Chronic epithelial kidney injury molecule-1 expression causes murine kidney fibrosis

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Chronic epithelial kidney injury molecule-1 expression causes murine kidney fibrosis

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Acute kidney injury predisposes patients to the development of both chronic kidney disease and end-stage renal failure, but the molecular details underlying this important clinical association remain obscure. We report that kidney injury molecule-1 (KIM-1), an epithelial phosphatidylserine receptor expressed transiently after acute injury and chronically in fibrotic renal disease, promotes kidney fibrosis. Conditional expression of KIM-1 in renal epithelial cells (Kim1RECtg) in the absence of an injury stimulus resulted in focal epithelial vacuolization at birth, but otherwise normal tubule histology and kidney function. By 4 weeks of age, Kim1RECtg mice developed spontaneous and progressive interstitial kidney inflammation with fibrosis, leading to renal failure with anemia, proteinuria, hyperphosphatemia, hypertension, cardiac hypertrophy, and death, analogous to progressive kidney disease in humans. Kim1RECtg kidneys had elevated expression of proinflammatory monocyte chemotactic protein-1 (MCP-1) at early time points. Heterologous expression of KIM-1 in an immortalized proximal tubule cell line triggered MCP-1 secretion and increased MCP-1–dependent macrophage chemotaxis. In mice expressing a mutant, truncated KIM-1 polypeptide, experimental kidney fibrosis was ameliorated with reduced levels of MCP-1, consistent with a profibrotic role for native KIM-1. Thus, sustained KIM-1 expression promotes kidney fibrosis and provides a link between acute and recurrent injury with progressive chronic kidney disease.

Introduction

Acute kidney injury (AKI) is characterized by a rapid decline in kidney function, often triggered by an ischemic or toxic insult. This clinical syndrome is associated with substantial short-term morbidity, mortality, and cost, but it had previously been assumed that patients surviving the episode made a full renal recovery (1). However, AKI is now appreciated to be markedly associated with increased risk of future chronic kidney disease (CKD), end-stage renal disease (ESRD) (2, 3), and long-term mortality (4). The population rate of AKI is increasing at greater than 7% per year (5, 6), and some estimates indicate that the incidence of AKI-related ESRD is equal to the incidence of ESRD from diabetes (7). The mechanisms that might explain the link between AKI and future CKD/ESRD are poorly understood, with peritubular capillary loss, a known consequence of AKI (8), is proposed to lead to chronic hypoxia and later development of tubulointerstitial fibrosis and CKD (9, 10). How chronic ischemia might trigger parenchymal loss at a molecular level is unresolved.

Kidney injury molecule-1 (KIM-1), originally identified as hepatitis A virus receptor (HAVCR1, also known as Tim-1), is a type 1 transmembrane protein strongly induced by ischemic and toxic insults to kidney. It also plays diverse roles in T and B cell biology (11). In healthy kidney, KIM-1 is undetectable, but after injury, it is induced more than any other protein, in which case it localizes to the apical surface of surviving proximal tubule epithelial cells (12). The extracellular KIM-1 Ig variable domain binds and internalizes oxidized lipid as well as phosphatidylserine exposed on the outer leaflet of luminal apoptotic cells (13, 14), thereby aiding in nephron repair and tissue remodeling through phagocytosis of cells and debris (15). KIM-1 is expressed in CKD (16–20) where it colocalizes with areas of fibrosis and inflammation (21), and its expression correlates directly with interstitial fibrosis in human allografts (22). Increased urinary KIM-1 is an independent predictor of long-term renal graft loss and is also elevated in human nondiabetic, proteinuric CKD (23, 24). The expression of KIM-1 in chronic and progressive kidney disease, settings without significant numbers of apoptotic cells in the tubule lumen, the epidemiologic association of AKI with future CKD (25), and the temporal and spatial association of KIM-1 with inflammation and fibrosis suggest that it might play a pathogenic role in linking AKI to CKD and renal fibrosis.

In this study, we examined the functional consequences of chronic KIM-1 expression in renal epithelial cells. To dissociate the effects of KIM-1 expression from the pleiotropic effects of
ischemic kidney injury used to induce KIM-1, we created a genetic model in which KIM-1 is expressed chronically in the absence of any injury stimulus. Using this model, we demonstrate here that chronic KIM-1 expression leads to inflammation, tubulointerstitial fibrosis characterized by elevated monocyte chemotactic protein-1 (MCP-1) levels and a murine CKD phenotype. In contrast, mice with mutant endogenous KIM-1 were protected from fibrosis in a mouse model of CKD and had a reduced level of MCP-1. Together, these results indicate that persistent KIM-1 expression after AKI promotes interstitial fibrosis and correlates with MCP-1 expression and further suggest that KIM-1 may represent a novel therapeutic target in CKD.

Methods

Materials and Methods

In a mouse model of CKD and had a reduced level of MCP-1. Together, these results indicate that persistent KIM-1 expression after AKI promotes interstitial fibrosis and correlates with MCP-1 expression and further suggest that KIM-1 may represent a novel therapeutic target in CKD.

Results

To determine the kinetics of KIM-1 induction during fibrotic disease, we examined the time course for KIM-1 expression in a rodent model of renal fibrosis, unilateral ureteral obstruction (UUO). KIM-1 protein was strongly upregulated 2 days after ureteral obstruction and fell thereafter, but remained significantly elevated at day 14 (Figure 1A). KIM-1 was expressed on the apical aspect of proximal tubule epithelia, in tubules surrounded by expanded interstitium with abundant interstitial smooth muscle actin–positive myofibroblasts (Figure 1B). To distinguish between KIM-1 expression as a cause or consequence of epithelial injury and fibrosis in vivo, we created a conditional Z/Kim1-AP transgene enabling Cre recombinase-dependent activation of KIM-1 and alkaline phosphatase (AP) expression (Figure 2, A–E). Crossing the Z/Kim1-AP mouse with Six2-GFPCre mice (hereafter referred to as Six2-GC) (27), generated bigenic Kim1RECtg (Kim1 renal epithelial cell transgenic) mice with KIM-1 and AP expression in metanephric mesenchyme–derived kidney epithelia. Kim1RECtg kidneys expressed the Z/Kim1-AP transgene primarily in cortical and outer medullary epithelia, with rare transgene expression in inner medulla (Figure 2E and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI45361DS1). Mosaic transgene activity was observed with 10%–20% of renal tubules positive for AP activity, with a similar fraction of positive podocytes. Note, however, that despite the AP expression pattern, KIM-1 protein was only seen in tubules and never in podocytes (Figure 3C and data not shown). A comparison of the distribution of endogenous KIM-1 after UUO versus AP expression in Kim1RECtg kidneys is presented in Table 1.

Kim1RECtg mice were born at expected Mendelian ratios and expressed Kim1 mRNA at birth (Figure 3A). KIM-1 protein was properly sorted to the apical membrane of cortical proximal tubule epithelia (Figure 3, B and C). There was no difference in the birth weights of transgenic versus littermate control mice (n = 3 Kim1RECtg or 7 littermate controls), but Kim1RECtg mice did not gain weight as quickly as littermate controls (Supplemental Figure 2). At birth, kidneys from Kim1RECtg mice were 23% smaller by weight, however, than those of littermate controls (Figure 3, D and E, P < 0.05, n = 5 Kim1RECtg or 13 control kidneys). This was associated with 43% fewer nephrons in Kim1RECtg kidneys without significant differences in glomerular diameter at P14 (Figure 3, F and G). Kidney histology at P1 showed a mild reduction in cortical thickness, with occasional microcysts that appeared to be glomerular (about 10% of total glomeruli; Figure 3H) and rare large cysts (fewer than 1 per section). A detailed histologic analysis at P15 revealed normal glomeruli including foot processes, however (Figure 3, I and J), as well as normal interstitium and vasculature. Focal coarse vacuolization and focal epithelial degeneration were noted only in Kim1RECtg mouse kidneys (n = 3 Kim1RECtg and 3 control kidneys; Tables 2 and 3). These coarse vacuoles, suggestive of local injury, were found in about 1% of tubules (Figure 3, K and L). There were no histologic differences in other organs of Kim1RECtg mice when compared with organs from littermate controls (data not shown). Thus, P15 kidneys from Kim1RECtg mice were characterized by reduced nephron endowment and rare tubular epithelial vacuolization, but kidney histology was otherwise normal.

At 5 weeks, kidneys from Kim1RECtg mice developed a patchy mononuclear interstitial infiltrate with occasional hylane casts and focal tubular damage. Kim1-1 continued to be expressed in a subset of tubular epithelial cells along the apical membrane (Supplemental Figure 3). By 12 weeks, interstitial inflammation was extensive, together with tubular dedifferentiation, microcystic tubular dilation, hylane casts, and fibrosis. This inflammatory, tubular injury, and fibrotic phenotype was observed in all Kim1RECtg mice older than 6 weeks that were examined (Figure 4A and Supplemental Figure 4). In the oldest mice, prominent periarterial inflammation was present resembling ectopic lymph nodes. Tubular injury scores confirmed these histologic observations (Figure 4B). Serum creatinine was equal between transgenic and control mice at P14, but...
rose progressively thereafter (Figure 4C), and kidneys from aged mice were shrunken with a cobblestone appearance typical of end-stage renal fibrosis (Figure 4D). *Kim1RECtg* mice died spontaneously of progressive renal failure at a median age of 11 weeks (Figure 4E).

Immunohistochemistry and collagen stains confirmed early focal fibrosis surrounding isolated tubules beginning at 4 weeks, whereas older mice exhibited extensive fibrosis, with abundant α-SMA-positive interstitial myofibroblasts and collagen fiber deposition (Figure 5A). In *Kim1RECtg* mice with established fibrotic disease, AP transgene expression was expressed in some, but not all, damaged and dilated tubules, consistent with transgene expression in 10%–20% of tubules (Figure 5B). We did not detect any interstitial cells that expressed the AP transgene, arguing against any direct contribution of injured epithelial cells to the myofibroblast population through epithelial-to-mesenchymal transition and consistent with the notion that epithelia are not capable of contributing directly to the interstitial myofibroblast pool (28, 29).

Given the progressive kidney disease exhibited by *Kim1RECtg* mice, we looked for extrarenal manifestations of CKD. Cardiac hypertrophy often accompanies CKD in humans and is linked to the very high cardiac mortality associated with CKD (30). We performed cardiac ultrasound in *Kim1RECtg* mice at age 10 to 12 weeks, a time when the renal phenotype is well established. At this time point, there was no increase in the systolic blood pressure (Figure 5, E–I), nor was there an increase in the interventricular septal thickness, left ventricular end-diastolic diameter, or left ventricular posterior wall dimensions in *Kim1RECtg* compared with littermate controls (Table 4). There was a significantly increased percentage fractional shortening, consistent with increased cardiac contractility, which is likely a consequence of the anemia that the mice develop (Figure 5J). In contrast, *Kim1RECtg* mice that survived past 6 months of age had clear evidence of left ventricular hypertrophy, measured as ventricular wall thickness-to-diameter ratio (Figure 5, C and D). If hypertension were the primary cause of the renal fibrosis in
Kim^{RECtg} mice then cardiac hypertrophy would have been expected to occur by the 10- to 12-week time point when renal injury and dysfunction were severe.

The presence of a normal blood pressure at 10 to 12 weeks is also consistent with the notion that hypertension was not simply a consequence of reduced nephron endowment, because it did not precede the renal phenotype, but rather that hypertension was a consequence of severe reduction in glomerular filtration rate (Figure 5E). The severe and progressive anemia developed by the Kim^{RECtg} mice (Figure 5F) was normocytic (mean corpuscular volume was not different between control and Kim^{RECtg} mice; data not shown). Late stages of CKD are characterized by hyperkalemia, hyperphosphatemia, and hypoalbuminemia. Thus, Kim^{RECtg} mice displayed all 3 of these characteristics in a progressive fashion over time (Table 5).

The phenotype is not a result of primary proteinuria, podocyte expression of KIM-1, or general toxicity of AP. Chronic proteinuria of any cause has been proposed to drive renal fibrosis. Kim^{RECtg} mice developed proteinuria after 4 weeks of age, subsequent to tubular damage and leukocyte influx (Figure 5G and Supplemental Figure 5). Importantly, there was no proteinuria in Kim^{RECtg} mice at P14, when podocyte foot processes and glomerular capillary endothelium were normal (Figure 3J). Since KIM-1 is not normally expressed in podocytes, we investigated whether the Kim^{RECtg} phenotype was

<table>
<thead>
<tr>
<th>Podocytes</th>
<th>Proximal tubules</th>
<th>Distal tubules</th>
<th>Collecting duct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous KIM-1</td>
<td>0</td>
<td>90%–100%</td>
<td>0</td>
</tr>
<tr>
<td>Kim^{RECtg} (AP)</td>
<td>10%–20%</td>
<td>10%–20%</td>
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</table>

Table 1 Comparison of endogenous Kim1 expression after UUO and AP expression in Kim^{RECtg} mice
a consequence of delayed toxicity to podocytes from glomerular KIM-1 expression. Mice with KIM-1–AP expression exclusively in podocytes (bigenic Podocin-Cre;Z/Kim1-AP) had expression of AP activity in 55% of glomeruli in a focal pattern (Supplemental Figure 5) with histologically normal glomeruli (data not shown). There was no effect on kidney size, and mice did not develop proteinuria even up to 6 months of age. Serum creatinine and hematocrit have the same values as in littermate controls (Figure 5, I and J). Taken together, these observations show that renal fibrosis was not a secondary consequence of abnormal glomerular development, early hypertension, or podocyte expression of KIM-1. We also evaluated the possibility that KIM-1 might directly regulate epithelial cytokine expression. To test this possibility, we stably expressed either vector alone (pcDNA-LLC) or KIM-1 (KIM-1–LLC) in LLC-PK1 porcine proximal tubule cells. The supernatant from these cultures encoding a panel of cytokines capable of being secreted by epithelial pattern recognition receptors that might mediate leukocyte recruitment. We detected a strong upregulation of mRNA encoding a panel of cytokines capable of being secreted by epithelial cells at 4 weeks (Figure 7C). Three of these cytokines, CXCL-1, MCP-1, and TGF-β, were upregulated at 2 weeks—a time point without evident histologic damage.

The inflammation observed in Kim1RECtg mice coupled with increased proinflammatory cytokine expression suggested the possibility that KIM-1 might directly regulate epithelial cytokine expression. To test this possibility, we stably expressed either vector alone (pcDNA-LLC) or KIM-1 (KIM-1–LLC) in LLC-PK1 porcine proximal tubule cells. The supernatant from these cultures was collected and assayed for cytokines. The supernatant from KIM-1–expressing, but not control, cultures showed significant elevations in the levels of TGF-β, MCP-1, and IL-6 (Figure 7, D–F).

We next asked whether conditioned medium from KIM-1–LLC cells might also induce chemotaxis. pcDNA-LLC and KIM-1–LLC cells were plated in lower wells of the Boyden chamber until confluency, then washed and incubated overnight with DMEM. 5 × 10⁵ phorbol ester-activated U937 cells or primary mouse BM-derived macrophages (MBMDM) were added to the upper wells. After 3 hours, cells that migrated through the filters were fixed, stained, and quantitated. In both cases, significantly more cells migrated in response to KIM-1–LLC medium compared with control (Figure 7, G and H). This effect could be largely abrogated by addition of a neutralizing anti–MCP-1 antibody (Figure 7I), consistent with the increased expression of MCP-1 detected in KIM-1–LLC supernatant.

Table 2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tubule histology</th>
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<tr>
<td></td>
<td>Reabsorption granules</td>
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<tr>
<td>Control 1</td>
<td>± (focal)</td>
</tr>
<tr>
<td>Control 2</td>
<td>1+ (focal apical)</td>
</tr>
<tr>
<td>Control 3</td>
<td>0</td>
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<tr>
<td>Transgenic 1</td>
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<tr>
<td>Transgenic 2</td>
<td>± (focal)</td>
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<tr>
<td>Transgenic 3</td>
<td>1+ (focal apical)</td>
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Table 3

<table>
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<tr>
<th>Genotype</th>
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<td>Control 3</td>
<td>± (focal)</td>
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<tr>
<td>Transgenic 2</td>
<td>± (focal)</td>
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<tr>
<td>Transgenic 3</td>
<td>1+ (focal)</td>
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</table>

GBM, glomerular basement membrane; Mes., mesangial.
Soluble fibronectin is a known inducer of proinflammatory cytokines in renal epithelial cells (33), and the KIM-1–expressing cell line had substantially elevated fibronectin expression compared with control cells. Moreover, in a third cell line that expressed a point mutant of a cytoplasmic tyrosine within a consensus phosphorylation sequence (Y350F), fibronectin levels matched those of control LLC-PK1 cells (Figure 7J).

These findings indicate that KIM-1 expression drives proinflammatory cytokine expression, suggesting a mechanism to explain enhanced leukocyte infiltration at early time points in the Kim1RECtg mouse model. KIM-1 also drives fibronectin expression in LLC-PK1 cells through a mechanism that requires cytoplasmic tyrosine Y350, suggesting that fibronectin upregulation may underlie the proinflammatory cytokine expression observed.

**KIM-1 mutant mice are protected from renal fibrosis in the UUO model.** The Kim1RECtg phenotype suggested that mice with mutant KIM-1 might be protected from kidney fibrosis. To test this hypothesis, we analyzed mice carrying a deletion of exon 3 of the KIM-1 locus (Kim1Δmuc), resulting in an in-frame deletion of the extracellular mucin domain. This mutation results in a smaller KIM-1 polypeptide that is defective in KIM-1–dependent phagocytic function (34). When subjected to UUO injury, KIM-1Δmuc protein was upregulated, but as expected, it migrated faster on Western blot than native KIM-1, reflecting the absence of the heavily glycosylated mucin domain encoded by exon 3 (Figure 8A).

Both control and Kim1Δmuc mice (n = 10 each) were subjected to UUO and sacrificed on day 10. Kim1Δmuc mice had significantly reduced interstitial collagen deposition as well as reduced tubular injury scores (Figure 8, B–D). mRNA levels of the myofibroblast marker αSMA were also reduced in Kim1Δmuc mice, in addition to levels of collagen 1α1 and fibronectin (Figure 8, E and F). Taken together, these findings indicate that Kim1Δmuc mice are protected from renal fibrosis in the UUO model. Since proinflammatory cytokine levels were increased in Kim1RECtg mice and KIM-1 expression regulates MCP-1, TGF-β, and IL-6 secretion in vitro, we analyzed the same panel of cytokines in Kim1Δmuc kidney samples before and after UUO. MCP-1 levels were significantly reduced in Kim1Δmuc compared with control at day 10 of UUO (Figure 8H), while other cytokine mRNAs were unchanged (data not shown). This result, combined with our previous data, strongly implicates MCP-1 as a mediator of KIM-1–dependent fibrosis in the mouse kidney.

**Discussion**

Resident kidney epithelial cells play an important role in detection of injury, regulation of the inflammatory and tissue repair responses, and mediation of interstitial fibrosis through paracrine mechanisms (35, 36). In this study, we hypothesized that KIM-1 might regulate kidney inflammation and fibrosis when its expression is prolonged because: (a) it is upregulated very early after kidney injury and is thus poised to serve as a sentinel of damage; (b) it is expressed in chronic fibrosing kidney disease, where it colocalizes with areas of fibrosis and inflammation (21, 22); (c) it is a phosphatidylserine receptor and may function in a manner similar to that of Toll-like receptors that are known to regulate innate immu-
Figure 5

CKD phenotype in Kim1RECtg. (A) Focal fibrotic changes at 4 weeks in Kim1RECtg that become progressively more severe with time. Scale bar: 50 μm. (B) AP expression identifies cells that have undergone Cre-mediated recombination. No AP-positive cells were found in fibrotic interstitium. Scale bar: 50 μm. (C) Concentric left ventricular hypertrophy in aged Kim1RECtg, trichrome stain. (D) Ventricular wall ratio (outer to inner diameter) was increased in aged (range, 12–45 weeks) Kim1RECtg (n = 5 for each group). *P = 0.03. (E) Younger Kim1RECtg (n = 3) do not have hypertension, but older Kim1RECtg (n = 5) do develop hypertension. *P = 0.0002. (F) Hematocrit in control (n = 10) or Kim1RECtg (n = 5) mice between 6 and 10 weeks or control (n = 13) and Kim1RECtg (n = 7) mice measured between 10 and 20 weeks of age. *P = 0.001; **P = 0.0001. (G) Total urinary protein is elevated in 8-week-old Kim1RECtg (n = 3–5) but not at 2 or 4 weeks compared with littermate controls (n = 4–6). *P = 0.01. (H) Urinary protein is not elevated in mice with expression of KIM-1 in podocytes alone at either 4 or 8 weeks. (I) Serum creatinine 13- to 20-week-old mice comparing control (n = 12) and Kim1RECtg (n = 8), control (n = 6) and Six2-GC;Z/AP (n = 7), or control (n = 4) and Podocin-Cre;Z/Kim1-AP (n = 4). *P = 0.006, NS. (J) Anemia was seen in Kim1RECtg but not control mice or mice in which KIM-1 was expressed in podocytes. Control refers to mice with neither transgene or 1 transgene for all groups. *P < 0.0001, NS.
The primary finding of the current report is that chronic KIM-1 expression in renal epithelial cells directly causes interstitial inflammation and fibrosis observed in Kim1RECtg mice are consistent with this hypothesis and, combined with the extrarenal manifestations described here, establish the Kim1RECtg mouse as what we believe to be a novel rodent model of progressive CKD and implicate the KIM-1 protein as a target for antifibrotic therapy in man (26).

The lower nephron endowment in our Kim1RECtg mouse model most likely reflects activation of KIM-1 expression in metanephric mesenchyme with effects on kidney development. Low nephron number is a recognized risk factor for the development of hypertension and possibly CKD (41). However, reduction in nephron number by 28%–40% results in no, or very mild, interstitial fibrosis with effects on kidney development. Low nephron number is a recognized risk factor for the development of hypertension and possibly CKD (41). However, reduction in nephron number by 28%–40% results in no, or very mild, interstitial fibrosis (41). However, reduction in nephron number by 28%–40% results in no, or very mild, interstitial fibrosis (41).

The stimulus for sustained KIM-1 expression after AKI or during CKD requires further investigation. The most likely explanation is that AKI itself causes peritubular capillary rarefaction (8), leading to chronic tubular hypoxia, which is a potent stimulus for KIM-1 expression (12). Chronic KIM-1 expression will promote further tubulointerstitial inflammation, capillary loss, and hypoxia, further inducing KIM-1 and creating a positive feedback loop of hypoxia and inflammation that culminates in tubulointerstitial fibrosis.

Our study suggests what we believe to be a novel role for chronic KIM-1 expression in the pathogenesis of renal fibrosis and through activation of the innate immune system and leukocyte recruitment. In contrast, very early induction of KIM-1 after AKI may serve an adaptive function to clear apoptotic and necrotic cells and debris and thereby decrease the early response of the immune system at a site of tissue damage. Persistent KIM-1 expression is perhaps maladaptive through chronic uptake of cell toxic components of the tubular lumen, thereby promoting chronic inflammation and ultimately renal fibrosis. Thus KIM-1 may represent a novel therapeutic target in fibrotic kidney disease, and antagonizing KIM-1 signaling might ameliorate renal fibrosis in CKDs.

**Methods**

*Mouse strains.* A KIM-1 cDNA was inserted into the NotI site of the Z/AP plasmid (48), and linearized Z/Kim1-AP transgene was introduced into FVB zygotes (Charles River Laboratories) by pronuclear injection. Three independent founder lines were obtained; all exhibited Six2-GC-dependent transgene expression, and the line with highest outer medulla and cortex transgene expression was selected for further analysis. The Z/Kim1-AP transgenic was maintained on an FVB × C57BL/6J (Jackson Laboratory) mixed background.

Z/Kim1-AP transgenic mice were crossed with the Six2-GC-Cre driver line (27) maintained on a CD-1 × Swiss Webster (Taconic) × C57BL/6J (Jackson Laboratory) mixed background. In other experiments, the Z/Kim1-AP

### Table 4

<table>
<thead>
<tr>
<th>Echocardiography in Kim1RECtg mice at 2.5–3 months</th>
<th>Control (n = 7)</th>
<th>Kim1RECtg (n = 3)</th>
</tr>
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<tr>
<td>Interventricular septal thickness (mm)</td>
<td>0.69 ± 0.06</td>
<td>0.75 ± 0.04</td>
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<tr>
<td>Left ventricular end-diastolic diameter (mm)</td>
<td>3.40 ± 0.33</td>
<td>3.37 ± 0.09</td>
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<tr>
<td>Left ventricular posterior wall thickness (mm)</td>
<td>0.76 ± 0.05</td>
<td>0.79 ± 0.04</td>
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<tr>
<td>Fractional shortening (%)</td>
<td>38.73 ± 3.55</td>
<td>46.71 ± 4.72a</td>
</tr>
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</table>

*P < 0.05.

### Table 5

<table>
<thead>
<tr>
<th>Biochemical parameters in Kim1RECtg mice</th>
<th>Control (n = 10)</th>
<th>Kim1RECtg (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUN (mg/dl)</td>
<td>23.4 ± 1.1</td>
<td>99.0 ± 25.0a</td>
</tr>
<tr>
<td>Cr (mg/dl)</td>
<td>0.12 ± 0.01</td>
<td>0.47 ± 0.1a</td>
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<td>Na (mM)</td>
<td>149 ± 0.9</td>
<td>148.1 ± 2.3</td>
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<td>K (mM)</td>
<td>5.9 ± 0.3</td>
<td>7.6 ± 0.8</td>
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<tr>
<td>Cl (mM)</td>
<td>104.3 ± 0.7</td>
<td>101.0 ± 1.6</td>
</tr>
<tr>
<td>Albumin</td>
<td>2.9 ± 0.1</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>Calcium (mg/dl)</td>
<td>9.2 ± 0.1</td>
<td>10.2 ± 0.2a</td>
</tr>
<tr>
<td>Phosphorus (mg/dl)</td>
<td>7.8 ± 0.3</td>
<td>10.14 ± 1.0</td>
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*P < 0.05.
transgenic mice were crossed with the Podocin-Cre driver line (49). The Z/AP reporter line was from JAX and was maintained on a 129/BL6 background. The Kim1 REC line has been described (34). The Z/AP and Z/Kim1-AP mice were genotyped using β-Geo–specific primers. The Sx2-ZGC allele was genotyped as described (29). The Podocin-Cre allele was genotyped using Cre primers. In all cases, littermate control phenotypes were compared. Littermate controls were used for all mouse experiments.

**Induction of renal fibrosis by UUO.** Male BALB/c mice aged 8 to 10 weeks weighing 20–22 g were purchased from Charles River Laboratories. Mice were anesthetized and the left kidney exposed by flank incision. The ureter was ligated at 2 points proximal to the kidney with 6-0 silk. Sham animals had kidney exposed, but ureter was not tied.

**Tissue preparation and histology.** Mice were anesthetized, sacrificed, and immediately perfused via the left ventricle with ice-cold PBS for 2 minutes. Kidneys were hemi-sectioned, and portions were snap frozen in liquid nitrogen. Other kidneys were fixed in 10% neutral buffered formalin at 4°C for 12 hours, processed, embedded in paraffin wax, sectioned, and air dried, and treated for immunofluorescence as described (50). Secondary antibodies were obtained from Dako. Sections were rehydrated and antigens retrieved using heated citrate. Staining was performed on formalin-fixed, paraffin-embedded 4-μm sections. Sections were mounted in Vectashield containing 4′,6-diamino-2-phenylindole (Vector Labs). Images were taken with a Nikon TE2000 microscope CoolSnap camera (Roper Scientific) and processed using IP Lab Software (BD Biosciences). Immunofluorescence images were obtained on a Nikon TE2000 or a Nikon C1 D-Eclipse confocal microscope using standard procedures.

Immunohistochemical stains (αSMA, CD3, neutrophil, KIM-1) were performed on formalin-fixed, paraffin-embedded 4-μm sections. Sections were rehydrated and antigens retrieved using heated citrate. Staining was visualized using horseradish peroxidase–coupled secondary antibodies (Vectastain Elite; Vector Labs). LacZ activity was measured by standard X-gal staining protocol on 7-μm frozen kidney sections that had been fixed in PLP for 2 hours and was carried out for 12–24 hours at 37°C; then stained with Nikon C1 D-Eclipse confocal microscope using standard procedures.

Figure 6
Leukocyte infiltration in Kim1 RECtg. (A) CD3+ lymphocyte infiltration begins at 4 weeks in Kim1 RECtg and is observed in tubulointerstitium, especially surrounding vessels in older animals. Scale bar: 50 μm. (B) F4/80-positive peritubular macrophages and dendritic cells are increased at 4 weeks in Kim1 RECtg. Scale bar: 50 μm. (C) Quantitation of infiltrating leukocytes and Ki67+ proliferating cells at 4 weeks in Kim1 RECtg compared with control kidneys (n = 3 for each). *P < 0.0001; **P = 0.0003. Neut., neutrophil; Lymph., lymphocyte; Mac., macrophage. (D) CD3-positive lymphocytes (brown) are frequently identified adjacent to KIM-1–positive (purple, *) tubules. Scale bar: 25 μm.
fuse if 50% or more of the tubule showed these changes. Semiquantitative analysis of glomeruli included glomerular histology as well as foot process morphology assessed by electron microscopy and was graded as follows: minimal: 1 (involving < 5% of glomerulus); mild: 2 (5%–24%); moderate: 3 (25%–49%); and severe: 4 (≥ 50%). For assessment of glomerular involvement, an average of 80–120 glomeruli per section were examined on multiple levels. All scoring was done in a blinded manner by an experienced renal pathologist. Semiquantitative analysis of tubular morphology in Kim1RECtg mice was also performed in a blinded fashion exactly as described (51).

Electron microscopy. Portions of kidneys were fixed in Karnovsky’s fixative and processed for electron microscopic studies by standard procedures. Semithin sections were stained with toluidine blue sections were counterstained with eosin (Sigma-Aldrich) and mounted. PAS, H&E, and Masson’s trichrome stains were performed using standard techniques. AP activity was measured using NBT/BCPIP, with a standard protocol that included a 30-minute incubation at 60°C to inactivate endogenous AP activity.

Histologic analysis. Histological analysis was performed on paraffin-embedded and serially cut kidney sections (3 μm) stained with H&E, PAS, and Masson’s trichrome. Analysis of tubules included the evaluation of epithelial histology. The degree of injury was scored semiquantitatively on a 0 to 4 scale for reabsorption granules, vacuolization, and epithelial degeneration as follows: 0, no lesion; 1, minimal (minor focal changes); 2, mild; 3, moderate; 4, severe. The distribution was graded as focal if 49% or less and diffuse if 50% or more of the tubule showed these changes. Semiquantitative analysis of glomeruli included glomerular histology as well as foot process morphology assessed by electron microscopy and was graded as follows: minimal: 1 (involving < 5% of glomerulus); mild: 2 (5%–24%); moderate: 3 (25%–49%); and severe: 4 (≥ 50%). For assessment of glomerular involvement, an average of 80–120 glomeruli per section were examined on multiple levels. All scoring was done in a blinded manner by an experienced renal pathologist. Semiquantitative analysis of tubular morphology in Kim1RECtg mice was also performed in a blinded fashion exactly as described (51).

Electron microscopy. Portions of kidneys were fixed in Karnovsky’s fixative and processed for electron microscopic studies by standard procedures. Semithin sections of each block were stained with toluidine blue

Figure 7 Persistent KIM-1 expression induces epithelial damage and proinflammatory cytokines in kidney epithelia in vivo and in vitro. (A) Shed KIM-1 is detectable in urine of Kim1RECtg at 4 weeks of age compared with controls. *P = 0.03. (B) Evidence of tubular injury in Kim1RECtg is also reflected by increasing urinary NAG in 4- and 8-week-old Kim1RECtg. *P < 0.01. (C) qPCR of kidney cortex cytokines, presented as fold increase in Kim1RECtg (n = 3) compared with controls (n = 3) at 2 weeks (black bars) or 4 weeks of age (white bars). At 2 weeks, there is mild induction of CXCL-1, MCP-1, and TGF-β1, with much higher levels of mRNA expression for all soluble cytokines by 4 weeks of age. *P < 0.05; **P < 0.006. (D–F) Porcine proximal tubule epithelial cells stably transfected with either pcDNA3 control plasmid (pcDNA-LLC) or KIM-1 plasmid (KIM-1–LLC) spontaneously express TGF-β1, MCP-1, and IL-6 (n = 3 for each). *P < 0.05; **P < 0.01. (G and H) Boyden chamber assay with pcDNA-LLC or KIM-1–LLC seeded on the bottom well and either U937 or bMDM applied to top filter. Migration was measured after 3 hours. *P < 0.05. Original magnification, x500. (I) Neutralizing antibody against MCP-1 abrogated KIM-1–LLC–dependent migration but not control IgG. *P < 0.05; **P < 0.01. (J) Cellular lysates from pcDNA-LLC, KIM-1–LLC, or LLC-PK1 cells transfected with KIM-1–Y350F (Y350-LLC) probed for fibronectin by Western blot demonstrate strong induction of fibronectin in KIM-1, but not Y350F–KIM-1–transfected cells.
transcribed with the M-MLV reverse transcriptase kit and Oligo dT primers (Promega). Real-time PCR was performed by TaqMan gene expression assays (Applied Biosystems) for detection of mRNA expression using GAPDH as the internal control. Qualitative RT-PCR was performed on 1/200th of the RT product using the following primer pairs (from 5′ to 3′): Cre forward: TTCCCGCAGAACCTGAAGATG, reverse: CCCCAGAAATGCCAGAT-TACG; KIM-1 forward: ATGAATCAGATTCAAGTCTTC, reverse: TCTGGTTGTAGTCCATGTG; GAPDH forward: TGGAGAAACCTGCCAAGTA, reverse: AAGAGTGGGAGTTGCTGTTG. The cycling conditions were as follows: Cre: melting temperature (Tm) 57°C and 35 cycles, KIM-1: Tm 55°C and 30 cycles; GAPDH: Tm 53°C and 25 cycles. PCR products were visualized on ethidium bromide containing, 2% agarose gels and photographed.

Quantitative PCR was performed using a Bio-Rad iCycler and the following primers (from 5′ to 3′): Col-1α1 forward: TGACTGGAAGAGCGGAGAGT, reverse: GTTCGGGCTGATGTACCAGT; CXCL1

Figure 8
The functional mutant Kim1<sup>Δmuc</sup> is protected from kidney fibrosis. (A) Control and Kim1<sup>Δmuc</sup> mice were subjected to UUO and sacrificed at day 2. Kidney lysates reveal KIM-1 protein at 75 kDa in the control, but at 55 kDa in the Kim1<sup>Δmuc</sup>, corresponding to the deletion of exon 3 (arrows). The arrowhead identifies a nonspecific Ig band. Proliferating cell nuclear antigen (PCNA) staining reflects increased cell proliferation after UUO. (B) Control or Kim1<sup>Δmuc</sup> kidney sections before or after UUO. There is reduced interstitial collagen in Kim1<sup>Δmuc</sup> reflected by Masson's trichrome stain, and reduced tubular injury (PAS). (C and D) Quantification of tubular atrophy and fibrosis index, respectively (n = 5 kidneys each condition). (E–G) qPCR of kidney cortex fibrosis, presented as fold increase of Kim1<sup>REC</sup> (n = 5) compared with control (n = 5) at 10 days after UUO. *P < 0.05. (H) Reduced MCP-1 mRNA by qPCR in Kim1<sup>Δmuc</sup> compared with control (n = 5). *P < 0.05.

Western blot analysis. Kidney tissues were lysed, and lysates were prepared as previously described (50). Membranes were incubated with 1 or more of the following primary antibodies: rabbit antibody to LacZ (1 in 5,000; Cappel), chicken antibody to GFP (1 in 1,000; AVES), rabbit antibody to KIM-1 (1 in 250) (12), rabbit anti-fibronectin (1 in 1,000; Abcam), proliferating nuclear cell antigen (1 in 1,000; Abcam) and ERK (1 in 1,000; Cell Signaling). Horseradish peroxidase–conjugated secondary antibodies were applied, and enhanced chemiluminescence (Amersham Biosciences) was used to detect proteins.

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Quantification of mRNA by reverse transcription PCR. Total RNA was isolated from snap-frozen kidneys with RNase columns (QIAGEN). Five micrograms of total RNA was treated with DNase I (Invitrogen) and reverse transcribed with the M-MLV reverse transcriptase kit and Oligo dT primers (Promega). Real-time PCR was performed by TaqMan gene expression assays (Applied Biosystems) for detection of mRNA expression using GAPDH as the internal control. Qualitative RT-PCR was performed on 1/200th of the RT product using the following primer pairs (from 5′ to 3′): Cre forward: TTCCCGCAGAACCTGAAGATG, reverse: CCCAGAAATGCCAGAT-TACG; KIM-1 forward: ATGAATCAGATTCAAGTCTTC, reverse: TCTGGTTGTAGTCCATGTG; GAPDH forward: TGGAGAAACCTGCCAAGTA, reverse: AAGAGTGGGAGTTGCTGTTG. The cycling conditions were as follows: Cre: melting temperature (Tm) 57°C and 35 cycles, KIM-1: Tm 55°C and 30 cycles; GAPDH: Tm 53°C and 25 cycles. PCR products were visualized on ethidium bromide containing, 2% agarose gels and photographed.

Quantitative PCR was performed using a Bio-Rad iCycler and the following primers (from 5′ to 3′): Col-1α1 forward: TGACTGGAAGAGCGGAGAGT, reverse: GTTCGGGCTGATGTACCAGT; CXCL1
forward: CTGGGATTTCACTCTAAGAACATC, reverse: CAGGGT-CAAGGCAAGCGCCT, CCLX2 forward: CCACCACCGGCTCAG, reverse: GGCCTACTCAGACTCGTC; CCLX10 forward: CCAAGT-GCTGCGCTTATTTTT; reverse: GGCTGCGAGGGATGATTCCAA; GAPDH forward: CAGTGGCTAGTATGCTCCACT, R: GGCCT-CACCCCACTTGTAGT; IL-1β forward: CCGGAAATGTTATCGCTC; reverse: CTGGGTCTTACGAGCT-CCATCTC; MCP-1 forward: TGACTGCGTCAAGGGTCTCT, R: TAAAGGTGGATGTTTGG; TGF-β forward: GCAAAATTTCTGG-CCGTTCAGTTTACACC; reverse: CGAAAGCCTGATTCCGCT; TFN-α forward: CACCTC-CAGTATCAGTATTCTCT, reverse: GCTACAGGTGGGGCTA-CAG; αSMA forward: CTGAGAGGACCACCTGAGA; fibronectin forward: ATGTTGACCCCTCCT-GATAGT, R: GGCCTAGTATTTGCAAGG.

Physiologic measurements. Serum creatinine was measured using a Beckman Creatinine Analyzer 2 by the Jaffe rate method. Hematocrit was calculated after centrifugation of a hematocrit capillary tube. Proteinuria was assessed by microsphere-based Luminex technology. NAG in urine was measured by colorimetric assay using a commercial kit (Roche). For serum electrolytes, CO2. To create stable cell lines expressing full-length KIM-1 (KIM-1–LLC), LLC-PK1 cells were transfected with pcDNA3–KIM-1 or pcDNA3–KIM-1–Y350F plasmid and the stable population was selected using G418 treatment.

Cell culture. Cos7 cells were transfected using Lipofectamine 2000 (Invitrogen). Porcine proximal tubular epithelial cells (LLC-PK1) cells were grown in DMEM supplemented with 10% FBS and maintained at 37°C in 5% CO2. To create stable cell lines expressing full-length KIM-1 (KIM-1–LLC), LLC-PK1 cells were transfected with pcDNA3–KIM-1 or pcDNA3–KIM-1–Y350F plasmid and the stable population was selected using G418 treatment (400 μg/ml). Control cell lines (pcDNA-LLC) stably expressing pcDNA3-neo were generated the same way. To generate mBDMD, femurs and tibias were removed from 20- to 25-g BALB/c mice. BM was isolated from these by standard sterile techniques and matured for 7 days in uncoated Petri dishes using DMEM/F12 medium with 10% FCS, penicillin (100 U/ml), and streptomycin (100 mg/ml) and conditioned with M-CSF from L929 cells. The U937 cell line, a human monomyelocytic cell line, was cultured in RPMI 1640 medium supplemented with 10% FCS, 1% penicillin/streptomycin, and 2 mM l-glutamine. Cells were subcultured 3 times a week and maintained at a concentration of 0.5–1.0 × 106 cells/ml. Monocytic differentiation of U937 cells was achieved by adding 10 nM PMA for 48 hours. PMA-differentiated U937 was washed 3 times by sterile PBS before experiments.

Cytokine measurement. Both KIM-1–LLC and pcDNA-LLC cells were grown to confluence, and supernatant was collected, centrifuged, and stored at −80°C until further analysis. Alternatively, mouse urine was collected with a metabolic cage and processed in a similar fashion. MCP-1, IL-6, and TGF-β microbead-based assays were developed and validated in the lab. Approximately 6000 beads/50 μl were incubated with 30 μl of sample or recombinant proteins (R&D Systems) for 1 hour, washed 3 times with PBST, and incubated in corresponding biotinylated antibodies (R&D Systems) for 45 minutes on an orbital shaker at 300 rpm. Beads were washed again with PBST and incubated for 15 minutes with streptavidin–PE solution (Invitrogen). The signal from the fluorochrome, which is directly proportional to the amount of antigen bound at the micro-bead surface, was captured using the Bio-Plex 200 system (Bio-Rad). Data were generated and interpreted using parametric logistic regression analysis.

Boyden chamber assay. Cell chemotaxis assay was performed in a modified Boyden chamber using 24-well flat-bottom tissue plates with 5-μm polystyrene/epoxytynyl membrane inserts (BD). The lower compartment of each chamber was filled with 500 μl of the conditioned medium. Membrane inserts were filled with 300 μl of cell suspension and placed in the pre-filled lower compartments. The chambers were then incubated for 3 hours in 37°C, 5% CO2. After incubation, nonmigrated cells in the upper wells were removed by scraping and the migrated cells were stained with eosin on the membrane. Adherent cells on the lower surface of the membrane were counted from 5 high power fields by light microscopy (×40). Data are presented as cells per high-power field. Neutralizing antibody against MCP-1 was from Sigma-Aldrich.

Statistics. All results are reported as mean ± SEM. All error bars on graphs represent SEM. Statistical tests are 2-tailed, unpaired t tests except for survival analysis (Figure 4E), which used the log rank test, and Figures 7B and Supplemental Figure 2, which used a repeated measures t test.

Study approval. All animal studies were approved by the Harvard Institutional Animal Care and Use Committee.

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