Targeted Deletion of p53 in the Proximal Tubule Prevents Ischemic Renal Injury

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ABSTRACT

The contribution of p53 to kidney dysfunction, inflammation, and tubular cell death, hallmark features of ischemic renal injury (IRI), remains undefined. Here, we studied the role of proximal tubule cell (PTC)-specific p53 activation on the short- and long-term consequences of renal ischemia/reperfusion injury in mice. After IRI, mice with PTC-specific deletion of p53 (p53 knockout [KO]) had diminished whole-kidney expression levels of p53 and its target genes, improved renal function, which was shown by decreased plasma levels of creatinine and BUN, and attenuated renal histologic damage, oxidative stress, and infiltration of neutrophils and macrophages compared with wild-type mice. Notably, necrotic cell death was attenuated in p53 KO ischemic kidneys as well as oxidant-injured p53-deficient primary PTCs and α-2-fetuin-treated PTC lines. Reduced oxidative stress and diminished expression of PARP1 and Bax in p53 KO ischemic kidneys may account for the decreased necrosis. Apoptosis and expression of proapoptotic p53 targets, including Bid and Siva, were also significantly reduced, and cell cycle arrest at the G2/M phase was attenuated in p53 KO ischemic kidneys. Furthermore, IRI-induced activation of TGF-β and the long-term development of inflammation and interstitial fibrosis were significantly reduced in p53 KO mice. In conclusion, specific deletion of p53 in the PTC protects kidneys from functional and histologic deterioration after IRI by decreasing necrosis, apoptosis, and inflammation and modulates the long-term sequelae of IRI by preventing interstitial fibrogenesis.


AKI is a clinical syndrome characterized by a rapid decline in GFR over a period of hours to days, leading to retention of metabolic waste products and disrupted fluid, electrolyte, and acid–base balance.1 Ischemic renal injury (IRI), which results from compromised perfusion of renal tissues, is the leading cause of AKI.2,3 Persistent perfusion deficit in the medulla, limited anaerobic glycolytic capacity, and targeted inhibition of glycolysis in the proximal tubule cell (PTC) make the proximal straight tubule the most vulnerable tubular segment to ischemic injury.4–7 Pathologically, IRI is characterized by apoptotic/necrotic cell death and inflammation in the outer medulla, which are proportional to the severity of renal ischemia.8,9 Pro- and antiapoptotic signaling pathways in the PTC are precisely regulated by essential factors, such as p53 and its proapoptotic targets, BCL2 family proteins and caspases.9–14 Therefore, it is crucial to understand the roles of these molecules in regulating tubular cell apoptosis in the pathogenesis of IRI.

The transcription factor p53, which was first identified as a tumor suppressor, performs many essential cell functions, such as halting the cell cycle, promoting senescence and apoptosis, and regulating cell metabolism.15 In response to various cell stresses and DNA damage, p53 controls the transcription of target genes that are usually key factors in cellular stress pathways.16 Although the transcriptional activation of p53 was considered the major mechanism by which p53 responds to cell stress, several studies suggest that transcription-independent
activity of p53 can potentiate apoptosis by directly interacting with members of BCL2 family proteins.\textsuperscript{12,17}

The involvement of p53 has been reported in nephrotoxic injury and IRI.\textsuperscript{18–20} Although previous studies showed that p53 levels are significantly increased in the medulla after IRI,\textsuperscript{9} the contribution of p53 to tubular cell death and kidney dysfunction is still unclear.\textsuperscript{8,9,20–22} Thus, considering the importance of p53 in promoting apoptotic/necrotic cell death, we hypothesized that knockout (KO) of p53 in the proximal tubule significantly reduces tubular cell death and kidney dysfunction after IRI. We tested this hypothesis using KO mice with the p53 gene specifically deleted in the proximal tubule.

RESULTS

Deletion of p53 in the Proximal Tubule Reduced p53 Expression after IRI

We analyzed the expression of p53 and one of its target genes, p21, after injury in whole-kidney tissues. Indeed, 24 hours after IRI, the expression levels of p53 (Figure 1, A and B) and p21 (Figure 1, A and C) were significantly increased in wild-type (WT) male littermates compared with sham-operated mice kidneys. However, the expression level of p53 was only slightly increased and p21 expression was not altered in p53 KO mice compared with sham-operated mice after IRI. The slightly increased levels of p53 in IRI-induced p53 KO may be because of p53 expression in cells other than the proximal tubules (PTs). Because of the difficulty in immunodetection of p53 in renal tissue, we carried out Western blot analysis for p53 in PTs isolated from p53 KO mice. Our data further confirmed successful deletion of p53 in the PT (Supplemental Figure 1). These data suggest that p53 is induced and involved in transcriptional regulation after IRI.

PT-Specific KO of p53 Reduced Deterioration of Renal Function after IRI

Ischemic kidneys from WT mice showed widespread necrosis, brush border blebbing, and sloughed cells in the proximal straight tubule, whereas these features were much less apparent in ischemic kidneys from p53 KO mice. The histologic changes after IRI were quantified by counting and scoring the percentage of tubules that displayed tubular necrosis, cast formation, and tubular dilation (Figure 2, C and D). The cumulative scores of histologic damage in the outer medulla at 1, 5, and 16 days as well as necrosis (Figure 2E) at 1 day were significantly lower in p53 KO kidneys compared with WT kidneys post-IRI, showing that gene ablation of p53 reduced tubular damage and cellular necrosis.

Renal Inflammation Was Reduced after IRI in p53 KO Mice

The infiltration of leukocytes in the outer medulla of WT and p53 KO mice at 1, 5, and 16 days post-IRI was assessed by

Figure 1. PT-specific KO of p53 reduced p53 and p21 expression after IRI. (A) Representative images of Western blot analysis showing expression levels of p53 and p21 at 24 hours (1 d) after IRI. (B and C) The expression levels of p53 and p21 in sham and IRI-induced kidneys were quantified (n=4 in each group). *P<0.05 compared with WT IRI. GAPDH served as a loading control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Figure 2. PT-specific KO of p53 reduced renal function deterioration and histologic damage after IRI. (A) Plasma creatinine and (B) BUN levels from WT and p53 KO mice (n=6) at 6 hours (h) and 1, 2, and 3 days after IRI. *P<0.05 compared with WT IRI. (C) WT or p53 KO mice underwent sham surgery or IRI. Renal histologic changes in the outer medulla after IRI were assessed by Periodic acid–Schiff (PAS) staining at 1, 5, and 16 days after IRI. Original magnification, ×400. (D) Histologic damage in the outer medulla assessed in PAS-stained kidney sections was scored by counting the percentage of tubules that displayed tubular necrosis, cast formation, and tubular dilation as follows: 0, normal; 1, <10%; 2, 10%–25%; 3, 26%–50%; 4, 51%–75%; 5, >75%. Ten fields (×200 magnification) per kidney were used for counting. *P<0.05 compared with WT IRI (n=6). (E) The number of necrotic tubules was counted in PAS-stained kidney sections at 1 day after IRI. *P<0.05 compared with WT IRI 1 day (n=6).
immunostaining for neutrophils and macrophages. As shown in representative photographs (Supplemental Figure 2), WT mice exhibited increased infiltration of neutrophils and macrophages in the outer medulla, which was attenuated in p53 KO mice. The numbers of positively stained cells were counted in a blinded manner, and quantitative data indicate that the accumulation of neutrophils and macrophages was reduced in the outer medulla of p53 KO mice compared with WT mice at all time points after IRI (Figure 3, A and B).

**Attenuated Oxidative Stress after IRI in p53 KO Mice**

Oxidative stress was assessed by lipid hydroperoxide levels in the kidney. Quantification of the whole-kidney lipid peroxide levels at 5 hours and 1 and 2 days post-IRI shows that its levels were significantly decreased in p53 KO mice compared with WT mice at all time points (Figure 3C).

**PT-Specific Deletion of p53 Decreased PARP1 Expression after IRI**

PARP1 can induce necrotic cell death after IRI. PARP1 expression was examined by Western blot analysis. PARP1 expression was significantly increased in WT kidneys after IRI, but its expression was downregulated in p53 KO mice (Figure 3D). This novel finding suggests that increased PARP1 function may be one of the mechanisms by which p53 activation regulates necrosis.

**Loss of p53 Reduced Renal Fibrosis after IRI**

To investigate whether deletion of p53 reduces renal fibrosis, collagen deposition in the kidneys of WT and p53 KO mice was measured using Sirius red staining and α-SMA immunofluorescence staining. Sixteen days after IRI, WT mice showed a dramatic increase of Sirius red-positive area in the kidneys compared with p53 KO mice, indicating that deletion of p53 reduces renal fibrosis in the late stage of IRI (Figure 5, A and B). No change in the Sirius red staining was seen at 5 days postinjury. α-SMA expression, however, was decreased in p53 KO mice compared with WT mice at 5 days after IRI, which was shown by immunostaining and Western blot analysis (Figure 5, C–F). p-Smad3, a downstream signaling molecule of TGF-β, was also increased in WT mice but significantly reduced in p53 KO mice at 16 days post-IRI (Figure 5, E and G).

**Loss of p53 Reduced Cell Cycle Arrest after IRI**

Cell cycle arrest at G2/M phase is associated with late kidney fibrosis in IRI. Histone H3 is phosphorylated during mitosis at Ser-10.
Phospho-H3 (p-H3) staining was performed to detect G2/M phase arrest. In WT mice, the number of p-H3-positive cells was significantly higher compared with the number of p-H3-positive cells in p53 KO mice at 1, 5, and 16 days after IRI (Fig. 5H, Supplemental Figure 4A). To confirm that the cells are truly arrested at the G2 phase and not in mitotic phase, we assessed the number of mitotic cells at the above time points. Mitotic entry is accompanied by the phosphorylation of several molecules, including mitotic protein monoclonal 2 (MPM-2), that may regulate the mitotic processes.24,25 Immuno- staining using antiphospho-Ser/Thr-Pro and MPM-2 antibody (05–368; EMD Millipore, Bedford, MA) showed that the number of cells at M phase was increased in WT mice 1 day after IRI compared with in KO mice, but it only accounted for a small fraction of the cells arrested at G2/M phase, indicating that most p-H3-positive cells were arrested at G2 phase (Supplemental Figure 4, B and C). The number of cells at M phase was negligible at 5 and 16 days in both WT and p53 KO mice tissues.

**Loss of p53 Reduced Necrotic Cell Death of PTC**

To further study the role of p53 in necrotic cell death, we used an in vitro H2O2-induced necrosis model. Necrosis was assayed using trypan blue staining and lactate dehydrogenase (LDH) release assay. After 1 hour of treatment of 1 or 2 mM H2O2, p53 KO primary PTCs had fewer trypan blue-positive cells compared with WT PTCs (Figure 6A). Pharmacological inhibition of p53 using pifithrin-α also reduced LDH release in LLC-PK1 cells after 5 mM H2O2 treatment (Figure 6B). These data indicate that deletion or inhibition of p53 reduces H2O2-induced necrosis.

**DISCUSSION**

Several studies have previously investigated the role of p53 in IRI. Kelly et al.9 first showed that p53 expression is increased in the renal medulla after IRI and that pharmacological inhibition of p53 using pifithrin-α can reduce renal injury in rats. Molitoris et al.20 showed that systemic administration of p53-targeted small interfering RNA in mice attenuates ischemic and cisplatin-induced AKI. In a recent report, Yang et al.26 administered pifithrin-α in a single dose 3 and 14 days after unilateral IRI in mice and showed that it relieved epithelial cell cycle arrest and inhibited fibrogenesis. However, two recent reports showed that p53 inhibition is detrimental to renal function in IRI. Dagher et al.21 administered pifithrin-α daily, starting at the time of bilateral IRI in rats and continuing for 7 days, which ultimately increased renal fibrosis. To add to the paradox, Sutton et al.22 showed that global p53 deficiency or pharmacological inhibition of p53 exacerbates the injury by increasing and prolonging leukocyte infiltration into the renal parenchyma. These differing results suggest that the role of p53 in the pathophysiology of IRI is much more complicated and still incompletely understood.

In this study, we used KO mice with p53 gene specifically deleted in the PTC to study its effect on the kidney damage after IRI. This strategy not only specifically targets the PT, the major injury site of IRI,6 but also excludes the normal function of p53.
Figure 5. Loss of p53 in PT reduced renal fibrosis and cell cycle arrest after IRI. (A) Collagen deposition detected by Sirius red staining in WT and p53 KO kidneys at 5 and 16 days after IRI. (B) The Sirius red-positive area was measured in four randomly chosen high-power (×200) fields per kidney using the National Institutes of Health ImageJ software. *P<0.05 compared with WT IRI (n=3). (C and D) Immunofluorescence staining of α-SMA in the outer medulla. The α-SMA-positive area was measured in four randomly chosen high-power (×200) fields per kidney. *P<0.05 compared with WT IRI (n=3). (E and F) Representative Western blot images and quantified data for expression of α-SMA in WT and p53 KO kidneys at 1, 5, and 16 days after IRI. *P<0.05 compared with WT IRI (n=3). (E and G) Representative Western blot films and quantified data for expression of p-Smad3 in WT and p53 KO kidneys at 1, 5, and 16 days after IRI. *P<0.05 compared with WT IRI (n=3). GAPDH served as a loading control. (H) Kidney sections were immunostained using p-H3 antibody. The number of p-H3-positive cells in WT and p53 KO kidneys at 1, 5, and 16 days after IRI was counted in 10 randomly selected high-power (×200) fields per kidney. *P<0.05 compared with WT IRI (n=4). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
in other cells within the kidney, like inflammatory cells recruited after IRI, in which absence of p53 may prolong inflammatory responses and increase renal injury. Our data show that specific deletion of p53 gene in the PT significantly improves kidney function, preserves renal histology, and reduces necrosis and apoptosis after IRI along with attenuated inflammatory response and late-term fibrogenesis. These findings support the concepts that p53 is a critical mediator of IRI and that its inhibition or its downstream signaling pathway prevents IRI and is a suitable therapeutic target.

A role for p53 in apoptosis is well established. p53 can upregulate the expression of several proapoptotic genes, including Bax, Fas/Apo-1, PERP, Siva, PUMA, and Noxa, under stress situations. Recently, Dong and colleagues, using two Bax-deficient mouse models, found that only conditional Bax deletion specifically from PTs could ameliorate IRI. Systemic deletion of Bax enhanced neutrophil infiltration without significant effect on kidney injury. Our data indicate that expression of Bax, Bid, and Siva are induced in ischemic kidneys but attenuated in p53 KO mice. The significance of Bid and Siva expression in IRI was previously reported. In a previous report, Dong and colleagues showed that Bid deficiency attenuated tubular disruption, tubular cell apoptosis, and caspase-3 activation during 48 hours of reperfusion. We previously reported that the expression of the proapoptotic p53 target Siva and its cognate receptor CD27 is highly upregulated and coexpressed in the ischemic rat and mouse renal tissues, indicating that Siva plays a critical role in apoptosis after IRI. It is likely that synergic functions of these proapoptotic molecules may lead to the execution of apoptosis after IRI.

Recently, Vaseva et al. showed that p53 may also be implicated in necrotic cell death through its interaction with cyclophilin D (CypD) in the inner membrane of mitochondria and subsequent opening of mitochondrial permeability transition pore (MPTP). Indeed, our data showed significantly reduced tubular necrosis and apoptosis and improvement of IRI along with attenuated oxidative stress and calcium mishandling. Our data indicate that p53 deletion can decrease oxidative stress, which could be a potential mechanism by which p53 inhibits necrosis in renal PTCs. Can p53 translocate to mitochondrial matrix and interact with CypD to open the MPTP after IRI? Our attempts to locate p53 to the mitochondria in various in vitro and in vivo models failed, suggesting that it may not be a prominent mechanism by which p53 induces necrosis in renal PTC (data not shown). However, our novel finding that p53 inhibition can downregulate PARP1 expression in renal PTC suggests that it could be an alternate mechanism by which p53 regulates necrosis after IRI. Activation of PARP1 is required for DNA repair, but excessive activation leads to necrotic cell death by depletion of intracellular ATP. We previously reported that pharmacological and genetic inhibition of PARP1 can prevent kidney dysfunction, oxidative stress, inflammation, and tubular necrosis but not apoptosis after ischemia/reperfusion and cisplatin nephrotoxicity. Furthermore, although bax/bak is generally regarded as an apoptosis inducer by regulating mitochondrial outer membrane permeabilization, recent evidence suggests that they may form the outer membrane channels of MPTP and contribute to necrotic cell death.

It is well established that neutrophils, monocytes/macrophages, and T cells play major roles in the pathophysiology of renal ischemia-reperfusion injury in animal models and human AKI. Blocking the activation or trafficking of proinflammatory leukocytes into the kidney is shown to prevent renal function deterioration and histologic damage. Recently, Sutton et al. used bone marrow transplantation to produce chimeric mice lacking p53 in leukocytes and showed that p53 deletion prevents leukocyte apoptosis and increases their potential for cytokine secretion, thus worsening the pathophysiology of IRI. This study also showed that systemic deletion of p53 can worsen the injury. Our studies using mice lacking p53 in PT, however, showed that it can profoundly decrease the infiltration of leukocytes and thus, protect the kidneys from IRI. The apparent paradox in the data from the two studies could be because of the variations in the experimental protocol and the differing effects of inhibition of p53 in specific cell types within the kidney.

The role of cell cycle arrest and tubular apoptosis on the fibrogenic response of kidney tissue has recently been established in different injury models of CKD. Defects in progression of the cell cycle after injury, such as arrest at G1/G0 or

**Figure 6.** Loss of p53 reduced PTC necrosis. (A) Percentage of trypan blue-positive cells after H2O2 treatment in primary PTCs derived from p53 KO compared with WT mice kidneys. *P<0.05 (n=4). (B) Percentage of LDH activity after 5 mM H2O2 treatment in LLC-PK1 cells with or without different concentrations of the p53 inhibitor ifitithin-α. *P<0.05 (n=4). Con, control with PBS or DMSO (dimethyl sulfoxide) treatment.
G2/M phase, cause tubular cells to switch to a profibrotic phenotype with increased expression and release of TGF-β, thereby promoting fibrosis.26 p53 is a major player in cell cycle regulation. The effect of pharmacological inhibition of p53 using pifithrin-α in fibrosis development after IRI was previously examined by two groups21,26 (the opposing results are detailed above). Although the exact cause of these differing results is not clear, the different species and the timing of p53 inhibition in the two experiments may have contributed. Our data indicate that G2/M cell cycle arrest occurs after IRI in WT mice but not p53 KO mice, which was shown by reduced p-H3 staining, and may contribute to the attenuation of fibrosis. PT-specific deletion of p53 downregulated p21 expression, suggesting that it may titrate the level of p21, which allows the cells to progress through G2 into M phase. However, our data show that p53 deletion can prevent inflammation and both necrotic and apoptotic cell deaths. Although it is unclear if the severity of the injury after an ischemic episode directly affects the progression of CKD, it is likely that the attenuated inflammation and injury in the p53 KO mice may contribute to the decreased fibrosis. Additional studies are needed to answer this question.

In summary, our data show that p53 has profound effects on tubular cell necrosis and apoptosis after IRI. Absence of p53 in the PT significantly preserves renal function and markedly reduces kidney damage, renal oxidative stress, inflammation, and long-term fibrosis. Targeting proximal tubular cell death by modulating p53 or its downstream signaling pathways may provide a more efficient therapeutic strategy for IRI.

CONCISE METHODS

Generation of PTC-Specific p53 KO Mice
Homozygous p53−/−mice (C57BL/6J background) were obtained from The Jackson Laboratories (Bar Harbor, ME). The breeding strategy for transgenic mice that expressed Cre recombinase under the control of kidney-specific Pepck promoter (Pepck-Cre) was reported elsewhere.43 KO mice with the p53 gene specifically disrupted in renal proximal tubular epithelial cells (genotype p53−/−, Cre+/−) were developed by mating p53−/−mice with Pepck-Cre transgenic mice. A routine PCR protocol was used for genotyping from tail DNA samples with the following primer pairs: Cre, 5′-CGGTGCTAACCAGGTTTTC-3′ and 5′-TGGGCGGCAATGGTCAAGTT-3′ and p53, 5′-GGTTAAACC-CAGCTTGACCA-3′ and 5′-GGAGGCAGAGACAGTTGGGAG-3′. Male littermates of the genotype p53−/−, Cre−/− were used as controls (WT). All animals were born at the expected Mendelian frequency and did not display any gross physical or behavioral abnormalities. Animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

Induction of IRI in Mice
IRI was induced in male mice as described previously.31,44 All animals were given free access to food and water. The mice were anesthetized by intraperitoneal administration of a cocktail containing ketamine (200 mg) and xylazine (16 mg) per 1 kg body wt. Ischemic injury was induced by bilateral renal pedicle clamping using microaneurysm clamps (Roboz Surgical Instrument, Gaithersburg, MD), and the core body temperature of mice was kept at 37°C. After 30 minutes of occlusion, the clamps were removed, and kidney reperfusion was verified visually. Sham-operated control animals underwent the same surgical procedure, except for the occlusion of the renal arteries. During the surgery, all animals were placed on a heating pad to maintain body temperature at 37°C. Blood samples were collected at the time of euthanasia or from the orbital sinus under isoflurane anesthesia at 0, 6, 24, and 48 hours post-IRI for measurement of serum creatinine and BUN. At the end of each time point (1, 5, or 16 days), renal tissue was collected, fixed in Bouins solution or snap frozen using liquid nitrogen, and stored at −80°C for future experiments.

Measurement of Serum Creatinine and BUN
Serum creatinine and BUN were measured to evaluate renal function using a Quantichrom assay kit (BioAssay Systems, Hayward, CA) according to the manufacturer’s protocol.

Morphologic Studies
WT and KO mice that underwent IRI were euthanized at 1 or 16 days. The kidneys then were processed at the University of Nebraska Medical Center histology core facility. Briefly, kidneys were fixed in formalin, embedded in paraffin, and cut into 5-μm sections. The tissue sections were then stained with Periodic acid–Schiff.

Immunofluorescence for Neutrophils
Formalin-fixed mouse kidney sections were processed for immunostaining as described previously.44 The slides were sequentially incubated with rabbit anti-mouse neutrophil antibody (Accurate, Westbury, NY) at a 1:100 dilution overnight at 4°C followed by FITC-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) at a 1:200 dilution for 1 hour at room temperature. Neutrophil infiltration was quantified by counting the number of stained cells per field.

Immunohistochemistry for Macrophages and p-H3 Staining
Formalin-fixed mouse kidney sections were processed for immunostaining by sequential incubation with rabbit anti-F4/80 antibody (18705–1-AP; Proteintech, Chicago, IL) and anti-p-H3 antibody (sc-8656-R; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:100 dilution overnight at 4°C followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Vector Laboratories) at a 1:200 dilution for 1 hour at room temperature. The color development was induced by diaminobenzamide reagent (Vector Laboratories) according to the manufacturer’s instructions. Macrophage infiltration and p-H3–positive cells were quantified by counting the number of stained cells per field.

Lipid hydroperoxide assays were performed in the kidney extracts using kits (BioVision, Mountain View, CA) according to the manufacturer’s instructions.

Apoptosis Detection by TUNEL Staining
TUNEL staining of kidney tissue sections was carried out using the In Situ Cell Death Detection Kit, Fluorescein (Roche, Indianapolis, IN) according to the manufacturer’s protocol.
Collagen Deposition by Sirius Red
The rehydrated paraffin sections were stained with Sirius red solution (0.1% Direct Red 80 and 1.5% picric acid; Sigma-Aldrich, St. Louis, MO) and washed two times in acidified water (0.5% acetic acid; Sigma-Aldrich). Then, the sections were dehydrated and cleared before being observed under the microscope.45

α-SMA Immunofluorescence Staining
Formalin-fixed mouse kidney sections were processed for immunostaining as described previously.44 The slides were sequentially incubated with mouse anti-α-SMA antibody (A5228; Sigma-Aldrich) at a 1:500 dilution overnight at 4°C followed by FITC-conjugated horse anti-mouse IgG (Vector Laboratories) at a 1:200 dilution for 1 hour at room temperature. α-SMA deposition was quantified by measuring α-SMA–positive area per field.

Western Blot Analyses
Briefly, whole-renal tissue extracts (80 μg protein/lane) were separated on 10% SDS-PAGE gels and then transferred to Immobilon membranes (EMD Millipore). The membranes were incubated with anti-p53 (2524; Cell Signaling Technology, Beverly, MA), anti-p21 (sc-6246; Santa Cruz Biotechnology), anti-p-Smad3 (ab51451; Abcam, Inc., Cambridge, MA), antiphospho-Ser/Thr-Pro, MPM-2 antibody (05–368; EMD Millipore), and anti-glyceraldehyde-3-phosphate dehydrogenase (sc-25778; Santa Cruz Biotechnology) antibodies overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies against the appropriate primary antibodies (1:5000; Vector Laboratories), exposed to Western Lightning Plus-ECL (NEL104001EA; PerkinElmer, Waltham, MA), and then developed with x-ray film. The area of each band was analyzed using the National Institutes of Health image software (ImageJ).

Proximal Tubular Cell Culture and In Vitro Experiment
Primary P7 epithelial cells were isolated from p53 KO or WT male mice and cultured as described previously.7,46 The porcine-derived proximal tubular cell line LLC-PK1 (ATCC, Rockville, MD) was cultured to 80%–90% confluent monolayer cultures as described previously.47 The cells were incubated with 1 or 2 mM H2O2 for 1 hour to induce necrosis.48 These concentrations and times were chosen for trypan blue staining as previously described.27 LDH release was measured enzymatically using a CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI).

Statistical Analyses
All data are expressed as means ± SEMs. One-way ANOVA was used to compare the mean values of all groups. An unpaired t test was used to compare the means of two different groups. A P value <0.05 was considered statistically significant.

ACKNOWLEDGMENTS
The authors thank Dr. Volker H. Haase for providing the Pepck-Cre transgenic mouse.

This study was supported by a University of Nebraska Medical Center predoctoral fellowship (to Y.Y.) and National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-083291 (to B.J.P.).

DISCLOSURES
None.

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