Mammalian Target of Rapamycin Mediates Kidney Injury Molecule 1-Dependent Tubule Injury in a Surrogate Model

Wenqing Yin,* Said Movahedi Naini,* Guochun Chen,* Dirk M. Hentschel,* Benjamin D. Humphreys,*† and Joseph V. Bonventre*‡

*Renal Division, Brigham and Women’s Hospital, Department of Medicine, Harvard Medical School, Boston, Massachusetts; †Harvard Stem Cell Institute, Cambridge, Massachusetts; and ‡Division of Health Sciences and Technology, Harvard-Massachusetts Institute of Technology, Cambridge, Massachusetts

ABSTRACT

Kidney injury molecule 1 (KIM-1), an epithelial phagocytic receptor, is markedly upregulated in the proximal tubule in various forms of acute and chronic kidney injury in humans and many other species. Whereas acute expression of KIM-1 has adaptive anti-inflammatory effects, chronic expression may be maladaptive in mice. Here, we characterized the zebrafish Kim family, consisting of Kim-1, Kim-3, and Kim-4. Kim-1 was markedly upregulated in kidney after gentamicin-induced injury and had conserved phagocytic activity in zebrafish. Both constitutive and tamoxifen-induced expression of Kim-1 in zebrafish kidney tubules resulted in loss of the tubule brush border, reduced GFR, pericardial edema, and increased mortality. Kim-1-induced kidney injury was associated with reduction of growth of adult fish. Kim-1 expression led to activation of the mammalian target of rapamycin (mTOR) pathway, and inhibition of this pathway with rapamycin increased survival. mTOR pathway inhibition in KIM-1-overexpressing transgenic mice also significantly ameliorated serum creatinine level, proteinuria, tubular injury, and kidney inflammation. In conclusion, persistent Kim-1 expression results in chronic kidney damage in zebrafish through a mechanism involving mTOR. This observation predicted the role of the mTOR pathway and the therapeutic efficacy of mTOR-targeted agents in KIM-1-mediated kidney injury and fibrosis in mice, demonstrating the utility of the Kim-1 renal tubule zebrafish models.


Kidney injury molecule 1 (KIM-1), also known as T cell Ig and mucin 1 or hepatitis A virus cellular receptor-1 (HAVCR1), is a type 1 transmembrane protein. In the normal mammalian kidney, KIM-1 expression is undetectable, but after acute injury its expression is induced abundantly in the proximal tubules, where it localizes to the apical surface of epithelial cells and also to the basolateral membrane when polarity is lost.1 Acting as a nonmyeloid phosphatidylserine (PS) receptor, the mammalian KIM-1 ectodomain binds and internalizes oxidized lipids as well as PS exposed on the outer surface of luminal apoptotic cells.2–4 KIM-1 clears the tubule lumen of debris following AKI, aiding in nephron repair and tissue remodeling. KIM-1 is also upregulated in a variety of animal models of CKD, including protein overload nephropathy,5 adriamycin-induced nephropathy,6 angiotensin II-induced renal damage,7 and murine polycystic kidney disease,8 where it colocalizes with areas of fibrosis and inflammation.9 It is upregulated in human CKD10 and its expression correlates directly with interstitial fibrosis in human allografts.11

Received May 7, 2015. Accepted September 8, 2015.
Published online ahead of print. Publication date available at www.jasn.org.

Correspondence: Dr. Joseph V. Bonventre, Renal Division, Harvard Institute of Medicine, 4 Blackfan Circle, Boston, MA 02115. Email: joseph_bonventre@hms.harvard.edu
Copyright © 2016 by the American Society of Nephrology

Recently, we reported a transgenic mouse model with nephron-specific overexpression of KIM-1. These mice develop tubular damage, reduced nephron number, and fibrosis, and have a high mortality rate compared with controls.12 These observations suggested a pathogenic role of KIM-1 in linking AKI to CKD and suggested KIM-1 as a novel therapeutic target in CKD. Embryonic overexpression of KIM-1 in these transgenic mice, however, resulted in reduced nephron numbers, raising the possibility that this might have contributed to the CKD phenotype.

Studies of CKD require intact organ systems to recapitulate the interplay of the multiple pathophysiologic processes involved in chronic inflammation and fibrosis; however, mice or other mammalian models are not suitable for high-throughput screening of therapeutic agents, due to the high cost and large time requirements of the screening. The zebrafish (Danio rerio), a small tropical freshwater fish, has become an excellent vertebrate model for studying human disease, genetics, and development.13,14 Despite the anatomic simplicity of the zebrafish pronephros (in larvae) and mesonephros (in adult fish), zebrafish models for studying AKI, polycystic kidney disease, nephropathies, and a range of ciliopathies have brought important insight to pathophysiologic processes in mammals.15–17 Rapid breeding and development, together with ease of genetic manipulation and optical transparency, make zebrafish an ideal model organism to develop high-throughput screening for therapeutic drug discovery.

In this study, using phylogenetic and genomic analysis, we characterized the zebrafish kim gene family, which consists of kim1, kim3, and kim4. Zebrafish Kim-1 protein shares a high degree of structural and functional homology with mammalian KIM-1. Similar to what is observed in mammals, zebrafish Kim-1 expression was markedly upregulated in the kidney tubules after injury. Kidney tubular cell expression of Kim-1 resulted in pathologic effects in the pronephros and mesonephros, and associated inhibition of fish growth in size. Kim-1–induced nephrotoxicity is inhibited by mTOR inhibition with rapamycin. This finding is recapitulated in the mouse overexpressing KIM-1 in the renal tubule, thus validating the zebrafish model as a surrogate for KIM-1–induced CKD in the mouse.

RESULTS

Identification of Zebrafish Kim-1 Family

By searching public protein and cDNA databases (European molecular biology laboratory [EMBL], Genbank, and University of California Santa Cruz [UCSC]), and using the National Center for Biotechnology Information (NCBI)/BLAST program, we identified the kim1 (EMBL accession number ENSDARG00000091692), kim3 (ENSDARG00000077257), and kim4 (ENSDARG00000040178) genes in zebrafish. Zebrafish Kim-1 has a putative signal peptide (SP) of 19 amino acids (aa), an Ig domain of 105 aa, a mucin domain of 172 aa, a transmembrane domain of 20 aa, and a cytoplasmic domain of 44 aa. Comparison of identity and similarity of each domain of zebrafish Kim-1 (zKim-1), zebrafish Kim-3 (zKim-3), and zebrafish Kim-4 (zKim-4) with their human and mouse orthologs, revealed that the zKim-1 Ig domain has the highest score of identity (approximately 40%) and similarity (approximately 57%) with mouse KIM-1 (mKIM-1) and human KIM-1 (hKIM-1) (Figure 1A). There was also a high degree of homology with the highest identity and similarity score between zKim-3, zKim-4, and their human orthologs in the Ig domain (hKIM-3 and hKIM-4) (Supplemental Figure 1, A and B).

Phylogenetic analysis placed zebrafish kim1 in the same branch as human KIM-1, alongside other mammalian KIM-1s (Figure 1B). Analysis of the genomic location of the zebrafish kim family, based on zv9 assembly,18 demonstrated that all three genes are located on chromosome 21, adjacent to each other, although the positional order of kim1, kim3, and kim4 on the chromosomes is not conserved between zebrafish chromosome 21 and human chromosome 5 (Figure 1, C and D). To identify possible splice variants of kim1 in zebrafish, we performed 3′ and 5′ rapid amplification of cDNA ends (RACE). kim1 has three splice variants. kim1-L is the longest splice variant and contains all exons except exon 4 (Figure 1D). kim1-S is the short splice variant and lacks exon 4 and 6. kim1-Ig is an Ig domain splice variant, which consists only of the Ig domain and incomplete mucin domain (exons 1–6).

Expression Pattern of Kim-1, Kim-3, and Kim-4 in Zebrafish After Injury

Kidney injury in mammals is associated with a marked upregulation of KIM-1 expression in proximal tubular cells. We previously reported a gentamicin–induced proximal tubular injury model in the zebrafish.15 To examine the expression of Kim-1 in zebrafish after injury, adult zebrafish (>90 days postfertilization [dpf]) were injected with gentamicin (5 μg/100 mg body wt). Quantitative RT-PCR was used to define the relative expression patterns for each kim family member at 2 days after gentamicin injection (Figure 2). After gentamicin–induced injury, kim1-L and kim1-S mRNA expression were higher in kidney compared with other organs, while kim3 and kim4 were expressed at low levels in all organs including the kidney. Immunofluorescence staining for Kim-1 was performed with a specific antibody that we produced against the Ig domain of Kim-1 (Supplemental Figure 2). Lotus tetragonolobus lectin (LTL) (proximal tubules marker) and Dolichos biflorus agglutinin (DBA; distal tubules and collective ducts marker) were used as markers of nephron segments.19,20 Kim-1 was expressed on the apical membrane of proximal tubular cells but not distal tubular or collecting duct cells after gentamicin–induced kidney injury (Figure 3A). The time course of expression of kim1 mRNA revealed that kim1-L and kim1-S transcripts in adult zebrafish kidney were upregulated by 2 days postgentamicin injection (dpi), and remained elevated until 8 dpi, after which they decreased to near-normal.
levels at 12 dpi, while the expression of kim1-Ig or kim3 and
kim4 transcripts was not altered by gentamicin administration
(Figure 3B). In larvae, the expression of kim1-L and kim1-S
transcripts were also upregulated at 8 dpf after gentamicin was
administered at 6 dpf (Supplemental Figure 3).

Zebrafish Kim-1 Is a Phagocytic Receptor for Apoptotic
Bodies and oxidized-LDL
As a PS receptor, mammalian KIM-1 confers on proximal
tubular cells the ability to recognize PS on apoptotic cells and
mediates the phagocytosis of apoptotic cells, necrotic cells, and
oxidized LDL (ox-LDL).2 The binding motif WFND has been
known to be essential for the phagocytic function of KIM-1.21

The alignment of zKim-1 with mKIM-1 and hKIM-1 protein
sequences demonstrated that the FND residues are conserved,
while the tryptophan (W) residue is replaced with leucine (L)
(Figure 3C). The WFND motif is completely conserved in
zKim-4. Similar to mammalian KIM-3, there is lack of con-
servation of the WF residues in zKim-3 (Figure 3C).

To determine whether zebrafish Kim-1 has phagocytic activity,
kim1-L was cloned from gentamicin-treated zebrafish
larvae. LLC-PK1 cells were transfected with expression plas-
mids encoding zebrafish kim1-L fused to EGFP (zkim1-EGFP)
or human KIM-1 fused to EGFP (hKIM-1-EGFP),2 and then
incubated with Dil-labeled apoptotic thymocytes or ox-LDL
for 2 hours (Figure 3D). Immunofluorescence staining

Figure 1. Homologies in Kim family among several species. (A) Protein sequence of zebrafish Kim-1 (zKim-1) was aligned with mouse
and human orthologs (mKIM-1, hKIM-1). Horizontal black lines indicate the Ig-like domain (Ig). (B) A phylogenetic tree of the Kim families.
Values on branches are percentages of replicate trees in which the genes clustered together in the bootstrap test (1000 replicates).
Branch lengths are drawn in units of 0.1 aa substitutions/site. (C) Detailed view of the human KIM-1 locus and corresponding introns and
exons, which map to chromosome 5q32.2. Genes are depicted with arrows to show the orientation. (D) The zebrafish kim family is
located on chromosome 21. Predicted protein structures of three kim1 splice variants based on the 3′-rapid amplification of cDNA ends
(RACE) and 5′ RACE sequences are presented. Numbers reflect exon and intron nucleic acid lengths (upper) and exon number (lower).
kim1-Ig, Ig domain only isoform; kim1-L, long isoform; kim1-S, short isoform.
showed engulfment of apoptotic thymocytes or ox-LDL (white arrowheads) by zKim-1-expressing cells (zKim-1-EGFP) and hKIM-1-expressing cells (hKIM-1-EGFP). To quantify the phagocytic capacity of zebra fish Kim-1, LLC-PK1 cells transfected with plasmids encoding EGFP, zkim1-EGFP, or hKIM-1-EGFP were cocultured for 2 hours at 37°C with fluorescent Dil-labeled apoptotic thymocytes. Undigested apoptotic cells were then washed away and live cells were lifted into a single-cell suspension with EDTA and trypsin. Two-color flow cytometry showed that, in hKIM-1-EGFP-PK1 and zKim-1-EGFP-PK1 cells, the number of phagocytosed apoptotic thymocytes was approximately 10-fold greater than cells expressing EGFP alone (51.5% ± 0.33% were positive for apoptotic cells in hKIM-1-EGFP-PK1 versus 53.5% ± 0.32% in zKim-1-EGFP-PK1 versus 5.89% ± 0.24% in EGFP-PK1) (Figure 3E). These data demonstrate that zebrafish Kim-1 is a phagocytic receptor for apoptotic cells and ox-LDL, indicating a conserved phagocytic function between zebra fish Kim-1 and the human ortholog.

Overexpression of Kim-1 in Pronephric Kidney Causes Renal Failure in Zebrafish

The cadherin 17 (cdh17) promoter was used to overexpress Kim-1 in kidney tubular epithelial cells in vivo. We generated a pTol-cdh17:kim1-RFP vector, encoding kim1-L fused with the RFP reporter (kim1-RFP). The pTol-cdh17:RFP plasmid was generated for comparison. Zebrafish eggs were injected with kim1-RFP or RFP-expressing constructs at the one-cell stage. At 48–72 hours after injection, we carefully selected fish that expressed Kim-1-RFP or RFP predominantly in the tubule, excluding those with significant nontubule (gut) expression. Approximately 100 RFP-positive fish were analyzed in each group. Kim-1-RFP-expressing fish began to develop pericardial edema at 2 dpf, which became more prominent at 3 dpf, compared with RFP-expressing fish and control fish (wild-type fish without injection) (Figure 4, A–C). Kim-1-RFP protein expression was confirmed by Western blot analysis (Figure 4D). Kim-1-RFP-expressing fish had a much higher incidence of pericardial edema at 3 dpf (47.9% ± 3.15% versus 5.5% ± 0.93% in RFP-expressing fish and 0.53% ± 0.35% in uninjected control fish) (Figure 4E). Survival was lowest in those fish expressing Kim-1 (Figure 4F).

To evaluate the functional consequences of Kim-1 overexpression, the clearance of 10 kDa dextran was measured to determine GFR in zebrafish. We injected wild-type larvae (control), and larvae expressing RFP or Kim-1-RFP with 2 ng tetramethylrhodamine-labeled 10 kDa dextran at 72 hours postfertilization (hpf) (0 hours) (Figure 4G). Fluorescence intensities were measured over the eye at 1, 7, 24, 31, and 43 hours after injection and compared with the baseline (immediately upon equilibration after injection, 0 hours). The renal clearance of tetramethylrhodamine-labeled 10 kDa dextran was significantly delayed in Kim-1-overexpressing fish, indicating reduced GFR (Figure 4H).
Persistent Expression of Kim-1 in Tubular Cells Results in Chronic Kidney Damage in Zebrafish

Because of genetic mosaicism caused by partially penetrant expression of the cdh17 promoter, in some of the transgenic zebrafish Kim-1 expression was detectable in only one of the two pronephric tubules (Supplemental Figure 4). To examine the effect of chronic expression of Kim-1 on the mesonephros, surviving transgenic larvae overexpressing Kim-1-RFP in one pronephric tubule were raised to adulthood. By 12 weeks of age, these fish were of smaller size and lower weight compared with age-matched RFP-expressing controls (Figure 5, A–F). Hematoxylin and eosin (H&E) staining of whole fish revealed smaller kidney parenchyma in Kim-1 transgenic zebrafish, compared with RFP reporter controls. Persistent Kim-1 expression was verified by immunofluorescence staining (Figure 5B). Dissection of kidneys, followed by histologic examination, revealed that Kim-1-overexpressing fish had fewer glomeruli, commensurate with reduced kidney area, but a normal density of glomeruli (Figure 5, E and F). Periodic acid–Shiff (PAS) staining and electron microscopy (EM) demonstrated tubular damage in Kim-1-overexpressing cells with loss of brush border and accumulation of vacuoles by light microscopy and phagosomes by EM (Figure 5, G and H).

Cre/Loxp Mediated Conditional Expression of Kim-1 in the Pronephros Causes Tubular Damage in Zebrafish

Because the fully functional glomerular barrier in zebrafish is formed at 72–96 hpf, it is possible that transgenic Kim-1 expression results in uptake of noxious factors from the glomerular filtrate that are not normally present in the tubule lumen after the glomerulus has fully matured. To address this possibility, we created a conditional Cre-mediated and tissue-specific overexpression model of Kim-1, in which Kim-1 is expressed in differentiated epithelial tubular cells after maturation of the glomerular filtration barrier. We first generated a stable transgenic zebrafish line expressing Cre-ERT2 in nephrons under control of the cdh17 promoter. The
schematic of this construct is shown in Figure 6A. To verify this transgenic line we crossed it with the ubi:loxP-EGFP-loxP-RFP line. Addition of 4-hydroxytamoxifen (4-OHT) at 4 dpf showed a robust expression of RFP in pronephric tubules, while addition of the vehicle, ethyl alcohol (EtOH), resulted in no expression of RFP (Figure 6B). The excision efficiency of the cdh17:Cre-ERT2 transgenic zebrafish line was high in each segment of the tubule (Supplemental Figure 5). Next, we generated a ubi:loxP-EGFP-loxP-Kim-1-RFP transgenic zebrafish, in which floxed-EGFP is driven by the ubiquitin promoter (ubi) (Figure 6C). Addition of 4-OHT results in Cre-mediated excision of EGFP and expression of Kim-1-RFP. One cell stage embryos from cdh17:Cre-ERT2 transgenic zebrafish line were injected with 25 ng of tetramethylrhodamine-labeled 10 kDa dextran, and serial fluorescence images were quantitated over the eye at 0, 1, 7, 24, 31, and 43 hpi (n=20 fish per condition). Data are expressed as mean±SEM. *P<0.05.

The Activation of mTOR in Kim-1 Constitutive Transgenic Zebrafish

We used our Kim-1 constitutive transgenic zebrafish model to gain insight into mechanisms of injury associated with Kim-1 expression and to identify potential therapeutic agents. At 3 months, the activation of caspase 3 was increased in Kim-1 constitutive transgenic zebrafish, consistent with increased tubular cell apoptosis associated with chronic Kim-1 over-expression (Figure 7A). The percentage of caspase 3-positive tubular cells was markedly increased in proximal (LTL+) and distal (DBA+) as well as LTL−/DBA− regions of Kim-1-expressing transgenic zebrafish when compared with non-Kim-1 expressing controls (Figure 7B).

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that plays a pivotal role in mediating cell size

Figure 4. Kim-1 overexpressing zebrafish larvae develop pericardial edema, renal failure, and have a higher mortality. (A–C) Bright field and RFP fluorescence images of uninjected zebrafish (control) and fish injected with Tol2(cdh17:RFP) or Tol2(cdh17:Kim-1-RFP) vector at the one-cell stage. Tol2(cdh17:Kim-1-RFP) zebrafish begin to develop detectable edema at 2 dpf. Black arrows indicate the edema area; white arrows indicate RFP or Kim-1-RFP expression in kidney tubules (scale bar, 50 μm). (D) Western blot analysis of RFP expression in Tol2(cdh17:RFP) or Tol2(cdh17:Kim-1-RFP) zebrafish. (E, F) Tol2(cdh17:RFP) or Tol2(cdh17:Kim-1-RFP) zebrafish were analyzed daily for edema formation and survival rate (the total number of tested fish is indicated). (G, H) At 96 hpf, wild-type (control) or Tol2(cdh17:RFP) or Tol2(cdh17:Kim-1-RFP) larvae were injected with 25 ng of tetramethylrhodamine-labeled 10 kDa dextran, and serial fluorescence images were quantitated over the eye at 0, 1, 7, 24, 31, and 43 hpi (n=20 fish per condition). Data are expressed as mean±SEM. *P<0.05.
and mass, proliferation, and survival.\textsuperscript{24–28} mTOR activity is low or absent in the normal kidney but increases markedly after AKI,\textsuperscript{29} diabetic nephropathy, and other forms of progressive CKD.\textsuperscript{30–34} We examined the activation of mTOR and evaluated whether inhibition of the mTOR pathway with rapamycin could protect against Kim-1-mediated kidney injury. As shown in Figure 7C, phosphorylated ribosomal S6 kinase (pS6K), the downstream target of mTOR, was increased in Kim-1 constitutive transgenic zebrafish at 3 months of age. pS6K localized to the apical surface of tubular cells that also expressed Kim-1 (Figure 7C). Compared with RFP-expressing controls, an increased number of pS6K-positive tubular cells were found in LTL+, DBA+, and LTL-/DBA– regions of Kim-1 transgenic zebrafish (Figure 7D).

Rapamycin Reduced Pericardial Edema and Mortality in Zebrafish Conditionally Overexpressing Kim-1 Expression of Kim-1 in the tubular epithelial cells of conditional transgenics was induced at 5 dpf. Rapamycin (50 nM) was added into the E3 medium at 9 dpf and maintained in the media after the addition. Time-course live images show that the development of both pericardial and yolk sac edema in tamoxifen-induced Kim-1 transgenic fish were reversed by rapamycin treatment at 10 dpf and 12 dpf (Figure 8A). Quantification of relative area of pericardial and yolk edema demonstrated that rapamycin treatment significantly reduced Kim-1-mediated pericardial and yolk edema formation (Figure 8B). To test the effects of rapamycin treatment on long-term mortality, conditional Kim-1 expression was induced at 5 dpf followed by addition of rapamycin at 5, 7, 9, or 11 dpf. Rapamycin was maintained in the media after the addition. These conditional Kim-1 transgenics died spontaneously at a median age of 21 days without rapamycin treatment, while rapamycin treatment of Kim-1 transgenic zebrafish significantly reduced mortality, with earlier treatment associated with more protection (Figure 8C).

Rapamycin Treatment in KIM-1 Transgenic Mice Ameliorated Serum Creatinine and Kidney Inflammation, and Diminished Fibrosis Next, we sought to examine whether this zebrafish model could predict mTOR pathway involvement in the pathophysiology of transgenic mice where KIM-1 is expressed in the kidney tubule derived from the Six-2+ metanephric mesenchyme (Kim1RECtg mice).\textsuperscript{12} The expression levels of mTOR pathway protein mRNAs were increased progressively in Kim1\textsuperscript{RECtg} mice at 2 and 4 weeks of age (Figure 9A). Enhanced levels of pS6K were present in Kim1\textsuperscript{RECtg} mice at 5 weeks (Figure 9B). Quantification of the number of pS6K-positive cells showed increased number in Kim1\textsuperscript{RECtg} mice at 5 weeks but not at 2 weeks of age, when compared with age-matched controls (Figure 9C). To test whether inhibition of mTOR by rapamycin protects against kidney injury and reduces the development of kidney fibrosis, Kim1\textsuperscript{RECtg} mice...
were treated with rapamycin by daily intraperitoneal (ip) injection (2 mg/kg per day, \( n = 6 \)) or saline (\( n = 6 \)) starting at 4 weeks of age for up to 6 weeks and mice were examined at 10 weeks of age. Staining with trichrome and PAS showed decreased kidney fibrosis, tubule dilatation, interstitial expansion, and interstitial mononuclear cell infiltration (Figure 9D).

The tubule injury scores and fibrosis index were lower in rapamycin-treated Kim1\(^{RECg}\) mice (\( P<0.05 \) and \( P<0.01 \) respectively; Figure 9E). Serum creatinine was lower and proteinuria less in rapamycin-treated mice at 10 weeks (\( P<0.01 \), Figure 9, F and G). In Kim1\(^{RECg}\) mice treated with rapamycin, there was a significant reduction in the number of the Ly-6G\(^+\) neutrophils; F4/80\(^+\) peritubular macrophages and dendritic cells (Figure 10A); Ki67\(^+\) tubular, glomerular, and interstitial proliferating cells; and CD3\(^+\) lymphocyte infiltration (Figure 10, B and C). Rapamycin decreased expression of pS6K, aSMA, fibronectin, and collagen-1 in Kim1\(^{RECg}\) mice (Figure 10, D–F). The mRNA levels of cortical cytokines, CXCR3, CXCL-10, IL-1\(\beta\), TNF-\(\alpha\), CXCL-1, IL-6, CXCL-2, MCP-1, and the marker of inflammation NGAL, were reduced (Figure 10G). Ly-6G\(^+\), collagen-1, and fibronectin mRNAs were also significantly reduced in Kim1\(^{RECg}\) mice treated with rapamycin (Figure 10, H–J).

**DISCUSSION**

KIM-1 is upregulated in a variety of human diseases and animal models,\(^{35}\) and is specifically expressed in injured proximal tubular epithelial cells, making it a sensitive and specific marker of proximal tubule injury in the kidney.\(^{1}\) In this study, we first identified and characterized the Kim/Tim family of proteins in zebrafish. Similar to mammalian KIM-1, zebrafish Kim-1 was markedly and specifically upregulated at the apical aspect of proximal tubular cells following kidney injury. The phagocytic function was also conserved in zebrafish Kim-1.

To evaluate whether chronic expression of Kim-1 resulted in kidney tubular injury in zebrafish, we generated transgenic zebrafish models in which Kim-1 was constitutively or conditionally expressed in the pronephric or mesonephric tubular epithelial cells. The tamoxifen-inducible conditional Kim-1 transgenic line was created to avoid potential early developmental consequences of Kim-1 expression. In both constitutive and conditional transgenic zebrafish, there was kidney tubular injury, reduced renal function, and systemic consequences including edema, smaller size, and an increased mortality rate.
These data indicate that zebrafish Kim-1 expression can model mammalian chronic KIM-1 expression and hence enable mechanistic studies of the consequence of KIM-1 expression using the power of genetic manipulation. Furthermore, given that zebrafish are amenable to high-throughput drug screening, these transgenic lines will be useful to identify inhibitors of Kim-1 and potentially effective therapies for CKD. As proof of principle, we used this zebrafish model to identify rapamycin as a protective agent in zebrafish and confirmed that this protection could also be seen in the mouse model of KIM-1 overexpression.

Acute tubular damage can facilitate the development and progression of CKD, which is associated with interstitial fibrosis in human and other mammalian species. Although temporary expression of KIM-1 after injury will help to eliminate the apoptotic cells and debris and partially restore renal function, persistent KIM-1 expression in renal epithelial cells induces inflammation, tubulointerstitial fibrosis, and a murine CKD phenotype. Here, we have demonstrated that persistent expression of Kim-1 in zebrafish tubular epithelial cells results in the development of pericardial edema, reduced GFR, tubular damage, and a higher mortality. The damaged pronephric tubules were marked with flattening and loss of tubular brush border and atrophic tubular epithelial cells with accumulation of vacuoles.

Compared with the zebrafish pronephros, the zebrafish mesonephros more closely resembles the human metanephros. Prolonged Kim-1 expression in mesonephric tubular epithelial cells led to a lower body weight and reduced overall zebrafish size. As zebrafish age, new nephrons are continually added to the kidneys. Persistent expression of Kim-1 in the mesonephros resulted in smaller kidneys. The smaller kidneys and reduced growth of zebrafish are reminiscent of the growth retardation associated with renal disease in children and suggests a permissive role of an increase in nephrons and kidney function for growth in size of fish.

To evaluate the effects of Kim-1 expression independent of any potential effects on development or glomeruli barrier maturation, we utilized a tamoxifen-induced conditional Kim-1 overexpression model in the pronephros. These Kim-1 transgenic zebrafish develop phenotypes similar to those seen with constitutive expression: pericardial and yolk sac edema and increased mortality rates.

mTOR, a serine/threonine kinase, is activated after AKI and has been implicated in renal regeneration and repair. In addition, the activation of mTOR also occurs in a variety of animal models of diabetic nephropathy and other progressive CKD. We hypothesized that Kim-1-induced injury may involve mTOR pathway activation, which could contribute to kidney fibrosis and progressive CKD. In the zebrafish, we observed that the expression of Kim-1 was associated with mTOR activation, and that the mTOR inhibitor, rapamycin, protected against Kim-1-induced injury. We then confirmed the power of the zebrafish model by demonstrating the protective effect of rapamycin translated to Kim-1-mediated kidney tubular injury in Kim1RECtg mice, where rapamycin treatment significantly reduced the kidney fibrosis and interstitial inflammatory response. In our Kim-1 transgenic zebrafish and mice models, mTOR activity was highly associated with Kim-1 expression. Kim-1 is known to directly interact with phosphatidylinositol-3-kinase (PI3K) pathway subunit p85 and regulate PI3K signaling in a phosphotyrosine-dependent manner. Recently, we demonstrated that Kim-1...
The PI3K/AKT/mTOR pathway is a well-known intracellular signaling pathway related to cell growth, proliferation, and survival.

Thus, the zebrafish models can be used to interrogate pathophysiologic mechanisms and potential therapeutic approaches which predict mechanisms and efficacy in mammals.

**CONCISE METHODS**

**Structural and Molecular Evolutionary Analyses**

Homology analysis with nucleic acid and protein databases (Genbank, EMBL, and SwissProt), including human expressed sequence tag databases, was performed using the BLAST algorithm from the NCBI (National Library of Medicine). Aa analysis for putative post-translation modification sites was performed with PROSITE.

Ig domains were predicted using the PROSITE database. The transmembrane domain was predicted using TMpred software (www.cbs.dtu.dk/services/TMPREDFORM.html), and O-linked glycosylation sites were predicted using the NETNGLYC 3.1 server (www.cbs.dtu.dk/services/NetOGlyc). The draft genome databases and expressed sequence tag databases distributed at Swiss-Prot protein databases, Expasy, Ensembl, University of California, Santa Cruz (UCSC) Genome Browser, The Institute of Genomic Research (TIGR), and NCBI database for Expressed Sequence Tags (dbEST) were employed to retrieve the Ig molecules. Multiple alignments of sequences were conducted using the Multi-Align software and Clustal W program (version 1.23).

Thus, the zebrafish models can be used to interrogate pathophysiologic mechanisms and potential therapeutic approaches which predict mechanisms and efficacy in mammals.

**Zebrafish Husbandry**

All animal husbandry adheres to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Wild-type zebrafish (D. rerio) were maintained on a 14/10 hour light–dark cycle at 28.5°C and fed twice daily. Fertilized eggs were raised in embryo medium at 28.5°C and staged according to the standard method as

*interaction with p85 and subsequent PI3K-dependent signaling pathway play an important role in kidney injury and repair.

The PI3K/AKT/mTOR pathway is a well-known intracellular signaling pathway related to cell growth, proliferation, and survival. Thus, it is likely Kim-1 directly binds to p85 and regulates mTOR activity through the AKT signaling pathway.

In conclusion, our study is the first to identify the Kim family genes in zebrafish and to characterize the function and expression of Kim-1 after injury. Kim-1 can potentially be used as a marker for tubular injury in zebrafish, as in rodents and humans. Conditional expression of Kim-1 in the transgenic zebrafish demonstrated a critical pathogenic role of Kim-1 in chronic kidney tubular injury. Kim-1 expression markedly reduced kidney function and fish survival, and retarded fish growth in the adults that did survive. We found that the mTOR pathway was activated in Kim-1 transgenic zebrafish and inhibition of this pathway significantly reduced Kim-1-mediated kidney injury and increased fish survival. We extended the zebrafish findings into mammalian models by examining the Kim-1-mediated mTOR pathway in mice and found that it was activated in Kim1RECtg mice. Inhibition of the mTOR pathway significantly ameliorated the Kim-1-mediated tubular injury and kidney fibrosis in Kim1RECtg mice. Thus, the zebrafish models can be used to interrogate pathophysiologic mechanisms and potential therapeutic approaches which predict mechanisms and efficacy in mammals.

**CONCISE METHODS**

**Structural and Molecular Evolutionary Analyses**

Homology analysis with nucleic acid and protein databases (Genbank, EMBL, and SwissProt), including human expressed sequence tag databases, was performed using the BLAST algorithm from the NCBI (National Library of Medicine). Aa analysis for putative post-translation modification sites was performed with PROSITE. Ig domains were predicted using the PROSITE database. The transmembrane domain was predicted using TMpred software (www.cbs.dtu.dk/services/TMPREDFORM.html), and O-linked glycosylation sites were predicted using the NETNGLYC 3.1 server (www.cbs.dtu.dk/services/NetOGlyc). The draft genome databases and expressed sequence tag databases distributed at Swiss-Prot protein databases, Expasy, Ensembl, University of California, Santa Cruz (UCSC) Genome Browser, The Institute of Genomic Research (TIGR), and NCBI database for Expressed Sequence Tags (dbEST) were employed to retrieve the Ig molecules. Multiple alignments of sequences were conducted using the Multi-Align software and Clustal W program (version 1.23).

Thus, the zebrafish models can be used to interrogate pathophysiologic mechanisms and potential therapeutic approaches which predict mechanisms and efficacy in mammals.

**Zebrafish Husbandry**

All animal husbandry adheres to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Wild-type zebrafish (D. rerio) were maintained on a 14/10 hour light–dark cycle at 28.5°C and fed twice daily. Fertilized eggs were raised in embryo medium at 28.5°C and staged according to the standard method as

*interaction with p85 and subsequent PI3K-dependent signaling pathway play an important role in kidney injury and repair.

The PI3K/AKT/mTOR pathway is a well-known intracellular signaling pathway related to cell growth, proliferation, and survival. Thus, it is likely Kim-1 directly binds to p85 and regulates mTOR activity through the AKT signaling pathway.

In conclusion, our study is the first to identify the Kim family genes in zebrafish and to characterize the function and expression of Kim-1 after injury. Kim-1 can potentially be used as a marker for tubular injury in zebrafish, as in rodents and humans. Conditional expression of Kim-1 in the transgenic zebrafish demonstrated a critical pathogenic role of Kim-1 in chronic kidney tubular injury. Kim-1 expression markedly reduced kidney function and fish survival, and retarded fish growth in the adults that did survive. We found that the mTOR pathway was activated in Kim-1 transgenic zebrafish and inhibition of this pathway significantly reduced Kim-1-mediated kidney injury and increased fish survival. We extended the zebrafish findings into mammalian models by examining the Kim-1-mediated mTOR pathway in mice and found that it was activated in Kim1RECtg mice. Inhibition of the mTOR pathway significantly ameliorated the Kim-1-mediated tubular injury and kidney fibrosis in Kim1RECtg mice. Thus, the zebrafish models can be used to interrogate pathophysiologic mechanisms and potential therapeutic approaches which predict mechanisms and efficacy in mammals.

**CONCISE METHODS**

**Structural and Molecular Evolutionary Analyses**

Homology analysis with nucleic acid and protein databases (Genbank, EMBL, and SwissProt), including human expressed sequence tag databases, was performed using the BLAST algorithm from the NCBI (National Library of Medicine). Aa analysis for putative post-translation modification sites was performed with PROSITE. Ig domains were predicted using the PROSITE database. The transmembrane domain was predicted using TMpred software (www.cbs.dtu.dk/services/TMPREDFORM.html), and O-linked glycosylation sites were predicted using the NETNGLYC 3.1 server (www.cbs.dtu.dk/services/NetOGlyc). The draft genome databases and expressed sequence tag databases distributed at Swiss-Prot protein databases, Expasy, Ensembl, University of California, Santa Cruz (UCSC) Genome Browser, The Institute of Genomic Research (TIGR), and NCBI database for Expressed Sequence Tags (dbEST) were employed to retrieve the Ig molecules. Multiple alignments of sequences were conducted using the Multi-Align software and Clustal W program (version 1.23).

Thus, the zebrafish models can be used to interrogate pathophysiologic mechanisms and potential therapeutic approaches which predict mechanisms and efficacy in mammals.

**Zebrafish Husbandry**

All animal husbandry adheres to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Wild-type zebrafish (D. rerio) were maintained on a 14/10 hour light–dark cycle at 28.5°C and fed twice daily. Fertilized eggs were raised in embryo medium at 28.5°C and staged according to the standard method as

*interaction with p85 and subsequent PI3K-dependent signaling pathway play an important role in kidney injury and repair.

The PI3K/AKT/mTOR pathway is a well-known intracellular signaling pathway related to cell growth, proliferation, and survival. Thus, it is likely Kim-1 directly binds to p85 and regulates mTOR activity through the AKT signaling pathway.

In conclusion, our study is the first to identify the Kim family genes in zebrafish and to characterize the function and expression of Kim-1 after injury. Kim-1 can potentially be used as a marker for tubular injury in zebrafish, as in rodents and humans. Conditional expression of Kim-1 in the transgenic zebrafish demonstrated a critical pathogenic role of Kim-1 in chronic kidney tubular injury. Kim-1 expression markedly reduced kidney function and fish survival, and retarded fish growth in the adults that did survive. We found that the mTOR pathway was activated in Kim-1 transgenic zebrafish and inhibition of this pathway significantly reduced Kim-1-mediated kidney injury and increased fish survival. We extended the zebrafish findings into mammalian models by examining the Kim-1-mediated mTOR pathway in mice and found that it was activated in Kim1RECtg mice. Inhibition of the mTOR pathway significantly ameliorated the Kim-1-mediated tubular injury and kidney fibrosis in Kim1RECtg mice. Thus, the zebrafish models can be used to interrogate pathophysiologic mechanisms and potential therapeutic approaches which predict mechanisms and efficacy in mammals.

**CONCISE METHODS**

**Structural and Molecular Evolutionary Analyses**

Homology analysis with nucleic acid and protein databases (Genbank, EMBL, and SwissProt), including human expressed sequence tag databases, was performed using the BLAST algorithm from the NCBI (National Library of Medicine). Aa analysis for putative post-translation modification sites was performed with PROSITE. Ig domains were predicted using the PROSITE database. The transmembrane domain was predicted using TMpred software (www.cbs.dtu.dk/services/TMPREDFORM.html), and O-linked glycosylation sites were predicted using the NETNGLYC 3.1 server (www.cbs.dtu.dk/services/NetOGlyc). The draft genome databases and expressed sequence tag databases distributed at Swiss-Prot protein databases, Expasy, Ensembl, University of California, Santa Cruz (UCSC) Genome Browser, The Institute of Genomic Research (TIGR), and NCBI database for Expressed Sequence Tags (dbEST) were employed to retrieve the Ig molecules. Multiple alignments of sequences were conducted using the Multi-Align software and Clustal W program (version 1.23).

Thus, the zebrafish models can be used to interrogate pathophysiologic mechanisms and potential therapeutic approaches which predict mechanisms and efficacy in mammals.

**Zebrafish Husbandry**

All animal husbandry adheres to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Wild-type zebrafish (D. rerio) were maintained on a 14/10 hour light–dark cycle at 28.5°C and fed twice daily. Fertilized eggs were raised in embryo medium at 28.5°C and staged according to the standard method as

*interaction with p85 and subsequent PI3K-dependent signaling pathway play an important role in kidney injury and repair.

The PI3K/AKT/mTOR pathway is a well-known intracellular signaling pathway related to cell growth, proliferation, and survival. Thus, it is likely Kim-1 directly binds to p85 and regulates mTOR activity through the AKT signaling pathway.

In conclusion, our study is the first to identify the Kim family genes in zebrafish and to characterize the function and expression of Kim-1 after injury. Kim-1 can potentially be used as a marker for tubular injury in zebrafish, as in rodents and humans. Conditional expression of Kim-1 in the transgenic zebrafish demonstrated a critical pathogenic role of Kim-1 in chronic kidney tubular injury. Kim-1 expression markedly reduced kidney function and fish survival, and retarded fish growth in the adults that did survive. We found that the mTOR pathway was activated in Kim-1 transgenic zebrafish
Figure 9. Rapamycin treatment ameliorated kidney injury and fibrosis in Kim1RECtg mice. (A) Real-time PCR demonstrates increased mRNA expression of mTOR pathway intermediates in Kim1RECtg mice relative to controls at 2 and 4 weeks. **P<0.01; ***P<0.001 (n=6 for each). (B) The immunofluorescence staining of pS6K in Kim1RECtg and control mice at 2 and 5 weeks of age. (C) Quantification of the number of pS6K-positive cells in Kim1RECtg or controls **P<0.01 (n=6 for each). (D) Trichrome and PAS staining of control or RAP-treated Kim1RECtg mice. At 4 weeks of age mice were given daily ip injection of RAP (2 mg/kg per day) for 6 weeks (n=6). Kidneys were taken at 10 weeks of age. Kidney fibrosis, tubule dilation, interstitial expansion, and interstitial mononuclear cell infiltration were decreased after RAP treatment of Kim1RECtg kidneys (scale bar, 50 μm). (E) Quantification of the tubule injury score and fibrosis index in Kim1RECtg after vehicle or RAP treatment (n=6 for each). (F) Serum creatinine at 4 weeks and 10 weeks of age in control (n=6) or Kim1RECtg mice (n=6), treated with vehicle or RAP. Data are expressed as mean±SEM. *P<0.05; **P<0.01; ***P<0.001. (G) Coomassie blue staining of control or RAP-treated Kim1RECtg mouse kidneys at 10 weeks of age shows the reduction of proteinuria with RAP treatment. DAPI, 4',6-diamidino-2-phenyindole.

Induction of AKI by Gentamicin and Renal Function Measurements

For the gentamicin and dextran-FITC injection, zebrafish larvae were anesthetized in a 1:20:1:100 dilution of 4 mg/ml tricaine (Sigma-Aldrich) and positioned on their back in a 1% agarose injection mold. Using a Nanoject II injection device (Drummond Scientific, Broomall, PA), 2.3 nl of a 10 mg/ml gentamicin (Sigma-Aldrich) stock solution and 5 ng of 40 kDa dextran-FITC (Molecular Probes), to confirm drug delivery, were injected into the cardiac venous sinus. Fish were then returned to egg water, where they regained motility quickly. Renal function was assayed as previously described. Briefly, 25 ng of tetramethylrhodamine-labeled 10 kDa dextran was injected into the cardiac venous sinus of control or Kim-1-overexpressing larvae at 72 hpf. With constant exposure time and gain, images were taken at 1, 7, 24, 31, and 43 hpi. More than ten fish were studied in each group. The fluorescence intensities over the eye were analyzed using the NIH ImageJ software. For adult zebrafish, gentamicin (5 μg/100 mg) and 40 kDa dextran-FITC (25 ng) were administered by ip injection.

Transgenic Zebrafish

To generate the Tol-cdh17:kim1-RFP construct, the zebrafish kim1-L coding sequence was cloned by RT-PCR. It was fused to the RFP coding sequence (kim1-L-RFP) and was inserted in place of the EGFP gene in the Tol-cdh17:EGFP construct (gift from Leonard I. Zon, Harvard Medical School), and inserted into PmiToII2 plasmid at EcoRV and Spel sites. Then the kim1-L-RFP sequence was inserted at SpeI and XhoI sites. For creating the cdh17:Cre-ERT2 construct, Cre-ERT2 (a gift from Dr. Ben Humphreys, Brigham and Women’s Hospital) replaced the EGFP gene in the Tol-cdh17:EGFP construct at KpnI and NotI restriction enzymes. For the Tol-cdh17:RAP construct, we replaced EGFP with RFP at BamHI and NotI sites. To generate the ubiqu:loxP-EGFP-loxP-kim1-RFP construct, ubiqu:loxP-EGFP-loxP sequence was cloned from the ubiqu:loxP-EGFP-loxP-RFP construct (gift from Weibin Zhou, University of Michigan), using KpnI and NotI restriction enzymes. For the Tol-cdh17:RAP construct, we replaced EGFP with RFP at BamHII and NotI sites. To generate the Tol-cdh17:cre-loxP-EGFP-loxP sequence, gentamicin (5 μg/100 mg) and 40 kDa dextran-FITC (25 ng) were administered by ip injection.

Rapamycin Treatment

In zebrafish, rapamycin (50 nM) was added into the E3 medium at 5, 7, 9, or 11 dpf and maintained in the media after the addition. In mice, the Kim1RECtg mice were treated with rapamycin by daily ip injection (2 mg/kg per day) or saline starting at 4 weeks of age for up to 6 weeks and mice were examined at 10 weeks.

Tissue Preparation and Histology

Zebrafish were euthanized in 300 mg/L tricaine. Tissues were fixed in 4% paraformaldehyde, embedded in paraffin wax, and sectioned and stained with H&E or PAS using standard procedures. For EM, kidneys were harvested, fixed with 2.5% glutaraldehyde, and sectioned.
Kidney area was measured on H&E-stained sections (5 μm) of five kidneys in each group by ImageJ software. Glomeruli in these sections were counted blinded to the genotype. The number of glomeruli per area of kidney was determined. The two-dimensional area of pericardial and yolk sac edema were measured on live images of fish in each group. Areas were quantitated using ImageJ software.

Mice were anesthetized, euthanized, 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 stained with PAS using standard procedures. PAS-stained paraffin sections were assessed by quantitative measurement of tubular injury in ten individual high-power fields (magnification, ×400) per kidney. A percentage of the area affected was estimated for the number of necrotic cells, loss of brush border, cast formation, and tubule dilation and was scored as follows: 0, 0%–5%; 1, 5%–10%; 2, 11%–25%; 3, 26%–45%; 4, 46%–75%; and 5, >76%. The fields analyzed in each section were selected at random and evaluations were made in a blinded fashion.

**Zebrafish Kim-1 Antibody**

From the zebrafish Kim-1 full protein sequence, we designed three anti-peptide antibodies against LQLNYRESHRFS, LYLISEKMTTDDVRM, and LFLRLRRYREQTI derived from Ig domain, mucin domain, and cytoplasmic domain, respectively. Antibodies were synthesized by Genemed Synthesis Inc. (San Antonio, Texas). Western Blot analysis (Supplemental Figure 2) demonstrates that the expression of Kim-1 was upregulated in gentamicin-treated adult zebrafish and hypoxia-exposed larval zebrafish, compared with wild-type adult fish and

![Figure 10.](image-url)
larval *kim1* morphants (*kim1* Mo, GTGCATTAAACACTCAC-CATTCTTC) (Supplemental Figure 2). The anti–Kim-1 antibody (1:100) raised against the Ig domain peptide was used throughout the manuscript for immunofluorescence staining.

**Immunofluorescence Staining**

Cryosections of 5 μm were mounted on Fisher Superfrost Plus microscope slides (Thermo Fisher Scientific), air-dried and prepared for immunofluorescence. TTL (lectin, 1:500; cat. no. L-1030; Vector Laboratories) and DBA (fluorescein, 1:500, cat. no. L-1321; Vector Laboratories) were used to identify proximal and distal tubules in zebrafish and mice. Primary antibodies against the following proteins were used: cleaved caspase 3, pS6K, fibronectin, and collagen I (rabbit polyclonal, 1:100, Cell Signaling Technology); RFP and Na+/K+ ATPase V9 (mouse monoclonal, 1:100, Abcam, Inc.); and ERK (1:1000; Cell Signaling Technology); mouse anti-RFP (1:200, Clontech); mouse anti-aSMA (1:200, Abcam, Inc.); pS6K (1:500; Cell Signaling Technology); and ERK (1:1000; Cell Signaling), followed by goat anti-mouse HRP (1:200, Jackson ImmunoResearch). Bands were visualized by chemiluminescence (Western Lightning; PerkinElmer).

**Expression Analysis of Genes by Quantitative RT-PCR**

Total RNA was isolated from snap-frozen kidney, brain, heart, eye, fin, intestine, pancreas, muscle, gills, and liver of five adult zebrafish with RNaseasy Kit. Of total RNA, 1 μg was treated with DNase RQ1 (Promega) and then reverse transcribed with the M-MLV reverse transcription Kit and Oligo dT primers (Promega). Real-time, quantitative RT-PCR was performed using a BioRad iCycler. Samples were in triplicate, and all experiments were repeated three times using separately prepared samples. Statistical analyses was performed using SPSS 11 software. Statistical significance for quantitative RT-PCR was analyzed by the Mann–Whitney *U* test.

**Histologic Analysis and EM**

Histologic analysis was performed on paraffin-embedded fish using 3-μm sections, stained with H&E or PAS. Portions of zebrafish larvae were fixed in Karnovsky fixative and processed for EM studies by standard procedures. Semithin sections of each block were stained with toluidine blue stain and examined by light microscopy to select for ultrathin sectioning. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by EM.

**Western Blot Analysis**

Kidneys were isolated and lysed as previously described.12 Membranes were incubated with rabbit anti–Kim-1 peptide antibodies against LQLNYRESHRSF (antibody #1) and LFLRRLRREQTI (antibody #3) (1:500, Genemed Synthesis Inc.); mouse anti-RFP (1:200, Clontech); mouse anti-aSMA (1:200, Abcam, Inc.); pS6K (1:500; Cell Signaling Technology); and ERK (1:1000; Cell Signaling), followed by goat anti-mouse HRP (1:200, Jackson ImmunoResearch). Bands were visualized by chemiluminescence (Western Lightning; PerkinElmer).

**Phagocytosis Assays**

To quantitate phagocytosis, hKIM-1-GFP-PK1, zKim-1-GFP-PK1, and GFP-PK1 cells (3×10^5 each) were plated in 3.8 cm^2 wells 24 hours prior to assay. 1×10^6 Dil-labeled apoptotic thymocytes or 50 mg/ml ox-LDL was added to confluent epithelial cell layers. After 2 hours at 37°C, cells were washed vigorously five times with ice-cold PBS/0.1% sodium azide (without Mg^2+/Ca^2+) to remove bound and uningested apoptotic cells or debris. Cells were fixed in 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100 and blocked with 0.1% BSA. Primary and appropriate second antibodies were applied to the cells, and imaging was performed with a Nikon Confocal microscope.
After incubation with apoptotic cells, the cells were washed, trypsinized, and stained with primary anti-EGFP antibody and FITC-conjugated secondary antibody, resuspended in FACS buffer and subjected to flow cytometry (FACSCalibur; BD Biosciences). Ingestion of Dil-labeled apoptotic cells was identified using green and red channels. Data were analyzed by FlowJo software version 9.4.7.

Statistical Analyses

Results were calculated as mean ± SEM. Analysis of variance was used to compare data among groups. Two-tailed, unpaired t tests were used to compare two groups. P values <0.05 (*P<0.05 and **P<0.01) were considered statistically significant.

ACKNOWLEDGMENTS

We thank Weinbin Zhou for the pTol-cdh17:EGFP plasmid and Dr. Leonard I. Zon for the ubi:switch plasmid. We thank Iain A. Drummond for help with various zebrafish techniques and Craig Brooks for helpful suggestions.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases/National Institutes of Health grants DK039773 and DK072381 to J.V.B.

DISCLOSURES

None.

REFERENCES

reference genome sequence and its relationship to the human ge-
20. McCampbell KK, Wingert RA: New tides: using zebrafish to study renal
Yamazaki S, Enomoto Y, Oki T, Akiba H, Abe T, Komori T, Monikawa Y,
Kiyonari H, Takai T, Okumura K, Kitamura T: TIM1 is an endogenous
ligand for LMIR5/CD300b: LMIR5 deficiency ameliorates mouse kidney
22. Kramer-Zucker AG, Wiessner S, Jensen AM, Drummond IA: Organiza-
tion of the pronephric filtration apparatus in zebrafish requires
Nephrin, Podocin and the FERM domain protein Mosaic eyes. Dev Biol
285: 316–329, 2005
23. Mosimann C, Kaufman CK, Li P, Pugach EK, Tamplin OJ, Zon LI: Ubiqui-
tin promoter in zebra transgene expression and Cre-based recombination driven by the
24. Hsu PP, Kang SA, Rameseder J, Zhang Y, Ottina KA, Lim D, Peterson TR,
Choi Y, Gray NS, Yaffe MB, Marto JA, Sabatini DM: The mTOR-regulated
phosphoproteome reveals a mechanism of mTORC1-mediated inhibition
H, Tempst P, Sabatini DM: mTOR interacts with raptor to form a nutrient-
sensitive complex that signals to the cell growth machinery. Mol Cell
127: 125–137, 2006
26. Bullschleger S, Loewth R, Hall MN: mTOR signaling in growth and
27. Hresko RC, Mueckler M: mTOR.Rictor is the Ser473 kinase for Akt/
protein kinase B in 3T3-L1 adipocytes. J Biol Chem 280: 40406–40416,
2005
28. Liebenthal W, Fuhrro R, Andry CC, Rennkhe H, Abemathy VE, Koh JS,
Valeri R, Levine JS: Rapamycin impairs recovery from acute renal failure:
role of cell-cycle arrest and apoptosis of tubular cells. Am J Physiol
Renal Physiol 281: F693–F706, 2001
29. Johnson SC, Rabinovitch PS, Kaeberlein M: mTOR is a key modulator of
pamycin prevents early steps of the development of diabetic ne-
pamycin pathway blockade slows progression of diabetic kidney disease
attenuates unilateral ureteral obstruction-induced renal fibrosis. Kid-
ney Int 69: 2029–2036, 2006
33. Dietmann F, Rovira J, Carerras J, Arellano EM, Bañón-Maneus E,
Ramírez-Bajo MJ, Gutiérrez-Dalmau A, Brunet M, Campistol JM: Mammalian target of rapamycin inhibition halts the progression of
proteinuria in a rat model of reduced renal mass. J Am Soc Nephrol 18:
2653–2660, 2007
16: 556–561, 2010
cell cycle arrest in G2/M mediates kidney fibrosis after injury. Nat Med
16: 535–543, 2010
T, Kuchroo V, Bonventre JV: KIM-1-mediated phagocytosis reduces acute
37. Zhou W, Boucher RC, Bollig F, Englert C, Hildebrandt F: Character-
ization of mesonephric development and regeneration using trans-
Dai C: Rheb/mTORC1 signaling promotes kidney fibroblast activation
F, Cordts T, Wanner N, Reichardt W, Kerjaschki D, Ruegg MA, Hall MN,
Moulin P, Busch H, Boerries M, Walz G, Artunc F, Huber TB: mTORC1
maintains renal tubular homeostasis and is essential in response to is-
Rictor/mTORC2 protects against cisplatin-induced tubular cell death
41. de Souza AJ, Oak JS, Jordanhazy R, DeKruyff RH, Fruman DA, Kane LP:
Ten cell Ig and mucin domain-1-mediated T cell activation requires
recruitment and activation of phosphoinositide 3-kinase. J Immunol
180: 6518–6526, 2008
42. Brooks CR, Yeung MY, Brooks YS, Chen H, Ichimura T, Henderson JM,
Bonventre JV: KIM-1/TIM-1-mediated phagocytosis links ATG5/ULK1-
dependent clearance of apoptotic cells to antigen presentation [pub-
lished online ahead of print August 17, 2015]. EMBO J doi:1015252/
embj.201489838
43. Loh AH, Brennan RC, Lang WH, Hickey RJ, Malkas LH, Sandoval JA:
Dissecting the PI3K signaling axis in pediatric solid tumors: Novel tar-
gets for clinical integration. Front Oncol 3: 93, 2013
44. Vanhaesebroeck B, Stephens L, Hawkins P: PI3K signalling: the path to
238, 2002
47. Corpet F: Multiple sequence alignment with hierarchical clustering.
48. Thompson JD, Higgins DG, Gibson TJ: CLUSTAL W: improving the
sensitivity of progressive multiple sequence alignment through se-
quence weighting, position-specific gap penalties and weight matrix
49. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S: MEGAS:
molecular evolutionary genetics analysis using maximum likelihood,
evolutionary distance, and maximum parsimony methods. Mol Biol

This article contains supplemental material online at http://jasn.asnjournals.
omlook/suppl/doi/10.1681/ASN.2015050500/DCSupplemental.