Loss of Klotho Contributes to Kidney Injury by Derepression of Wnt/β-Catenin Signaling

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ABSTRACT

Aging is an independent risk factor for CKD, but the molecular mechanisms that link aging and CKD are not well understood. The antiaging protein Klotho may be an endogenous antagonist of Wnt/β-catenin signaling, which promotes fibrogenesis, suggesting that loss of Klotho may contribute to CKD through increased Wnt/β-catenin activity. Here, normal adult kidneys highly expressed Klotho in the tubular epithelium, but various models of nephropathy exhibited markedly less expression of Klotho. Loss of Klotho was closely associated with increased β-catenin in the diseased kidneys, suggesting an inverse correlation between Klotho and canonical Wnt signaling. In vitro, both full-length and secreted Klotho bound to multiple Wnts, including Wnt1, Wnt4, and Wnt7a. Klotho repressed gene transcription induced by Wnt but not by active β-catenin. Furthermore, Klotho blocked Wnt-triggered activation and nuclear translocation of β-catenin, as well as the expression of its target genes in tubular epithelial cells. Investigating potential mediators of Klotho loss in CKD, we found that TGF-β1 suppressed Klotho expression and concomitantly activated β-catenin; conversely, overexpression of Klotho abolished fibrogenic effects of TGF-β1. In two mouse models of CKD induced by unilateral ureteral obstruction or adriamycin, in vivo expression of secreted Klotho inhibited the activation of renal β-catenin and expression of its target genes. Secreted Klotho also suppressed myofibroblast activation, reduced matrix expression, and ameliorated renal fibrosis. Taken together, these results suggest that Klotho is an antagonist of endogenous Wnt/β-catenin activity; therefore, loss of Klotho may contribute to kidney injury by releasing the repression of pathogenic Wnt/β-catenin signaling.


The prevalence of CKD in the general population is high and is increasing worldwide.1,2 CKD often progresses to end-stage renal failure, a devastating condition that requires renal replacement for the survival of the afflicted patients. Several studies suggest that CKD is becoming a public health problem on a global scale.2 This trend of increasing CKD prevalence is associated with the expansion of the aging population in the last several decades. Consistent with this notion, many studies have demonstrated that aging is an independent risk factor for the development and progression of CKD.3–5 However, little is known about the molecular mechanism that links aging to the evolution of kidney deficiency.

α-Klotho, referred to as Klotho hereafter, is a novel antiaging protein that is highly expressed in the tubular epithelium of normal adult kidneys.6–8 Klotho is encoded as a single-pass transmembrane protein with a molecular weight of 130 kDa, and it functions as the co-receptor for fibroblast growth factor-23 (FGF-23), a bone-derived hormone that plays a critical role in phosphate homeostasis.9 In addition to this full-length transmembrane form,
alternative splicing and/or proteolytic shedding of the extracellular domain produce the truncated, secreted form of Klotho. Of particular interest, secreted Klotho can exert its biologic actions in distant tissues, indicating its potential as an endocrine hormone. Clinical studies have demonstrated that both transmembrane and secreted forms of Klotho are markedly downregulated in human CKD, suggesting that loss of Klotho is associated with the pathogenesis of kidney injury. The cause-effect relationship of Klotho loss and CKD, however, is largely elusive. Furthermore, the mechanism by which Klotho deficiency may contribute to CKD remains to be determined.

Wnt proteins are a family of highly conserved, secreted, extracellular signal molecules that play an essential role in nephron formation and renal development. Accumulating evidence indicates aberrant activation of Wnt signaling in many kinds of CKD, including obstructive nephropathy, adriamycin nephropathy, and diabetic nephropathy. Wnt proteins interact with their receptors and co-receptors and induce downstream signaling events, which leads to dephosphorylation and stabilization of β-catenin. Stabilized β-catenin controls the expression of a battery of downstream genes that are implicated in matrix production and fibrosis, such as Snail1, plasminogen activator inhibitor-1 (PAI-1), fibronectin, fibroblast-specific protein 1, and matrix metalloproteinase. In this context, inhibition of Wnt/β-catenin signaling could be a rational strategy for therapeutic intervention of CKD. Indeed, previous studies have shown that blockade of Wnt/β-catenin by a variety of approaches ameliorates proteinuria, kidney injury, and renal fibrosis.

Earlier studies demonstrate that Klotho deficiency contributes to stem and progenitor cell dysfunction and depletion in normal aging by increasing Wnt signaling, consistent with the notion that Klotho could be an endogenous Wnt antagonist. These observations suggest that loss of Klotho might play a role in promoting kidney injury by de-repression of Wnt/β-catenin signaling. To test this hypothesis, we have investigated the regulation of Klotho, its interplay with Wnt/β-catenin, and its role in protecting kidneys from fibrotic injury.

RESULTS

Klotho Is Downregulated in Various Models of CKD
Klotho is highly expressed in normal adult kidneys, predominantly in renal tubular epithelium. To identify the specific tubular segments that express Klotho, we carried out double immunostaining for Klotho and various segment-specific tubular markers. As shown in Figure 1A, co-localization of Klotho and aquaporin-1, a proximal tubular marker, was clearly evident, confirming its presence in the proximal convoluted tubules in adult kidneys. Klotho (red) was similarly co-localized with the sodium chloride cotransporter (green), a marker of distal convoluted tubules. Furthermore, Klotho was also co-stained with aquaporin-3, a marker for collecting-duct epithelium. This staining pattern indicates that Klotho is widely and ubiquitously expressed in all major tubular segments along the nephron in adult kidneys.

To investigate the regulation of Klotho in CKD, we used three mouse models of fibrotic kidney disease induced by unilateral ureteral obstruction (UUO), adriamycin or ischemia/reperfusion injury (IRI), respectively. These models are widely used and represent different causes that lead to interstitial fibrosis. As shown in Figure 1B, ureteral obstruction induced a dramatic loss of renal Klotho because its protein level was suppressed by 90% in the obstructed kidneys at 14 days after UUO compared with sham controls. Similarly, renal Klotho levels were significantly downregulated at 5 weeks after adriamycin injection (Figure 1C). In a mouse model of IRI, renal Klotho was almost completely lost at 7 days after surgery (Figure 1, D and E), a time point when renal fibrotic lesions emerge. These results suggest that loss of Klotho is a common finding in diseased kidneys after a variety of injuries.

Loss of Renal Klotho Is Associated with Activation of β-Catenin
We next examined the kinetics of Klotho expression in different CKD models. As shown in Figure 2, both Klotho protein and mRNA were markedly downregulated as early as 3 days after UUO. The loss of renal Klotho progressed in a time-dependent fashion (Figure 2). Of note, loss of Klotho was associated with induction of β-catenin, the principal intracellular mediator of canonical Wnt signaling. As shown in Figure 2A, β-catenin protein also localized predominantly to renal tubular epithelium, and its abundance increased in a time-dependent manner in this model, as previously reported. Co-immunostaining for Klotho (red) and active β-catenin (green) demonstrated that β-catenin was activated only in the tubules that were deficient in Klotho (Figure 2B, arrow), but not in tubules that retained this protein (Figure 2B, arrowhead). Further linear regression analysis revealed an inverse correlation between renal Klotho mRNA and β-catenin levels in the obstructed kidneys (Figure 2D).

To further establish the correlation between Klotho depletion and β-catenin induction, we used adriamycin nephropathy, a model of human FSGS that develops chronic tubulointerstitial lesions as a secondary consequence of glomerular injury. As shown in Figure 2, E and F, renal Klotho protein and mRNA were gradually downregulated, which was again accompanied by tubular induction of β-catenin. Linear regression analysis confirmed a close association between loss of Klotho and activation of β-catenin in this model as well.

Klotho Binds to Wnts and Inhibits Wnt-Mediated Gene Transcription
We further investigated the potential connection between loss of Klotho and activation of Wnt/β-catenin signaling in diseased kidneys. To this end, we examined the interaction between Klotho and various Wnt ligands, such as Wnt1, Wnt4,
and Wnt7a, all of which are upregulated in the fibrotic kidneys.17,18 Human proximal tubular epithelial cells (HKC-8) were transfected with the V5-tagged, full-length, membrane form of Klotho expression plasmid (pV5-mKlotho) and hemagglutinin (HA)-tagged Wnt1. As shown in Figure 3A, Wnt1 was detectable in the immunocomplexes precipitated by anti-Klotho antibody, suggesting a physical interaction between Klotho and Wnt1. Of note, although HKC-8 cells were transfected with the expression vector encoding full-length Klotho (pV5-mKlotho), the majority of Klotho detected was the truncated, secreted form with 70 kDa in size (Figure 3A), suggesting that these cells possess the full capacity to proteolytically cleave membrane Klotho into the secreted form. Results were similar when HKC-8 cells were transfected with pV5-mKlotho and either Wnt4 (Figure 3B) or Wnt7a expression vector (data not shown), suggesting that Klotho is able to bind multiple Wnt ligands. To determine whether the truncated, secreted form of Klotho is responsible for binding to Wnt, we transfected HKC-8 cells with the expression vector encoding the secreted form of Klotho (pV5-sKlotho). As shown in Figure 3C, the secreted form of Klotho was fully capable of interacting with Wnt1. Of note, Klotho/Wnt1 interaction was also detectable when cell culture medium was used (Figure 3D), suggesting that such an interaction could take place in the extracellular space.

We next assessed the functional consequence of Klotho on Wnt-mediated gene transcription in a β-catenin responsive TOPFlash luciferase reporter assay. As shown in Figure 3E, transfection of Wnt1 expression vector (pHA-Wnt1) markedly induced luciferase reporter activity, as expected. However, co-transfection of Klotho blocked Wnt1-mediated reporter gene expression in a dose-dependent fashion. Of note, Klotho did not affect the luciferase activity induced by the N-terminally truncated, constitutively activated β-catenin (Figure 3F). These results suggest that inhibition of Wnt/β-catenin signaling by Klotho is mediated by targeting Wnts, but not β-catenin.

Figure 1. Klotho is downregulated in various models of CKD. (A) Co-localization of Klotho and various segment-specific tubular markers in normal adult kidneys. Kidney cryosections were immunostained for Klotho (red) and various segment-specific tubular markers (green). Segment-specific tubular markers used are as follows: proximal tubule, aquaporin-1 (AQP1); distal tubule, thiazide-sensitive NaCl cotransporter (NCC); and collecting duct, aquaporin-3 (AQP3). Arrowheads indicate positive tubules with co-localization of Klotho and specific tubular makers. Scale bar, 50 μm. (B) Western blot analyses of renal expressions of Klotho in sham and obstructed kidneys at 14 days after UUO. (C) Renal Klotho expression in adriamycin (ADR) nephropathy. Klotho expression was assessed in the kidneys at 5 weeks after adriamycin injection. CTL, control. (D) Western blot analyses of renal Klotho expression at 7 days after ischemia/reperfusion injury (IRI). Numbers (1 through 5) indicate each individual animal in a given group. (E) Quantitative determination of relative abundance of renal Klotho in different disease models as indicated. Relative Klotho levels (control = 1.0) were presented after normalization with actin. *P<0.05 versus normal controls.
Klotho Represses Wnt/β-Catenin Target Genes in Vitro

We further examined the ability of Klotho to antagonize Wnt/β-catenin signaling and target gene expression in tubular epithelial cells. As shown in Figure 4A, expression of Wnt1 in HKC-8 cells induced β-catenin activation and its nuclear translocation (Figure 4A, arrows). However, co-transfection of Klotho expression vector completely prevented Wnt1-triggered β-catenin nuclear translocation (Figure 4A). Results were similar when nuclear and cytoplasmic β-catenin was detected after subcellular fractionation (Figure 4, B and C). These data indicate that Klotho is able to effectively block Wnt-mediated β-catenin activation in tubular epithelial cells.

To further confirm the efficacy of Klotho in antagonizing Wnt/β-catenin signaling, we examined the expression of β-catenin target genes in HKC-8 cells. As shown in Figure 4D, transient transfection with HA-tagged Wnt1 expression...
vector in HKC-8 cells induced β-catenin activation, as demonstrated by an increased level of dephosphorylated, active β-catenin. Wnt1 also induced PAI-1 and Snail1, two direct downstream targets of canonical Wnt signaling. However, co-transfection with Klotho expression vector in HKC-8 cells dose-dependently suppressed Wnt1-mediated β-catenin activation and PAI-1 and Snail1 expression (Figure 4, D–G).

Inhibition of Klotho by TGF-β1 De-represses Its Fibrogenic Actions

To identify the pathologic cues responsible for mediating Klotho loss in diseased kidneys, we tested whether Klotho expression is regulated by TGF-β1, a fibrogenic cytokine that is upregulated in almost all cases of CKD. However, co-transfection with Klotho expression vector in HKC-8 cells dose-dependently suppressed Wnt1-mediated β-catenin activation and PAI-1 and Snail1 expression (Figure 4, D–G).

Figure 3. Klotho binds to Wnt and blocks Wnt-mediated gene transcription. (A) Co-immunoprecipitation demonstrates that Klotho binds to Wnt1 in tubular epithelial cells. HKC-8 cells were transfected with V5-tagged, full-length, membrane Klotho expression vector (pV5-mKlotho) and HA-tagged Wnt1 expression vector (pHA-Wnt1). Cell lysates were immunoprecipitated with anti-Wnt1 or anti-Klotho, followed by immunoblotting with anti-Wnt1 or anti-Klotho, respectively. (B) Klotho also binds to Wnt4. Co-immunoprecipitation shows that full-length, membrane Klotho also binds to Wnt4 in HKC-8 cells. (C) The extracellular KL1 domain of Klotho is sufficient for mediating Wnt/Klotho interaction. HKC-8 cells were transfected with truncated, secreted Klotho expression vector (pV5-sKlotho) and Wnt1 expression vector (pHA-Wnt1). (D) Interaction of Klotho and Wnt1 occurs in the extracellular space. HKC-8 cells were transfected with pV5-mKlotho and pHA-Wnt1. Cell culture medium was immunoprecipitated with anti-V5 antibody, followed by immunoblotting with anti-HA or anti-V5, respectively. Cell lysates were immunoblotted with antibodies against Klotho and actin. (E) Klotho inhibits Wnt1-mediated β-catenin gene transcription in a dose-dependent manner. HKC-8 cells were co-transfected with TOP-Flash reporter plasmid and different amounts of full-length Klotho plasmid (pV5-mKlotho) as indicated in the absence or presence of Wnt1 expression vector. *P<0.05 versus pHA-Wnt1 alone (n=3). (F) Klotho does not inhibit activated β-catenin–triggered gene transcription. HKC-8 cells were co-transfected with TOP-Flash reporter plasmid and Klotho plasmid in the absence or presence of the N-terminally truncated, constitutively activated β-catenin expression vector (pDel-β-cat). IB, immunoblotting; IP, immunoprecipitation.

expression is not mediated by TNF-α because TGF-β1 did not induce its expression (Figure 5E). It appeared that Smad3 signaling is required for the TGF-β1–mediated suppression of Klotho because TGF-β type 1 receptor inhibitor SB431542 or Smad3 inhibitor SIS3 were able to largely restore Klotho expression in HKC-8 cells (Figure 5, F and G). Therefore, TGF-β1 is identified as the pathologic culprit that triggers Klotho suppression in diseased kidneys.

We previously reported that TGF-β1 can activate β-catenin in HKC-8 cells. However, a comprehensive analysis of the mRNA expression of all 19 Wnts by RT-PCR revealed that TGF-β1 did not induce their expression in tubular epithelial cells (Figure 5H). The mRNA of several Wnts was undetectable in HKC-8 cells in basal conditions, and TGF-β1 incubation also failed to induce their expression (data not shown). These data suggest that β-catenin activation by TGF-β1 in tubular epithelial cells is not mediated by an increased Wnt expression; rather, it is probably mediated by the loss of Klotho. To test this hypothesis, HKC-8 cells were transfected with Klotho expression vector, followed by incubation with TGF-β1. As shown in Figure 5,
expression of Klotho was able to dose-dependently inhibit TGF-$\beta_1$-mediated $\beta$-catenin activation, as well as PAI-1 and Snail1 induction. Furthermore, Klotho also inhibited TGF-$\beta_1$-mediated induction of fibronectin and $\alpha$-smooth muscle actin ($\alpha$-SMA) in HKC-8 cells (Figure 5, M and N).

Expression of Klotho in Vivo Attenuates Renal Fibrosis in Obstructive Nephropathy

Given the ability of Klotho in antagonizing Wnt/$\beta$-catenin in vitro, we next tested whether exogenous Klotho protects kidneys from development and progression of CKD in vivo. To this end, mice were intravenously administered the expression vector encoding the secreted form of Klotho (pV5-sKlotho) through hydrodynamic-based gene delivery, an approach that is routinely used in our laboratory for in vivo expression of a variety of genes. As shown in Figure 6A, secreted Klotho was dramatically induced in the kidneys after intravenous injection of naked pV5-sKlotho plasmid. Quantitative determination of urinary Klotho by a specific ELISA also showed a substantial increase of this protein in mouse urine at 24 h after plasmid injection (Figure 6B). We then investigated the effect of exogenous Klotho in mouse model of obstructive nephropathy. A shown in Figure 6C, we used both preventive and therapeutic protocols, in which Klotho plasmid was injected

Figure 4. Klotho inhibits Wnt-mediated $\beta$-catenin activation and represses its target genes in vitro. (A) Immunofluorescence staining shows that ectopic expression of Klotho blocked the Wnt1-mediated $\beta$-catenin activation and its nuclear translocation. HKC-8 cells were transfected with Wnt1 expression plasmid (pHA-Wnt1) and Klotho expression vector (pV5-mKlotho) as indicated. Cells were then immunostained for $\beta$-catenin and DAPI, the nuclear marker. Arrows indicate nuclear staining of $\beta$-catenin. (B and C) Klotho inhibits Wnt1-mediated $\beta$-catenin nuclear translocation. Nuclear (B) and cytoplasmic (C) protein was prepared from HKC-8 cells co-transfected with various plasmids as indicated and immunoblotted with antibodies against $\beta$-catenin, TATA-binding protein (TBP), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). n-$\beta$-catenin, nuclear $\beta$-catenin; c-$\beta$-catenin, cytoplasmic $\beta$-catenin. The ratios of nuclear $\beta$-catenin to TATA-binding protein and cytoplasmic $\beta$-catenin to GAPDH are presented in the bottom panels. (D–G) Klotho dose-dependently inhibits Wnt1-mediated $\beta$-catenin activation and its target genes, such as PAI-1 and Snail1. Western blot analyses (D) and quantitative data for active $\beta$-catenin (E), PAI-1 (F), and Snail1 (G) are shown. Different doses of pV5-mKlotho (1, 2 and 4 $\mu$g/well) were used. *$P<0.05$ versus pHA-Wnt1 alone.
Figure 5. TGF-β1 represses Klotho expression and Klotho inhibits the TGF-β1–mediated induction of various fibrogenic genes in vitro. (A–D) Western blot analyses show that TGF-β1 inhibited Klotho expression in a dose- and time-dependent manner. HKC-8 cells were incubated with different doses of TGF-β1 for 24 hours (A and B) or the same concentration of TGF-β1 (2 ng/ml) for various periods of time (C and D) as indicated. Representative Western blots (A and C) and quantitative data (B and D) are presented. *P<0.05 versus controls (n=3). (E) RT-PCR shows that TGF-β1 repressed Klotho mRNA expression in tubular epithelial cells. HKC-8 cells were treated with TGF-β1 (2 ng/ml) for various periods of time as indicated. Expression of Klotho, TNF-α, and β-actin mRNA was analyzed. (F and G) Repression of Klotho by TGF-β1 depends on Smad3 signaling. HKC-8 cells were pretreated with TGF-β type I receptor inhibitor (SB431542; 10 μM) or Smad3 inhibitor (SIS3; 10 μM) for 1 hour, followed by incubation with TGF-β1. Representative Western blot (F) and quantitative data (G) are presented. *P<0.05 versus controls (n=3); †P<0.05 versus TGF-β1 alone (n=3). SB, SB431542. (H) TGF-β1 does not induce Wnt expression in tubular epithelial cells. HKC-8 cells were treated with TGF-β1 (2 ng/ml) for various periods of time as indicated. Expression of various Wnt mRNA was assessed by RT-PCR. (I–L) Western blot analyses and quantitative data show that Klotho abolished TGF-β1–mediated fibrogenic actions. HKC-8 cells were transfected with empty vector (pcDNA3) or Klotho expression vector (pV5-mKlotho) as indicated, followed by incubation with TGF-β1 (2 ng/ml). Western blot (I) and quantitative data for active β-catenin (J), PAI-1 (K), and Snail1 (L) are presented. (M and N) Klotho abolished TGF-β1–mediated fibrogenic actions. HKC-8 cells were transfected with empty vector (pcDNA3) or Klotho expression vector (pV5-mKlotho) as indicated, followed by incubation with TGF-β1 (2 ng/ml). Total cell lysates were immunoblotted with specific antibodies against fibronectin, α-SMA and actin, respectively. *P<0.05 versus pcDNA3 controls; †P<0.05 versus TGF-β1 alone (n=3).
either 1 day prior to ureteral obstruction (UUO/K-1), or 3 days after the surgery (UUO/K+3), respectively. As shown in Figure 6, D and E, in either protocol, expression of secreted Klotho in vivo was able to suppress renal β-catenin expression in the obstructed kidneys at 7 days after UUO. Consistently, exogenous Klotho also inhibited renal expression of Snail1 and PAI-1 (Figure 6, D, F and G), two Wnt/β-catenin target genes that are directly implicated in renal fibrogenesis.

We further examined the effects of exogenous Klotho on kidney injury and renal fibrosis in the UUO model. As shown in Figure 7A, immunohistochemical staining revealed that expression of Klotho in vivo substantially inhibited the expression of α-SMA, the molecular signature protein for myofibroblasts. Klotho also reduced the expression and deposition of major interstitial matrix proteins, including fibronectin and collagen I in the obstructed kidneys (Figure 7A). Quantitative determinations by using Western blot analyses of whole kidney lysates produced similar results (Figure 7, B–D). Consistently, Masson trichrome staining revealed that expression of Klotho in vivo attenuated renal fibrotic lesions in obstructive nephropathy in both preventive and therapeutic protocols (Figure 7, E and F).

**Klotho Ameliorates Kidney Injury in Adriamycin Nephropathy**

To further confirm the therapeutic efficacy of Klotho in CKD, we used adriamycin nephropathy, another clinically relevant model. As shown in Figure 8A, mice were injected with pV5-sKlotho plasmid at 1 and 2 weeks after adriamycin injury, time points when robust albuminuria and glomerular injury are established in this model. At 3 weeks after adriamycin administration, albuminuria and kidney injury were assessed. As shown in Figure 8B, urine albumin levels were markedly elevated at 3 weeks after adriamycin injection. Expression of Klotho in vivo tended to reduce albuminuria, but this finding was not statistically significant (P=0.095; n=7). Kidney histology as shown by periodic acid-Schiff (PAS) staining revealed significant lesions, characterized by dilated tubules loaded with proteins, and interstitial inflammation and expansion, at 3 weeks after adriamycin injection (Figure 8C). Delivery of secreted Klotho largely abolished these morphologic lesions (Figure 8C). Secreted Klotho also significantly improved kidney function, as reflected by a reduced serum creatinine level (Figure 8D). As shown in Figure 8E, adriamycin caused significant downregulation of nephrin and Wilms tumor protein (WT1) in glomerular podocytes, characteristic features of this model, and expression of secreted Klotho largely preserved both nephrin and WT1 expression. Results were similar when nephrin protein was quantitatively analyzed by Western blot (Figure 8, F and G).

We further examined the expression of β-catenin, as well as the expression of its target genes, such as fibronectin, PAI-1, and Snail1, by Western blotting and immunostaining (Figure 9, A–D). Of note, delivery of secreted Klotho appeared to largely

Figure 6. Ectopic expression of secreted Klotho in vivo inhibited renal β-catenin activation and the expression of its target genes. (A) Western blot analyses show Klotho expression in mouse kidneys at 24 h after intravenous injection of naked plasmid encoding secreted Klotho (pV5-sKlotho). Secreted Klotho was readily detected in the kidneys after injection of pV5-sKlotho plasmid. (B) ELISA shows increased Klotho protein in the urine after injection of pV5-sKlotho plasmid. Urine was collected at 24 hours after plasmid injection. Urinary Klotho was expressed as ng/mg creatinine. *P<0.05 versus pcDNA3 (n=6). (C) Experimental design. Arrows indicate the injection of pV5-sKlotho plasmid. Narrow arrows indicate the timing of UUO surgery. Both preventive protocol (UUO/K-1) and therapeutic protocol (UUO/K+3) were used. (D–G) Western blot analyses and quantitative data show renal expression of β-catenin and its target genes, such as Snail1 and PAI-1, in different groups as indicated. Numbers (1 and 2) indicate each individual animal in a given group. *P<0.05 versus sham controls; †P<0.05 versus UUO alone (n=5–7).
restore renal endogenous full-length Klotho expression and inhibited β-catenin and its target gene expression in the injured kidneys (Figure 9A). Furthermore, Masson trichrome staining demonstrated fewer fibrotic lesions and reduced collagen deposition in the kidneys receiving exogenous Klotho (Figure 9, E and F). Taken together, these results indicate that expression of Klotho in vivo is able to ameliorate kidney injury and renal fibrosis in both UUO and adriamycin nephropathy models.

**DISCUSSION**

The results presented in this study demonstrate that loss of Klotho, an antiaging protein that is highly expressed in all major tubular segments of normal adult kidneys, is a common pathologic feature in multiple models of CKD with distinct causes. We show that loss of Klotho de-represses Wnt/β-catenin signaling because Klotho physically binds to and sequesters Wnt ligands in the kidneys. This leads to activation of a fibrogenic signaling pathway that plays a crucial role in triggering the expression of a battery of profibrotic genes, such as Snail1, PAI-1, and fibronectin. More important, expression of secreted Klotho in vivo is able to inhibit Wnt/β-catenin signaling and the expression of its target genes and attenuates renal fibrotic lesions in two models of CKD induced by UUO and adriamycin. These findings illustrate that Klotho is an endogenous antagonist that safeguards the kidneys against the pathogenic Wnt/β-catenin signaling. Our studies also...
provide novel insights into the mechanism by which Klotho protects the kidneys from developing fibrosis after a wide variety of insults.

Klotho is a type I single-pass transmembrane protein composed of a large extracellular region with two homologous KL1 and KL2 domains, a transmembrane segment, and a short intracellular domain. Whereas the full-length, membrane-bound form of Klotho functions as an obligatory co-receptor for FGF23, the extracellular domain of Klotho is often cleaved by secretases and released into the extracellular space, including the bloodstream and urine, and this can potentially function as an endocrine factor. Notably, kidney tubular cells appear to possess all components of the molecular machinery to spontaneously convert membrane Klotho into the secreted form because the majority of Klotho is the truncated, secreted form even when HKC-8 cells were transfected with expression vector encoding full-length Klotho (pV5-mKlotho). We show that Klotho physically binds to multiple Wnt ligands, such as Wnt1, Wnt4, and Wnt7a (Figure 3), all of which are upregulated in injured kidneys. This is consistent with an earlier report demonstrating that Klotho suppresses cell senescence through the binding and inhibition of Wnt3a. Although not tested, it is conceivable to speculate that Klotho might be able to bind all members of the Wnt family proteins. Of interest, such Klotho/Wnt interaction is apparently mediated by the extracellular KL1 domain of Klotho, as expression of secreted...
Klotho (pV5-sKlotho), which contains only the KL1 domain but lacks the KL2, transmembrane, and intracellular domains, is sufficient for mediating its interaction with Wnt (Figure 3C). Furthermore, Klotho/Wnt interaction is detectable in the cell culture medium (Figure 3D), suggesting that their binding occurs in the extracellular space. Not surprisingly, Klotho binding inevitably leads to functional sequestration of Wnt ligands and blocks Wnt-mediated β-catenin activation and its target gene expression (Figure 4). Therefore, we conclude that Klotho, via its KL1 domain, antagonizes Wnt/β-catenin signaling through physical interaction and functional sequestration.

One of the interesting observations in this study is the mutual antagonism of Klotho and TGF-β1. Loss of Klotho is a common pathologic finding in different models of CKD (Figure 3C). Furthermore, Klotho/Wnt interaction is detectable in the cell culture medium (Figure 3D), suggesting that their binding occurs in the extracellular space. Not surprisingly, Klotho binding inevitably leads to functional sequestration of Wnt ligands and blocks Wnt-mediated β-catenin activation and its target gene expression (Figure 4). Therefore, we conclude that Klotho, via its KL1 domain, antagonizes Wnt/β-catenin signaling through physical interaction and functional sequestration.

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through induction of Wnt ligands in this cell type. Of note, overexpression of Klotho is sufficient to inhibit TGF-β1–mediated β-catenin activation and the subsequent induction of profibrotic genes, such as Snail1, PAI-1, fibronectin, and α-SMA (Figure 5). Therefore, although TGF-β1 inhibits Klotho expression, Klotho in turn functionally blocks the fibrogenic actions of TGF-β1. Such reciprocal antagonism between TGF-β1 and Klotho suggests that loss of Klotho would lead to the initiation of a vicious cycle between Klotho deficiency and TGF-β1-β-catenin activation in diseased kidneys.

As a potent endogenous Wnt antagonist, Klotho is unique in that its expression is downregulated in the fibrotic kidneys. There are several secreted antagonists of Wnt signaling, including soluble Frizzled-related protein, Wnt inhibitory factor, and a family of Dickkopf (DKK) proteins.42–44 Most of them are actually the target of Wnt/β-catenin signaling itself, and therefore their expression is induced, rather than diminished, in the injured kidneys. For instance, all four members of DKK family proteins are upregulated in the obstructed kidneys after UUO.17 The fact that Klotho is deficient in diseased kidneys suggests that Klotho expression is not positively controlled by Wnt/β-catenin. In this regard, supplementation by exogenous Klotho to the diseased kidneys is particularly well suited as a rational strategy to antagonize Wnt/β-catenin and to inhibit fibrotic lesions. Furthermore, the mechanism and modes of action of different Wnt antagonists are quite divergent. While soluble Frizzled-related protein functions as a Frizzled receptor decoy, DKK proteins specifically inhibit the canonical Wnt signaling by binding to the Wnt co-receptors, the low density lipoprotein (LDL) receptor-related protein 5 and 6 (LRP5/6). Through binding to multiple Wnt ligands, it is conceivable that Klotho could possess the potential to antagonize both canonical and non-canonical Wnt signaling.

This study provides direct and compelling evidence for the therapeutic efficacy of exogenous Klotho in reducing kidney injury and fibrosis in CKD in vivo. Using both UUO and adriamycin nephropathy models, we have demonstrated that in vivo expression of Klotho by a hydrodynamics-based gene transfer approach ameliorates renal fibrotic lesions. As discussed elsewhere,17,35,36 this approach leads to substantial expression of transgene in the kidneys (Figure 6). Of note, we used the expression vector encoding secreted Klotho because this form of Klotho is sufficient for binding and sequestering Wnt proteins (Figure 3C). Therefore, regardless of where the exogenous Klotho is produced, the very fact that it is a secreted protein makes it accessible to the kidneys through diffusion and/or bloodstream, a scenario similar to the intransverse injection of exogenous Klotho protein.

The ability of Klotho to ameliorate kidney injury in different CKD models is impressive and is corroborated by recent observations in Klotho transgenic or knockout mice.12,39,45 By using different treatment protocols (Figures 6 and 8), our studies for the first time show that supplementation of exogenous Klotho at late time points when kidney lesions are already established is still therapeutically effective. The renal protection elicited by Klotho is associated with its inhibition of β-catenin activation and suppression of Snail1, PAI-1, and fibronectin, suggesting that delivery of exogenous Klotho is able to reinstate the repression of Wnt/β-catenin signaling. Of interest, delivery of exogenous Klotho is also able to induce (Figure 6A) or restore the endogenous, full-length Klotho expression in vivo (Figure 9A). This is probably due to the fact that exogenous Klotho antagonizes TGF-β1–mediated suppression of native Klotho expression. Therefore, it is plausible to conclude that administration of exogenous Klotho confers renal protection by inhibiting Wnt/β-catenin signaling, as well as by restoring endogenous Klotho expression. However, we cannot exclude the possibility that Klotho may exert its beneficial action by other mechanisms as well. In that regard, an earlier report indicates that Klotho also directly binds to and inhibits the TGF-β type II receptor.45

In summary, we have shown that loss of Klotho is a common pathologic feature in a wide variety of CKD and is associated with renal activation of β-catenin. As Klotho binds to Wnt and functions as a potent, endogenous Wnt antagonist, loss of Klotho inevitably promotes kidney injury by de-repression of Wnt/β-catenin signaling. In this context, administration of exogenous Klotho could be a rational strategy for inhibiting pathogenic Wnt/β-catenin signaling and for the treatment of fibrotic kidney disorders.

**CONCISE METHODS**

**Animal Models**

Male CD-1 and BALB/c mice, weighing approximately 20–22 g, were purchased from Harlan Sprague-Dawley (Indianapolis, IN). CD-1 mice were used for generating the UUO model by an established protocol, as described previously.27 Briefly, under general anesthesia, complete UUO was carried out by double-ligating the left ureter using 4-0 silk following a midline abdominal incision. Sham-operated mice had their ureters exposed, manipulated but not ligated. Mice were randomly assigned to one of four groups (n=5): (1) sham control, (2) UUO 3 days, (3) UUO 7 days, and (4) UUO 14 days. For the adriamycin nephropathy model, BALB/c mice were administered a single intravenous injection of adriamycin (doxorubicin hydrochloride; Sigma, St. Louis, MO) at 10 mg/kg body weight. Groups of mice (n=5) were euthanized at 1, 3, and 5 weeks after adriamycin injection, respectively. Renal IRI was established as described elsewhere.29 Briefly, after mice were anesthetized, a midline abdominal incision was made and bilateral renal pedicles were clamped for 35 minutes using microaneurysm clamps. During the ischemic period, body temperature was maintained between 35–37.5°C using a temperature-controlled heating system. After removal of the clamps, reperfusion of the kidneys was visually confirmed. Mice were killed at 7 days post-IRI, and kidney tissues were collected for various analyses.

For studying the effects of exogenous Klotho on CKD, two sets of experiments were performed. The detailed experimental design was presented in Figures 6C and 8A, respectively. In vivo expression of
secreted Klotho in mice was carried out by a hydrodynamic-based gene delivery approach, as described previously.35 Briefly, groups of mice were administered human secreted Klotho expression plasmid (pV5-sKlotho)38 (Addgene, Cambridge, MA) or empty vector (pcDNA3) by rapid injection of a large volume of DNA solution through the tail vein. In the first set of experiments, four groups of CD-1 mice were used: (1) sham control (n=5), (2) UUO mice injected with empty vector pcDNA3 (n=6), (3) UUO mice injected with pV5-sKlotho plasmid 1 day before surgery (n=7), and (4) UUO mice injected with pV5-sKlotho plasmid 3 days after surgery (n=7). The mice were euthanized at day 7 after UUO, and kidney tissues were collected for subsequent analyses. In the second set of experiments, three groups of BALB/c mice were used: (1) normal control (n=6), (2) adriamycin mice injected with pcDNA3 vector (n=7), and (3) adriamycin mice injected with pV5-sKlotho plasmid at weeks 1 and 2 (n=7). At 3 weeks after adriamycin injection, all mice were euthanized. Urine and kidney tissue were collected for various analyses. All animal studies were performed by use of the procedures approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

Cell Culture and Treatment
Human proximal tubular epithelial cells (HKC, clone 8) were provided by Dr. L. Racusen (Johns Hopkins University, Baltimore, MD). Cell culture was carried out according to the procedures described previously.27 HKC-8 cells were treated with human recombinant TGF-β1 (R & D Systems, Minneapolis, MN) at 2 ng/ml for various periods of time, except as otherwise indicated. For some experiments, HKC-8 cells were pretreated with TGF-β receptor type 1 inhibitor SB431542 (Tocris Bioscience, Bristol, United Kingdom) at 10 μM or Smad3 inhibitor SIS3 (EMD Millipore, Billerica, MA) at 10 μM for 1 hour, followed by incubation with TGF-β1. Whole-cell lysates were prepared and subjected to Western blot analyses. HKC-8 cells were transiently transfected with various expression vectors, such as HA-tagged Wnt1 expression plasmid (pHA-Wnt1; Upstate Biotechnology), human membrane Klotho (pV5-mKlotho; Addgene) or secreted Klotho (pV5-sKlotho) by using Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY).

**Histology and Immunohistochemical Staining**
Paraffin-embedded mouse kidney sections (3 μm thickness) were prepared by a routine procedure. Sections were stained with hematoxylin-eosin, PAS reagents by standard protocol. Kidney sections were also subjected to Masson trichrome staining for assessing collagen deposition and fibrotic lesions. Quantification of the fibrotic area was carried out by computer-aided morphometric analysis (MetaMorph, Universal Imaging Co., Downingtown, PA), as described previously.27 Immunohistochemical staining was performed using routine protocols.29 Antibodies used were as follows: goat polyclonal anti-Klotho (AF1819, R&D System), rabbit polyclonal anti-β-catenin (ab15180; Abcam, Cambridge, MA) and mouse monoclonal anti-α-SMA (A2547; Sigma, St. Louis, MO). Incubation with primary antibodies at 4°C overnight, the slides were then stained with horseradish peroxidase–conjugated secondary antibody (Jackson Immuno-Research Laboratories, West Grove, PA). Nonimmune normal IgG was used to replace primary antibodies as a negative control, and no staining occurred. Slides were viewed under a Nikon Eclipse E600 microscope equipped with a digital camera (Melville, NY).

**Immunofluorescence Staining and Confocal Microscopy**
Kidney cryosections were fixed with 3.7% paraformaldehyde for 15 minutes at room temperature. HKC-8 cells cultured on coverslips were fixed with cold methanol:acetone (1:1) for 10 minutes at −20°C. After blocking with 10% donkey serum for 30 minutes, the slides were immunostained with primary antibodies against Klotho (R&D System) and β-catenin (Abcam) as aforementioned, as well as rabbit polyclonal antifibronectin (F3648, Sigma), rabbit polyclonal anticalcificener-I (234167; EMD Millipore), mouse polyclonal anti–PAI-1 (sc-5297; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti–α-actin (AB2129; EMD Millipore), rabbit polyclonal antithio-oxid-sensitive NaCl cotransporter (NCC) (AB3553; EMD Millipore), rabbit polyclonal anti–α-actin (AB3067; EMD Millipore), guinea pig polyclonal antinephrin (20R-NP002, Fitzgerald Industries International, Concord, MA), rabbit polyclonal anti-WT1 (sc-192; Santa Cruz Biotechnology), and mouse monoclonal anti–active β-catenin (05–665, Millipore). The slides were then stained with Cy3- or Cy2-conjugated secondary antibody (Jackson Immunoresearch Laboratories), and mounted with Vectashield antifade mounting media by using DAPI to visualize the nuclei (Vector Laboratories, Burlingame, CA). Slides were viewed under a Leica TCS-SL confocal microscope.

**Western Blot Analysis**
Protein expression was analyzed by Western blot analysis as described previously.29 The primary antibodies used were as follows: anti-Klotho (R&D System), mouse monoclonal anti–active β-catenin (05–665; Millipore), anti–PAI-1 (Santa Cruz), rabbit polyclonal anti-Snail1 (ab17732; Abcam), antifibronectin (Sigma), anti–α-SMA (Sigma), mouse monoclonal anti–TATA binding protein (ab818; Abcam), rabbit monoclonal anti–GAPDH (#2118, Cell Signaling, Denver, MA), mouse monoclonal anti–pan-specific actin (MAB1501; Millipore), antinephrin (Fitzgerald Industries International), and rabbit polyclonal anti-WT1 (ab15251; Abcam, Cambridge, MA).

**Immunoprecipitation**
The interaction of Klotho and Wnts in HKC cells was determined by co-immunoprecipitation, as previously described.18 HKC-8 cells were co-transfected with Klotho plasmid vectors (pV5-mKlotho or pV5-sKlotho) and different Wnt plasmids for 24 hours. Cell lysates were immunoprecipitated overnight at 4°C with anti-Klotho antibody (R&D System) and protein A/G plus agarose (sc-2003; Santa Cruz Biotechnology). The precipitated complexes were washed three times with lysis buffer and boiled for 5 minutes in SDS sample buffer, followed by immunoblotting with rabbit polyclonal anti-Wnt1 (ab15251, Abcam), rabbit polyclonal anti-Wnt4 (sc-13962), and goat polyclonal anti-Wnt7a (sc-26361; Santa Cruz Biotechnology), respectively. For detection of Klotho/Wnt interaction in cell culture medium, HKC-8 cells were transfected with pV5-mKlotho and pHA-Wnt1 plasmids, and the culture medium was collected at 36 hours.
after transfection, followed by immunoprecipitated overnight at 4°C with rabbit polyclonal anti-V5 epitope tag antibody (AB3792, Millipore) and protein A/G plus. The immunocomplexes were blotted with rabbit polyclonal anti-HA tag antibody (ab9110, Abcam). Cell lysates were also immuno blotted with antibodies against Klotho and actin, respectively.

**Nuclear and Cytoplasmic Fractionation**

For preparation of nuclear protein, HKC-8 cells were collected and washed twice with cold PBS. Nuclear and cytoplasmic protein was prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents, according to the protocols specified by the manufacturer (Thermo Scientific, Rockford, IL).

**RT and Real-Time 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 of complementary DNA was synthesized using 1 μg of RNA in 20 μl of reaction buffer containing AMV-RT and random primers at 42°C for 60 minutes. Real-time PCR was performed using a Plantinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen). The sequences of the primer pairs for different human Wnts as well as other genes were shown in Supplemental Table 1. Primer pairs for both mouse and human Klotho were described previously. 46

**Transfection and Luciferase Assay**

The effect of Klotho on β-catenin–mediated gene transcription was assessed by using the TOPFlash reporter plasmid containing two sets of three copies of the T cell factor–binding site at the upstream of the thymidine kinase minimal promoter and luciferase cDNA (Millipore, Billerica, MA). HKC-8 cells were co-transfected using Lipofectamine 2000 reagent with TOPFlash plasmid and Wnt1 expression vector (pHA-Wnt1) or N-terminally truncated, constitutively activated β-catenin expression vector (pDel–β-cat) in the absence or presence of membrane Klotho expression vector (pV5-mKlotho) at the concentrations as indicated. An internal control reporter plasmid (0.1 μg) Renilla reniformis luciferase driven under thymidine kinase promoter (Promega, Madison, WI) was also co-transfected for normalizing the transfection efficiency. Luciferase assay was performed using a dual luciferase assay system kit according to the manufacturer’s protocols (Promega). Relative luciferase activity (arbitrary units) was reported as fold induction over the controls after normalizing for transfection efficiency.

**Urinary Albumin and Creatinine Assay**

Urinary albumin was measured by using a mouse Albumin ELISA Quantitation kit, according to the manufacturer’s protocol (Bethyl Laboratories, Inc., Montgomery, TX). Urine creatinine was determined by a routine procedure as described previously. 18 Urinary albumin was standardized to urine creatinine and expressed as mg/mg urinary creatinine. Serum creatinine level was determined by use of a QuantiChrom creatinine assay kit (DICT-500, Bioassay systems, Hayward, CA), according to the protocols specified by the manufacturer.

Klotho ELISA

Klotho protein level in urine was determined by using a specific ELISA kit for Klotho (E97757Hu; USCN Life Science Inc., Wuhan, China). Urinary Klotho was measured according to the protocols specified by the manufacturer, and expressed as ng per mg creatinine.

**Statistical analyses**

All data examined were expressed as mean ± SEM. Statistical analysis of the data were carried out using SigmaStat software (Jandel Scientific, San Rafael, CA). Comparison between groups was made using one-way ANOVA followed by Student-Newman-Kuels test. P<0.05 was considered to represent a statistically significant difference.

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**DISCLOSURES**

None.

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