Kiellin/Chordin-Like Protein Attenuates both Acute and Chronic Renal Injury

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ABSTRACT
The secreted kielin/chordin-like (KCP) protein, one of a family of cysteine-rich proteins, suppresses TGF-β signaling by sequestering the ligand from its receptor, but it enhances bone morphogenetic protein (BMP) signaling by promoting ligand-receptor interactions. Given the critical roles for TGF-β and BMP proteins in enhancing or suppressing renal interstitial fibrosis, respectively, we examined whether secreted KCP could attenuate renal fibrosis in mouse models of chronic and acute disease. Transgenic mice that express KCP in adult kidneys showed significantly less expression of collagen IV, α-smooth muscle actin, and other markers of disease progression in the unilateral ureteral obstruction model of renal interstitial fibrosis. In the folic acid nephrotoxicity model of acute tubular necrosis, mice expressing KCP survived high doses of folic acid that were lethal for wild-type mice. With a lower dose of folic acid, mice expressing KCP exhibited improved renal recovery compared with wild-type mice. Thus, these data suggest that extracellular regulation of the TGF-β/BMP signaling axis by KCP, and by extension possibly other cysteine-rich domain proteins, can attenuate both acute and chronic renal injury.


Progressive renal diseases, which can lead to end stage and ultimately require dialysis and transplantation, are increasing in frequency and correlate with the rise of diabetes, obesity, and hypertension among populations in the United States and Europe. Renal interstitial and/or glomerular fibrosis is common to most all progressive renal diseases, allograft nephropathy, and aging.1 Yet effective therapies for chronic fibrosis are still not forthcoming.

Among the most well studied signaling pathways in renal fibrotic disease are those of the TGF-β superfamily,2 the most relevant of which are the TGF-βs, bone morphogenetic proteins (BMPs), and Activins. In animal models, increased renal fibrosis correlates with increased expression of TGF-β.3 In mice that overexpress a TGF-β transgene4 or are treated with recombinant TGF-β,5 increased tubular interstitial fibrosis and tubular atrophy were observed over time. In loss-of-function studies, the severity of renal histopathology was reduced in experimental glomerular nephritis models by treating animals with antibodies against TGF-β6 or a soluble type II TGF-β receptor.7 Significantly, genetic deletion of the downstream TGF-β signaling molecule Smad3 also results in protection against renal interstitial fibrosis in the unilateral ureteral obstruction (UOO) model,8,9 suggesting that TGF-β and its effectors drive the initiation and progression of fibrotic disease. BMPs are thought to counteract the profibrotic effects of TGF-β in animal models of renal disease. In the kidney, BMP7 has been studied in detail and was shown to alleviate and even reverse fibrosis, either through direct application or by stimulation of the BMP receptor ALK3.10,11

Regulation of TGF-β superfamily signaling occurs at multiple levels, within and outside of the cell, and offers multiple potential avenues of intervention. The active TGF-β family ligand is a

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disulfide-linked homo- or heterodimer that is processed from large inactive precursors. A diverse family of secreted proteins can inhibit TGF-β superfamily signaling by sequestering ligands away from receptors. In the Golgi, processing of the TGF-β proprotein results in the formation of a latent complex. The latency associated protein LAP1, which is the cleaved amino terminus of the TGF-β proprotein, together with latent TGF-β binding protein 1 (LTBP) and the active TGF-β homodimer constitute the large latent complex, which associates with the extracellular matrix and can be released and activated by proteolytic cleavage. Extracellular inhibitors of BMP signaling include vertebrate chordin, which binds directly to BMPs through the cysteine-rich (CR) domains containing CXXCXC and CCXXC motifs. Whereas chordin blocks BMP/receptor interactions, the CR domain protein KCP enhances BMP/receptor interactions to increase the efficacy of signaling. Similarly, the CR domain protein connective-tissue growth factor also enhances TGF-β-mediated signaling while suppressing the BMP-dependent pathway.

We previously identified KCP (Crim2) as a protein with 18 CR domains that is expressed in the developing kidney at both early and late stages. KCP expression corresponds to the formation of epithelial structures within the intermediate mesoderm and to the formation of the proximal tubules in the more developed metanephric kidney. Unlike chordin or connective-tissue growth factor, KCP enhances BMP-mediated signaling by facilitating the binding of BMP7 to BMP receptor 1A. Conversely, KCP is able to inhibit TGF-β signaling by blocking ligand-receptor interactions. Mice homozygous for a mutant KCP allele show no gross developmental abnormalities but KCP mutations enhance the renal developmental phenotype in mutants of CV2, another gene that encodes a multi-CR domain activator of BMPs. However, Kcp−/− mice exhibit enhanced susceptibility to developing renal interstitial fibrosis in two different animal models, a process regulated by both BMPs and TGF-β. Such animals exhibit lower BMP signaling and higher TGF-β signaling upon renal injury.

In this report, we address whether altering the balance between the TGF-β and BMP signaling pathways can be achieved by ectopic or overexpression of the secreted KCP protein to change the course of renal fibrosis. Transgenic mice were engineered to express KCP protein in renal proximal tubule cells and subjected to UUO or acute tubular necrosis. Although KCP expression by itself had few measurable deleterious affect, KCP transgenic mice were significantly more resistant to interstitial fibrosis and renal injury. These studies point to a novel renal protective function for KCP. That the TGF/BMP signaling cascades can be shifted by a secreted protein opens new therapeutic avenues of intervention for both acute and chronic renal disease.

RESULTS

Creation of KCP Transgenic Mice

The KCP protein is expressed in developing kidneys but is not detected at appreciable levels in healthy adult kidneys until they are subject to injury. To test whether ectopic or overexpression of KCP would ameliorate renal damage, we created a strain of transgenic mice that express KCP in the kidney using a Pepck promoter fragment (Figure 1). The Pepck promoter, reported to be active in renal proximal tubular epithelia, was fused to a human Igk light chain signal peptide to enhance secretion of the downstream KCP protein. A carboxy-terminal myc epitope tag was fused to KCP so that the transgenic protein could be detected. Founder animals were mated to wild-type (WT) and subsequent F1 generations genotyped.
for the transgene. Kidneys and other tissues were analyzed for mycKCP expression by Western blotting and immunohistochemistry (Figure 1). Transgenic KCP expression could be detected in newborn kidney and liver extracts but not in other tissues. In the adult kidney, mycKCP was found primarily in proximal and distal tubules, with staining around the parietal glomerular epithelia with few cells in the glomerular tuft. The strongest expressing founder strain was viable and fertile and used for subsequent renal injury studies.

To directly examine whether exogenous, transgenic KCP could alter the response to signaling by either BMPs or TGF-β, we isolated primary renal epithelial cells from adult kidneys that carried the KCP transgene and from age-matched WT controls. Cells were plated onto plastic, passaged once, and their epithelial nature confirmed by staining for the tight junction marker ZO-1, aquaporin-1 (AQP1), and E-cadherin (Figure 2A). Cells from the KCP transgenic mice also showed strong mycKCP staining in the endoplasmic reticulum, consistent with the processing of the secreted KCP protein. Primary epithelial cells were then treated with increasing doses of TGF-β and whole cell lysates examined for P-Smad after 1 hour (Figure 2B). Quantitation showed a clear inhibition of P-Smad3 accumulation in the KCP expressing cells at the lowest dose of TGF-β (Figure 2B, 0.5 ng/ml). At higher doses, there was no significant difference in P-Smad3 levels between WT and KCP expressing cells. The dose response for BMP7 was measured with an antibody against P-Smad1/5/8 (Figure 2C). At 10 and 100 ng/ml of BMP7, KCP expressing cells showed significantly higher levels of P-Smad1/5/8 compared with WT cells. These data are consistent with previous observations that KCP enhances BMP signaling and suppresses TGF-β signaling.

**UUO Model**

To test whether transgenic KCP protein could affect the progression of renal interstitial fibrosis, we subjected WT control and KCP transgenic mice to UUO. Both obstructed and contralateral kidneys were examined before and 7 and 14 days after obstruction of a single ureter. Western blots of protein lysates from whole kidneys showed increased expression of α-smooth muscle actin (αSMA) at 7 and 14 days after UUO in WT obstructed kidneys and increased levels of P-Smad3 (Figure 3). However, the obstructed kidneys from the KCP transgenic mice had lower levels of αSMA and P-Smad3 compared with the WT controls at 7 and 14 days after UUO (Figure 3). However, levels of P-Smad1 were slightly higher in KCP transgenic mice at 7 and 14 days after UUO compared with WT controls. Kidney injury molecule 1 (Kim1) was also measured as an indicator of renal damage and recovery. Strikingly, Kim1 levels were higher in KCP transgenic mice at 7 and 14 days after UUO.

Interstitial fibrosis was further characterized by immunostaining of kidney sections and quantitative morphometry. Antibodies against αSMA, collagen IV, and Kim1 were used to characterize the degree of interstitial fibrosis at 7 and 14 days after UUO (Figures 4 and 5A). WT UUO kidneys showed more αSMA and collagen IV positive staining at both 7 and 14 days after UUO compared with KCP transgenics. Expansion of extracellular matrix, fibroblasts, and myofibroblasts correlate with decreased renal function and increased interstitial area in the UUO model. The overall amounts of interstitial matrix expansion in WT and KCP kidneys were confirmed by morphometric analysis of multiple sections from at least three independent animals. KCP transgenic mice showed a significant 50% reduction of αSMA and collagen IV surface area staining.
The KCP gene encodes a large, secreted protein capable of enhancing BMP signaling and suppressing TGF-β and Activin signaling. These opposing functions are thought to occur outside of the cell through enhancing or inhibiting receptor ligand interactions. Given the central role for BMPs and activins in renal fibrosis, the KCP gene likely functions to modulate BMP signaling and thereby affect the balance between cell proliferation and matrix deposition. This balance is critical for the repair process following renal injury, and KCP expression may provide a means to modulate this balance in favor of improved recovery and reduced fibrosis.


eur kidney function, acute injury can lead to fibrosis and scarring at sites of injury, especially if regenerating epithelial cells fail to re-populate the damaged tubules completely. Thus, we tested WT and KCP transgenic mice for the ability to recover after folic acid injection. Initial experiments used a slightly higher dose (275 mg/kg) to induce significant renal injury in 2- to 3-month-old mice. At this dose, none of the WT mice recovered from renal injury and all died within 72 hours (Figure 6A). However, 85% of the KCP transgenic mice survived the higher dose of folic acid and recovered sufficient renal function to survive beyond 9 months of age. However, at this dose of folic acid, we did not analyze fibrosis in the survivors because we had no WT mice for comparison.

At the lower dose of folic acid (250 mg/kg), survival for both WT and KCP transgenics was equivalent and the animals were followed postrecovery to assess short- and long-term damage. Kidneys were isolated from untreated or folic acid–treated animals at 2 days, 14 days, and 28 days postinjection and were assayed using Western blotting and immunohistochemistry. Quantitative morphometry (Figure 6B) of immunostainings (Figure 7) was done at all time points. At 2 days, induction of Kim1 expression (Figure 6D) and increased collagen IV and αSMA (Figures 6, B and C, and 7) was evidence for acute injury. KCP transgenic mice had less collagen IV positive surface area but similar levels of αSMA. By 14 days, levels of αSMA and collagen IV were decreasing, with KCP transgenics showing less αSMA positive surface area. By 28 days, both collagen IV and αSMA interstitial areas were almost back to untreated levels. Differences in P-Smad levels could also be seen at certain time points. In KCP transgenic kidneys, levels of P-Smad1 were higher at 28 days in KCP transgenics, but P-Smad3 levels were lower at 14 days. These data suggest that KCP expression in adult kidneys can lessen the severity of acute tubular injury and accelerate the recovery phase.

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**Acute Tubular Necrosis Model**

To assess the effects of KCP on an alternative model of renal injury, we utilized the folic acid nephrotoxicity model of AKI. A single injection of 250 mg/kg body weight of folic acid results in ARF within 24 hours, followed by a period of recovery. Despite recovery of renal function, acute injury can lead to fibrosis and scarring at sites of injury, especially if regenerating epithelial cells fail to re-populate the damaged tubules completely. Thus, we tested WT and KCP transgenic mice for the ability to recover after folic acid injection. Initial experiments used a slightly higher dose (275 mg/kg) to induce significant renal injury in 2- to 3-month-old mice. At this dose, none of the WT mice recovered from renal injury and all died within 72 hours (Figure 6A). However, 85% of the KCP transgenic mice survived the higher dose of folic acid and recovered sufficient renal function to survive beyond 9 months of age. However, at this dose of folic acid, we did not analyze fibrosis in the survivors because we had no WT mice for comparison.

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TGF-β in renal fibrosis, we hypothesized that excess KCP in renal proximal tubules could delay the progression of chronic interstitial fibrosis in animal models. Thus, we generated lines of transgenic mice that express KCP under the control of a Pepck promoter fragment that was previously shown to work well in the kidney. Transgene expression was detected in liver and kidney, with renal expression limited to proximal tubules and parietal epithelial cells of Bowman’s capsule. These KCP transgenics showed significantly less accumulation of collagen IV and αSMA in the interstitium of obstructed kidneys in the UUO model of fibrosis. In an AKI model, KCP transgenic mice showed significantly less mortality at high doses of the nephrotoxin folic acid and showed some limited signs of accelerated recovery. These data provide evidence that modulation of the TGF-β superfamily of ligands, through the enhanced expression of secreted accessory proteins, may provide alternative therapies to direct application of BMPs, receptor stimulation, or the direct inhibition of TGF-β signaling.

Several lines of evidence suggest that KCP attenuates renal injury. Levels of P-Smad3 were generally lower in KCP transgenics at 7, 14, and 28 days after obstruction, whereas levels of P-Smad1 were slightly higher at 7 and 14 days after obstruction. This observation was consistent with our understanding of KCP function and suggests that the signaling paradigm was shifted from less TGF-β to more BMPs. Experiments in isolated primary epithelial cells confirmed both the enhanced sensitivity of KCP cells to BMP7 and the inhibitory effects of KCP at low doses of TGF-β. We also noted increased Kim1 protein levels in KCP transgenic mice at 7 and 14 days after obstruction. Although Kim1 was identified as a biomarker for injured kidney tubules, it is unclear whether its expression is harmful or beneficial. Of note is the recent finding that Kim1 binds to LMIR5/CD300b to recruit neutrophils to sites of tissue injury. Deletion of LMIR5 suppresses the inflammatory response in AKI to suppress tubular necrosis, suggesting that Kim1 enhances the response to injury. However, our observation that Kim1 expression increases in KCP transgenic kidneys and that this increase correlates with a reduction in interstitial fibrosis would suggest that Kim1 is beneficial and may work in the recovery or regeneration phase after injury.

In the folic acid model of acute injury, the effects of KCP transgenic expression on the recovery phase were less pronounced at normal dose, compared with the UUO model. Nevertheless, lower collagen and αSMA levels were observed at specific time points after injury, suggesting that KCP could provide some enhancement of recovery. The most significant effect of transgenic KCP was on the initial survival at higher doses of folic acid. Using 275 mg/kg of folic acid, a single dose induced serious renal failure and death in 100% of WT mice within 4 days, whereas 85% of KCP transgenic mice survived and recovered. This result suggests significant protection in KCP mice against the initial acute injury within the first 24–48 hours. This protective effect could be due to enhanced regeneration in the initial 24- to 48-hour period after injury or a reduction in cell death immediately after injury.

Figure 4. Attenuation of fibrosis in KCP transgenic kidneys after UUO. Representative sections are shown from control, 7 day UUO, and 14 day UUO kidneys for WT and KCP transgenics. (A) Kim1 (green) and αSMA (red) staining show decreased interstitial αSMA and increased epithelial Kim1 expression in KCP transgenic kidneys. (B) Collagen IV (red) and Kim (green) staining shows increased and more diffuse collagen IV staining in WT kidneys at 7 and 14 days after UUO and persistent Kim1 expression in KCP transgenic kidneys.
The relationship between chronic renal disease and AKI is becoming clearer, because maladaptive repair and cumulative scarring after repeated acute injury may underlie the chronic condition. The failure to fully re-populate the injured proximal tubules, the infiltration of macrophages and neutrophils, the expansion of interstitial fibroblasts, and prolonged hypoxia from damaged vasculature can all contribute to focal regions of fibrosis after the acute phase is resolved but that cumulatively can lead to a slowly progressing form of renal disease depending on the frequency and duration of subsequent acute injury incidents. Thus, any reduction in acute injury can potentially reduce the frequency of chronic disease even if such acute phases are subclinical. Indeed, repeated bouts of acute injury could accentuate the protective effects of KCP in transgenic mice, a hypothesis that merits further testing.

The use of transgenic expression, while convenient as a proof of principle, suffered from several caveats. We noted that transgenic KCP expression was not uniform on histologic sections and also varied over time after injury. The Pepck promoter is responsive to conditions that may alter KCP expression in our mice, such as local metabolic acidosis. The size and complexity of KCP is such that it becomes impractical to produce and administer the protein to laboratory animals. However, our results demonstrate that KCP and perhaps related CR domain proteins can be therapeutic if expressed or administered in the right context. Our studies provide a conceptual basis for the use of CR domains or derivatives to attenuate profibrotic pathways that depend on continued TGF-β signaling and/or counteraction by BMPs.

**CONCISE METHODS**

**Animals**

Mice were kept according to National Institutes of Health guidelines and all procedures approved by the University Committee on Use and Care of Animals at the University of Michigan. The transgene was linearized and injected into fertilized mouse eggs by the University of Michigan Transgenic Animal Core Facility. After implantation into pseudopregnant female mice, offspring were genotyped at weaning with PCR primers specific for the Pepck promoter and KCP coding region. For the UUO model, mice were anesthetized by isoflurane inhalation. Through a midline abdominal incision, the right ureter was exposed and tied off at the mid-ureteral level with fine suture materials (4-0 silk) to induce a complete obstruction. Mice were allowed to recover from anesthesia and were supplied food and water *ad libitum* until the indicated time of sacrifice (7, 14, and 28 days). For each time point, five mice were taken of each genotype. Both obstructed and contralateral kidneys were harvested for RNA, histology, and protein analyses. Contralateral and obstructed kidneys were removed and cut in half. One-half of each kidney was fixed in 4% paraformaldehyde and embedded in paraffin blocks. The other half of each kidney was fresh frozen and used for protein and RNA analysis. In addition, blood samples were obtained from the abdominal aorta.

For the acute tubular necrosis model, 20 WT and 20 KCP transgenic mice of similar ages were injected intraperitoneally with a single dose of 275 mg/kg of folic acid in a total volume of 500 μl of 0.15 M NaHCO₃. Subsequent experiments utilized a single 250 mg/kg dose of folic acid to induce acute tubular necrosis; all control mice received only NaHCO₃. Mice that received the dose of 250 mg/kg were sacrificed at 2, 14, or 28 days after injury. Mice were put under constant observation for the first 48 hours after injection. At each endpoint, mice were anesthetized with intraperitoneal injection of ketamine/xylazine. Sera were collected, and one kidney was removed and fresh frozen for protein and RNA analysis and the other was perfused fixed for immunohistochemistry and histology.

**Immunostaining**

Kidneys were perfused fixed or fixed overnight in 4% paraformaldehyde and paraffin embedded. Immunofluorescence detection was done using modifications of published protocols. Paraffin sections of 5 μm were rehydrated and stained with mouse anti-Myc-KCP (9E10; BaCO) or monoclonal anti-Myc-FITC (Sigma), rabbit anti-Myc-KCP, rabbit anti-Laminin (1.9393; Sigma), monoclonal anti-α-SMA-Cy3 conjugated (catalog # C6193; Sigma), rabbit anti-collagen IV
Primary Renal Epithelial Cell Cultures

Primary renal epithelial cells were isolated from the cortex of 5- to 6-week-old female mice. Briefly, the medulla was manually removed and cortex was digested by Liberase DH (Roche) in DMEM (Lonza). The tissue fragments were sieved through a 212-μm pore size mesh. After three washes with cold DMEM, cells were expanded in UltraMDCK serum-free medium (Roche) supplied with 0.5× insulin-transferring-ethanolamine-selenium (Lonza), 60 μg/L EGF (R&D systems), 10⁻⁹ M triiodothyronine, and 1× antibiotic antimycotic (Gibco). Recombinant human TGF-β1 and BMP7 were from R&D Systems. Protein lysates were harvested 1 hour after addition of factors and analyzed by SDS/PAGE and Western blotting.

Western Blot and RNA Analyses

A quarter of the flash-frozen kidney was homogenized with a polytron and protein extracts were prepared as described. Equal amounts of protein were separated on 8% SDS gels, and Western blotted using the following antibodies: mouse anti-αSMA (Sigma), rabbit anti-P-Smad1/5/8 (catalog # 9511; Cell Signaling), mouse anti-Smad1 (catalog # sc-7965; Santa Cruz Biototechnology), rabbit anti-P-Smad3 (catalog # ab52903; Abcam), rabbit anti-Smad3 (Cell Signaling), goat anti-Kim1 (anti-mTIM1, catalog # AF1817; R&D Systems), mouse antimyc (9E10; BaCO), rabbit anti-KCP, mouse anti-β-tubulin (Sigma), and rabbit anti-α-actin (catalog # A2066; Sigma Aldrich).

RNA Analyses

A quarter of each frozen kidney was lysed in TRIzol using a polytron homogenizer and RNAs extracted following the manufacturer’s instructions. Isolated RNAs were used for the semiquantitative real-time quantitative PCR were done following the previously described protocol. Approximately 2–3 μg total RNA was reverse-transcribed into cDNA with a SuperScript First-Strand Kit (Invitrogen). The cDNA products were diluted five times and amplified with the iTaq SYBR Green Master Mix (Bio-Rad) in a Prism 7500 (Applied Biosystems). Primers pairs for quantitative PCR were as follows: Wnt11: 5'-GGGCCAAGTTTTCCGATGCT, 5'-TTCGTGCTGACAGGTAGCG; ZEB1: 5'-TCAAGTAAACACCACCTG, 5'-TGGCGAGGAACACTGAGA; PAI1: 5'-ACATGTTTAGTGCAACCCTG, 5'-GGTCTATAACCATCTCCGTG; Snai1: 5'-GGAAGCCCAACTATAGCGA, 5'-AGCGAGGTCAGCTCTACG; and GAPDH: 5'-ACCACAGTCCATGCCATCAC, 5'-TCCACACCCTGGTGTGCAG.

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DISCLOSURES

None.
Figure 7. Reduced fibrosis after AKI in KCP transgenic kidneys. Representative sections are shown from WT and KCP transgenic kidneys before and after folic acid injection and stained with the indicated antibodies. (A) Collagen IV is red and Kim1 is green. (B) αSMA is red and Kim1 is green. The times after folic acid injection are indicated below.

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