls; †P<0.05 versus vehicle (n=6). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
proof of principle that strategies to manipulate this signaling may be effective approaches to prevent AKI to CKD progression in the afflicted patients.

**CONCISE METHODS**

**Mouse Ischemic AKI Models**

Male C57BL/6 mice weighing approximately 22–25 g were purchased from Harlan Sprague–Dawley (Indianapolis, IN). Mice were subjected to bilateral renal IRI by an established protocol as described previously. For studying moderate and severe AKI induced by IRI, two different protocols with 20- or 30-minute ischemia time were used. Briefly, under general anesthesia induced by intraperitoneal injection of a mixture of ketamine hydrochloride (100 mg/kg body wt) and xylazine hydrochloride (10 mg/kg), a midline abdominal incision was made, and bilateral renal pedicles were clipped for 20 or 30 minutes using microaneurysm clamps (item no. 18051–35; Fine Science Tools, Cambridge, UK) for moderate or severe AKI, respectively. After removal of the clamps, reperfusion of the kidneys was visually confirmed. The incision was then closed in two layers. During the ischemic period, body temperature was maintained between approximately 37°C and 38°C using a temperature–controlled heating system. Animals were then administered intraperitoneally with buprenorphine at 0.05 mg/kg body wt. Mice were euthanized at 1, 3, and 10 days post-IRI, respectively. Serum and kidney tissues were collected for various analyses. It should be noted that the severity of ischemic AKI in murine models is influenced by several factors as recently reviewed. In addition to the duration of ischemia, experimental conditions, including temperature control, animal strain, sex, and age, are important variables. In this study, surgery was performed by the same individual (D.Z.), and all other experimental conditions were identical between groups except the ischemia time. All animal experiments were approved by the Laboratory Animal Committee at the Southern Medical University and the Institutional Animal Care and Use Committee at the University of Pittsburgh.

**Manipulation of Wnt/β-Catenin Signaling In Vivo**

To manipulate Wnt/β-catenin signaling during AKI-CKD progression in vivo, a UIRI model was used. This model avoids animal loss after severe AKI induced by bilateral IRI, because one kidney is intact at the time of injury. The UIRI model is well established and particularly suited to study AKI-CKD progression, because kidney function can be assessed after removing the intact kidney by nephrectomy. The
details of UIRI protocol are described in Figure 4A. Briefly, 10-week-old male C57BL/6 mice were subjected to UIRI by a similar protocol to bilateral IRI, except that only one renal pedicle was clamped for 30 minutes. Briefly, under general anesthesia, a midline abdominal incision was made, and the left renal pedicle was clamped using microaneurysm clamps. After removal of the clamps, reperfusion of the kidneys was visually confirmed, and the incision was then closed. During the ischemic period and recovery, body temperature was maintained between approximately 37°C and 38°C using a temperature-controlled heating system. Animals were then administered intraperitoneally with buprenorphine at 0.05 mg/kg body wt. At 5 days after UIRI, mice were subjected to a single intravenous injection of Wnt1 expression plasmid (pHA-Wnt1; Upstate Biotechnology) at 1 mg/kg body wt (Figure 4A) using the hydrodynamic–based gene transfer technique as reported previously.37 For inhibition of Wnt/β-catenin signaling, at 5 days after UIRI, mice were subjected to daily intraperitoneal injections of ICG-001 (Chembest, Shanghai, China) at 5 mg/kg body wt for 6 days (Figure 7A). At 10 days after UIRI, mice were subjected to unilateral nephrectomy to remove the intact contralateral kidney. Mice were euthanized at 11 days after UIRI. Serum and kidney tissues were collected for various analyses.

**Cell Culture and Wnt-CM**

HKC-8 cells and their culture conditions were described previously.44 NRK-49F cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained as described previously.47 HKC-8 cells were transfected with a mixture of Wnt expression vectors using Lipofectamine 2000 according to the protocol specified by the manufacturer (Invitrogen, Grand Island, NY). The Wnt expression vectors used were the pHA-Wnt1, V5-tagged Wnt2 expression vector (pV5-Wnt2), pV5-Wnt3a, pHA-Wnt4, and pV5-Wnt16. At 16 hours after transfection, cells were incubated in serum-free medium, and the supernatant of the transfected HKC-8 cells was harvested after an additional 48 hours of incubation as Wnt-CM. Serum–starved NRK-49F cells were then administered intraperitoneally with buprenorphine at 0.05 mg/kg body wt. At 5 days after UIRI, mice were subjected to daily intraperitoneal injections of ICG-001 (Chembest, Shanghai, China) at 5 mg/kg body wt for 6 days (Figure 7A). At 10 days after UIRI, mice were subjected to unilateral nephrectomy to remove the intact contralateral kidney. Mice were euthanized at 11 days after UIRI. Serum and kidney tissues were collected for various analyses.

**Serum Creatinine and BUN Assay**

Serum creatinine levels were measured using the QuantChrom Creatinine Assay Kit (DICT-500; Bioassay Systems, Hayward, CA) as described previously.17 The level of serum creatinine was expressed as milligrams per 100 ml. BUN levels were determined by using the QuantiChrom Urea Assay Kit according to the manufacturer’s protocol (DIUR-500; Bioassay Systems).

**Histology and Immunohistochemical Staining**

Paraffin-embedded mouse kidney sections (4-μm thickness) were prepared by a routine procedure. Kidney sections were subjected to MTS for assessing collagen deposition and fibrotic lesions as described previously.46 Immunohistochemical staining was performed using routine protocol. Antibodies used were as following: rabbit polyclonal anti-Wnt1 (ab15251; Abcam, Inc., Cambridge, MA), rabbit polyclonal anti-β-catenin (ab15180; Abcam, Inc.), mouse monoclonal anti-α-SMA (ab5694; Abcam, Inc.), mouse monoclonal anti-MMP-7 (GTX17854; GeneTex, Inc.), and goat polyclonal anti-renin (sc-27320; Santa Cruz Biotechnology, Santa Cruz, CA).

**Immunofluorescence Staining**

Kidney cryosections or cells cultured on coverslips were fixed with 3.7% paraformal for 15 minutes at room temperature. After blocking with 10% donkey serum for 30 minutes, the slides were immunostained with primary antibodies against fibronectin (F3648; Sigma-Aldrich, St. Louis, MO), collagen III (234189; EMD Millipore), and vimentin (V5255; Sigma-Aldrich). To visualize the primary antibodies, slides were stained with cyanine Cyanine dye-2– or Cyanine dye-3–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Slides were viewed under a Nikon Eclipse E600 Microscope equipped with a digital camera.

**Western Blot Analysis**

Protein expression was analyzed by Western blot analysis as described previously.48 The primary antibodies used were as follow: rabbit polyclonal antibronectin (F3648; Sigma-Aldrich), mouse monoclonal anti-β-catenin antibody (610154; BD Transduction Laboratories, San Jose, CA), mouse monoclonal antia-α-SMA antibody (A2547; Sigma-Aldrich), mouse anti–PAI-1 antibody (Sc-5297; Santa Cruz Biotechnology), rabbit polyclonal anti–Wnt1 (ab15251; Abcam, Inc.), mouse anti–α-tubulin (T9026; Sigma-Aldrich), mouse antiantiactin (MAB1501; Chemicon, Billerica, MA), and rabbit antiglyceraldehyde-3-phosphate dehydrogenase (2118s; Cell Signaling Technology, Danvers, MA).

**RT-PCR and Real-Time RT-PCR**

Total RNA isolation was carried out using the TRIzol RNA Isolation System (Life Technologies, Grand Island, NY) according to the manufacturer’s instruction. The first–strand cDNA synthesis was carried out by using a Reverse Transcription System Kit (Promega, Madison, WI). qRT-PCR was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) as described previously.23,48 The PCR reaction mixture in a 25-μl volume contained 12.5 μl 2× SYBR Green PCR Master Mix (Applied Biosystems), 5 μl diluted reverse transcription product (1:10), and 0.5 μM sense and antisense primer sets. The sequences of the primer pairs used in RT-PCR or qRT-PCR are given in Supplemental Table 1. Expression of various genes was determined by the comparative CT method (2–ΔΔCT). The mRNA levels of various genes were calculated after normalizing with β-actin.

**Statistical Analyses**

All data examined are expressed as means±SEMs. Statistical analyses of the data were performed using SigmaStat software (Jandel Scientific, San Rafael, CA). Comparison between groups was made using one-way ANOVA followed by Newman–Kuels test. P<0.05 was considered statistically significant.
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DISCLOSURES

None.

REFERENCES


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