FoxO3 activation in hypoxic tubules prevents chronic kidney disease

Ling Li, … , Qais Al-Awqati, Fangming Lin


Acute kidney injury (AKI) can lead to chronic kidney disease (CKD) if injury is severe and/or repair is incomplete. However, the pathogenesis of CKD following renal ischemic injury is not fully understood. Capillary rarefaction and tubular hypoxia are common findings during the AKI to CKD transition. We investigated the tubular stress response to hypoxia and demonstrated that a stress responsive transcription factor, FoxO3, was regulated by prolyl hydroxylase. Hypoxia inhibited FoxO3 prolyl hydroxylation and FoxO3 degradation, thus leading to FoxO3 accumulation and activation in tubular cells. Hypoxia-activated Hif-1α contributed to FoxO3 activation and functioned to protect kidneys, as tubular deletion of Hif-1α decreased hypoxia-induced FoxO3 activation, and resulted in more severe tubular injury and interstitial fibrosis following ischemic injury. Strikingly, tubular deletion of FoxO3 during the AKI to CKD transition aggravated renal structural and functional damage leading to a more profound CKD phenotype. We showed that tubular deletion of FoxO3 resulted in decreased autophagic response and increased oxidative injury, which may explain renal protection by FoxO3. Our study indicates that in the hypoxic kidney, stress responsive transcription factors can be activated for adoptions to counteract hypoxic insults, thus attenuating CKD development.

Find the latest version:

http://jci.me/122256/pdf
FoxO3 Activation in Hypoxic Tubules Prevents Chronic Kidney Disease

Ling Li¹, Huimin Kang¹,², Qing Zhang³, Vivette D. D’Agati⁴, Qais Al-Awqati⁵ and Fangming Lin¹,*

Departments of ¹Pediatrics, ⁴Pathology and ⁵Internal Medicine, Columbia University Vagelos College of Physicians and Surgeons, New York, NY 10032, USA

²Department of Pediatrics, Fujian Medical University Union Hospital, Fuzhou, Fujian, China

³Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC 27599, USA

*Corresponding author

Name: Fangming Lin

Address: Division of Pediatric Nephrology

Department of Pediatrics

Columbia University Vagelos College of Physicians and Surgeons

622 West 168th Street, PH17-102F

New York, NY 10032, USA

Phone: (212) 305 0793

Email: FL2300@columbia.edu

Conflict of Interest: The authors have declared that no conflict of interest exists.

Word count: 11,878, including legends, table and references
Abstract

Acute kidney injury (AKI) can lead to chronic kidney disease (CKD) if injury is severe and/or repair is incomplete. However, the pathogenesis of CKD following renal ischemic injury is not fully understood. Capillary rarefaction and tubular hypoxia are common findings during the AKI to CKD transition. We investigated the tubular stress response to hypoxia and demonstrated that a stress responsive transcription factor, FoxO3, was regulated by prolyl hydroxylase. Hypoxia inhibited FoxO3 prolyl hydroxylation and FoxO3 degradation, thus leading to FoxO3 accumulation and activation in tubular cells. Hypoxia-activated Hif-1α contributed to FoxO3 activation and functioned to protect kidneys, as tubular deletion of Hif-1α decreased hypoxia-induced FoxO3 activation, and resulted in more severe tubular injury and interstitial fibrosis following ischemic injury. Strikingly, tubular deletion of FoxO3 during the AKI to CKD transition aggravated renal structural and functional damage leading to a much more profound CKD phenotype. We showed that tubular deletion of FoxO3 resulted in decreased autophagic response and increased oxidative injury, which may explain renal protection by FoxO3. Our study indicates that in the hypoxic kidney, stress responsive transcription factors can be activated for adaptions to counteract hypoxic insults, thus attenuating CKD development.
Introduction

When acute renal failure was recognized as a syndrome more than 70 years ago, it was considered to be an acute injury to the kidney, which more often than not resulted in recovery of renal function. A separate syndrome of chronic renal failure became known and the two were thought to differ in etiology and natural history. Recent epidemiological and case controlled studies using new definitions of the two syndromes indicate that acute kidney injury (AKI) and chronic kidney disease (CKD) are interconnected (1). AKI increases the risk for CKD by 8.8-fold and the risk for end-stage renal disease (ESRD) needing dialysis or kidney transplantation by 3.3-fold (2).

The pathogenesis of AKI leading to CKD has been under intensive investigation, with many investigators using animal models of ischemia-reperfusion injury (IRI). Mild ischemic injury triggers intrinsic repair mechanisms that result in replacement of damaged cells and complete functional recovery. (3, 4). With more severe injury, the repair processes fail to completely ameliorate the damage and animals develop chronic kidney disease (5, 6). The severity of ischemic injury induced by clamping of renal blood vessels can be readily modified by changing the duration of warm ischemia. A severe injury induced by 45 minutes of ischemia results in profound tubular damage with loss of brush border in proximal tubules, diffused cell detachment from the basement membrane, extensive cast formation in tubular lumens, and intense interstitial inflammation. Repair of tubular epithelia following severe injury is largely mediated by dedifferentiation, proliferation, migration and re-differentiation of surviving tubular cells. Most published animal studies of IRI have investigated renal repair for short periods of time following the acute ischemic phase. Long-term follow-up studies have shown that, following severe IRI, the kidneys develop tubular atrophy (7) resulting in impaired nephron architecture and function. Tubular atrophy and fibrosis are the hallmarks of CKD. Sequential examinations for weeks in a model of severe IRI has allowed us to investigate the pathogenesis of AKI to CKD transition at
cellular and molecular levels. We aim to understand the cellular defense mechanism that promotes survival and repair. Given that ischemia is a strong stress signal, autophagy could be a mechanism since it is an evolutionally conserved stress response. Autophagy recycles intracellular constituents and removes damaged organelles to maintain cellular homeostasis. In kidneys with severe injury and incomplete repair, chronic hypoxia develops, likely as a result of peritubular capillary rarefaction (8, 9). Hypoxia and metabolic perturbation are one of the major inducers of autophagy (10). We reasoned that autophagy in response to hypoxia could be at the root of renal injury and repair. With chronic hypoxia, autophagy as a compensatory mechanism may not be sufficient to allow renal structure and function to return to the pristine stage.

Here, we investigate the relationship between hypoxia and autophagy and find that hypoxia activates a stress-responsive transcription factor FoxO3, a major regulator of epithelial autophagy in the kidney, as we have recently demonstrated (11). We provide evidence to show that, like Hif-1α transcription factors, FoxO3 can be hydroxylated at proline residues in the presence of molecular oxygen. During renal hypoxia, prolyl hydroxylation and FoxO3 degradation are inhibited, thereby increasing FoxO3 protein abundance and leading to its activation and stimulation of stress responses including epithelial autophagy. Furthermore, tubular deletion of FoxO3 during the AKI to CKD transition reduces autophagy and aggravates oxidative damage leading to profound pathological changes resembling the CKD phenotype. Our results demonstrate a previously unrecognized function of the pleotropic transcription factor FoxO3 in regulating renal stress responses and ameliorating the severity of CKD.
RESULTS

*FoxO3 transcription factor is activated in hypoxic renal tubules*

To understand the pathogenesis of the AKI to CKD transition, we examined acute and long term responses of mouse kidneys following severe injury induced by 45 min of IRI. While acute injury triggers cell proliferation and tubular repair (Supplemental Figure 1), renal repair is often incomplete. Specifically, there was evidence of partial recovery at 1 w. At 2 w, focal atrophic tubules began to appear as kidneys transitioned to CKD. At 4 w, CKD developed with areas of tubular atrophy, interstitial fibrosis and chronic inflammation (Figure 1A). The density of renal microvessels identified by the endothelial marker endomucin was found to be markedly reduced. This reduction in microvessels was associated with tubular hypoxia shown by increased intensity of the hypoxia-dependent pimonidazole protein adducts (Figure 1B). Since hypoxia is a potent inducer for autophagy, we examined the conversion of LC3I to LC3II, a commonly used biochemical assay for autophagy, during the period of CKD development. We found that the ratio of LC3II/I was significantly increased (Figure 1C). We demonstrated that there was an increase in autophagy using the autophagy reporter *CAG-RFP-EGFP-LC3 (CREL)* mice (12) by showing increased fluorescent dots in tubules surrounding areas of low capillary density (Figure 1D).

Next, we investigated hypoxia-induced autophagy through the activation of a stress-responsive transcription factor FoxO3 that has been shown to regulate renal epithelial autophagy in kidneys with obstructive injury (11). We found that FoxO3 was activated in renal tubules during the AKI to CKD transition (Figure 1E). At 4 w post-IRI, 29.1 ± 2.9% of tubular cells expressed nuclear FoxO3 from a baseline of 5.9 ± 1.2% in uninjured kidneys, suggesting activation of the FoxO3 transcription factor during chronic hypoxia.

*Hypoxia increases FoxO3 protein abundance by inhibiting its prolyl hydroxylation*
To exert its effect, FoxO3 needs to be located in the nucleus where it acts as a transcription factor. To understand the role of hypoxia in FoxO3 activation, we used primary cultures of renal tubular cells grown in a medium known to promote proximal tubular cell growth (13). Cells were infected with adeno-FoxO3-GFP or control adeno-GFP viruses. Using a viral titer that produced low infection efficiency, an average of 17.7% cells expressed cytoplasmic GFP under normal culture conditions at 24h post-infection. Exposing cells to 1% O\(_2\) resulted in nuclear accumulation of FoxO3-GFP that peaked at 30 min, at which time point 65.6 ± 4% of infected cells showed strong nuclear signal (Figure 2A-B). Immunostaining of endogenous FoxO3 (without FoxO3 overexpression) indicated abundant nuclear FoxO3 in cells exposure to 1% O\(_2\) (Figure 2C). Endogenous FoxO3 protein levels showed significant increases in response to hypoxia for 30 min to 2h (Figure 2D), while FoxO3 mRNA levels did not change at 60 min (Supplemental Figure 2). These results suggest that hypoxia may regulate FoxO3 at the post-translational level. One of the best studied post-translational regulations of FoxO3 is phosphorylation by Akt at S253, which is the crucial step causing p-FoxO3 nuclear export to the cytoplasm where it is degraded (14-17). We found that the accumulation of nuclear FoxO3 protein was accompanied by increased p-FoxO3 at S253. However, we did not detect significant effect of hypoxia on Akt and p-Akt levels (Figure 2E), suggesting that nuclear FoxO3 accumulation under hypoxic conditions is unlikely due to reduced FoxO3 nuclear export, and the increased p-FoxO3 may not be due to the change in Akt activity. Furthermore, while proximal tubules of the kidney had undetectable level of p-FoxO3 under baseline or at 4 w post-IRI, nuclear FoxO3 increased from 6.0±3.15% at baseline to 28.28±15.6% at 4 w post-IRI (Figure 2F), supporting the conclusion that other mechanisms rather than Akt signaling is playing a role in the post-translational regulation of FoxO3 protein abundance.

While the hypoxia-induced increase in FoxO3 protein levels could be Hif-dependent, recent studies suggest that FoxO3 can be directly affected by hypoxia. FoxO3 was found to be
hydroxylated at Pro426 and Pro437 by PHD1 that depends on oxygen, \(\alpha\)-ketoglutarate, iron and ascorbic acid for its activity, similar to the regulation of \(\alpha\) subunit of Hif proteins. Hydroxylation of FoxO3 prevents its binding to the USP9x deubiquitinase and promotes its proteasomal degradation (18). To examine whether hypoxia directly affects FoxO3 in kidney epithelia, we immunoprecipitated proteins isolated from renal epithelial cell cultures using the FoxO3 antibody followed by blotting with a pan-hydroxyl proline antibody. We found that there was reduced prolyl hydroxylated FoxO3 in cells grown in hypoxic conditions (1% O2). Remarkably, treating cells grown in normal growth factor- and nutrient-rich conditions with a hypoxia mimetic dimethyloxalylglycine (DMOG) that inhibited PHD enzymes also caused a 60% reduction in hydroxylated FoxO3 and a 39% increase in FoxO3 protein levels (Figure 2G). Similarly, depriving cells of amino acids and glucose increased the abundance of FoxO3 protein. Conversely, supplementing starved cells with a cell permeable analogue of \(\alpha\)-ketoglutarate, dimethyl \(\alpha\)-ketoglutarate (DMKG), resulted in a 57% decrease in FoxO3 protein (Figure 2H). PHD1, 2 and 3 enzymes, which are known to be expressed in renal tubular cells (19) showed no change in protein levels in cells exposed to hypoxia (Supplemental Figure 3). Taken together, our results indicate that FoxO3 protein can be regulated by the O2- and \(\alpha\)-ketoglutarate-dependent PHD enzymes in kidney epithelial cells. Hypoxia or a deficiency in \(\alpha\)-ketoglutarate, a metabolic intermediate generated by the TCA cycle, prevents FoxO3 hydroxylation and reduces its degradation.

Next, we used a newly generated antibody to detect OH-FoxO3 at Pro437 (Millipore, ABE1848). First, we confirmed that the antibody specifically recognized hydroxylated FoxO3 peptide (420-444) at Pro437 but not at a nearby Pro426 or non-hydroxylated FoxO3 peptide (supplemental Figure 4). Using this antibody, we detected the presence of OH-FoxO3 under normal culture conditions with 21% O2. Treating these cells with MG132 that inhibited ubiquitin proteasomal degradation resulted in an increase in OH-FoxO3, while treating cells with a
combination of MG132 and PHD enzyme inhibitor DMOG or exposing cells to 1% O2 resulted in a decrease in OH-FoxO3 (Figure 2I).

To test the involvement of Phd isoforms in FoxO3 hydroxylation, we used siRNA technology to knocked down Phd1 (>80%), Phd2 (>80%) or Phd3 (>60%) in renal epithelial cells cultured under 21% O2, and measured OH-FoxO3 protein levels 72 h after transfecting with siRNA reagents. The results showed a significant reduction of OH-FoxO3 protein when Phd1, Phd2 or Phd3 was knocked down (Figure 2J). Our results confirm that FoxO3 is a substrate for Phd1 as reported in MEFs and cancer cell lines (18), and provide new information that in primary cultures of renal epithelial cells, FoxO3 can also be prolyl hydroxylated by Phd2 and Phd3 isoforms. Like primary cultures exposure to 21% O2 or 1% O2 (Supplemental Figure 3), the isoform protein levels showed no significant differences between uninjured and injured kidneys 4 w post-IRI (Supplemental Figure 5), suggesting that the availability of oxygen and factors affecting enzymatic activities plays a more important role in FoxO3 hydroxylation.

**Hif-1α contributes to FoxO3 activation and CKD protection**

Since studies have also shown that FoxO3 can be activated by Hif-dependent pathways in a variety of cell types (20-23), we examined the role of Hif-1α in FoxO3 activation in the hypoxic kidneys. We found that Hif-1α protein levels increased during the AKI to CKD transition period (Figure 3A). These results are in a good agreement with the fact that renal hypoxia stabilizes Hif-1α and Hif-2α proteins and these proteins act as central players in hypoxia adaptation through transcriptional activation of genes that promote cell survival and oxygen delivery (24-27). In the kidney, Hif-1α is predominantly expressed by tubular epithelial cells, although low level of expression has been detected in the Foxd1-derived stromal cell lineages in the interstitial compartment (28-30). To test whether hypoxia-induced FoxO3 activation is dependent on Hif-1α, we took the Pax8-rtTA (31) and Tet-O-Cre (32) approach to delete Hif-1α by treating mice
harboring the Hif-1α floxed alleles (Jackson Laboratory) with doxycycline or vehicle for 2 w. Cells with tubular deletion of Hif-1α and controls were designated Hif-1α<sup>hub</sup> and Hif-1α<sup>ctl</sup>, respectively. Deletion of Hif-1α was demonstrated by the absence of its gene at the genomic level (Supplemental Figure 6A). Primary cultures were obtained in mouse kidneys with no injury. As expected, both Hif-1α<sup>hub</sup> and Hif-1α<sup>ctl</sup> cells had undetectable level of Hif-1α under culture conditions with 21% O<sub>2</sub>. Exposing cells to 1% O<sub>2</sub> for 1h resulted in robust accumulation of Hif-1α protein in Hif-1α<sup>ctl</sup> cells. The protein also reached detectable level in Hif-1α<sup>hub</sup> cells, which could be due to the presence of Foxd-1 derived fibroblasts in these cultures. In contrast, FoxO3 protein was present under normoxic conditions and its levels increased further under hypoxia in cells with and without Hif-1α deletion, though relatively lower levels were seen in cells with Hif-1α deletion (Figure 3B). Our results suggest that hypoxia can regulate FoxO3 protein levels in the absence of Hif-1α protein and that Hif-1α contributes to its regulation as well. Because Hif-1α is not known to interact with FoxO3 at the protein level, we measured FoxO3 mRNA and detected increases of FoxO3 mRNA in Hif-1α<sup>ctl</sup> cells exposed to 1% O<sub>2</sub> for 10 and 30 min but not with further exposure for 60 min. Deleting Hif-1α attenuated hypoxia-induced increase in FoxO3 mRNA (Figure 3C). Similarly, a control experiment with a known Hif-1 regulated gene phosphoglycerate kinase 1 (Pgk-1) showed gradual increases of Pgk-1 mRNA in Hif-1α<sup>ctl</sup> cells but not in Hif-1α<sup>hub</sup> cells after exposure to 1% O<sub>2</sub> (Supplemental Figure 6B). These results suggest Hif-1α stimulation of FoxO3 at the transcriptional level.

Hif-1α was found to protect kidneys from acute ischemic and hypoxic injury when analyses were performed during the early repair phase (29, 33, 34). Here, we aimed to study the role of Hif-1α during AKI to CKD transition. In order to avoid mortality and be able to measure renal functional changes from the surviving kidney, mice were subjected to a moderate to severe injury with 35 min of IRI to the left kidney along with right nephrectomy. We deleted Hif-1α in renal
tubules at days 8-21 (2 weeks total) following IRI to preserve Hif-1α effects during the first week after IRI. Similar to cell culture studies, deleting Hif-1α led to lower nuclear FoxO3 expression in proximal tubules 4 w post-IRI (Figure 3D), further supporting the contribution of Hif-1α to FoxO3 regulation. Analyses at 4 w post-IRI indicated higher tubular injury scores (54.3 ± 6.8 in Hif-1α\textsuperscript{tub} vs. 10.5 ± 2.4 in Hif-1α\textsuperscript{ctl}) along with higher urinary albumin and N-gal excretions in Hif-1α\textsuperscript{tub} mice (Figure 3E-G). Interstitial inflammation and fibrosis were also more severe, although no significant differences in microvascular density were detected between Hif-1α\textsuperscript{tub} and Hif-1α\textsuperscript{ctl} mice (Figure 3H). Furthermore, immunoblot analysis indicated lower levels of FoxO3 protein in Hif-1α\textsuperscript{tub} kidneys compared to that of Hif-1α\textsuperscript{ctl} kidneys (relative protein density 12.5 ± 4.2 in Hif-1α\textsuperscript{tub} vs. 35.6±2.1 in Hif-1α\textsuperscript{ctl}). Interestingly, deleting Hif-1α led to no significant changes in the ratio of LC3II/I (Figure 3I). Taken together, our results suggest that HIF1α plays a role in the late phase of tubular repair after IRI and that its renal protection may be due to mechanisms other than autophagy.

**Tubular FoxO3 deletion during AKI to CKD transition accelerates CKD development**

To study the biological function of FoxO3 activation in the transition from AKI to CKD, we bred mice harboring floxed FoxO3 alleles (FoxO3\textsuperscript{f/f}) (35) to mice carrying Pax8-rtTA and Tet-O-Cre transgenes. We deleted FoxO3 by giving mice doxycycline or control by giving vehicle in the drinking water for 2 weeks (days 8-21) starting 1w post-IRI, after mice recovered from the initial injury. We designated mice with tubular deletion of FoxO3 as FoxO3\textsuperscript{tub}, and the mice without FoxO3 deletion as FoxO3\textsuperscript{ctl}. FoxO3 protein from FoxO3\textsuperscript{tub} kidney lysates was significantly reduced to 16% of that of controls (Figure 4A). The incomplete deletion might be due to lower blood flow to the post-ischemic kidneys, which affected delivery of doxycycline to induce cre recombination. Tubular deletion of FoxO3 severely aggravated tubular damage 4 w post IRI as indicated by significantly higher injury scores with a loss of proximal tubular brush borders, tubular cast...
formation and atrophy (229 ± 5.3 vs. 22 ± 3.6 in FoxO3<sup>ctl</sup>, n= 5) (Figures 4B). Tubular dilation was also more pronounced (Supplemental Figure 7). Interestingly, chronic hypoxic injury did not result in significant proliferation in the tubules located in the cortex and outer medulla in FoxO3<sup>ctl</sup> mice, as only 2.63 ± 0.54 cells per field under 200x magnification expressed Ki67 compared to 2.23 ± 0.09 cells per field in normal kidneys. FoxO3 deletion doubled the number of proliferating tubular cells (4.53 ± 0.6 cells per field) and led to more expression of proximal tubular injury marker Kim1 (Figure 4B). The excretion urinary N-gal was 4-fold higher when FoxO3 was deleted. Additionally, urinary albumin excretion was increased in FoxO3<sup>tub</sup> mice (53.2 ± 3.1 mg/g vs. 18.4 ± 4.1 mg/g in FoxO3<sup>ctl</sup>). Serum creatinine, a frequently used but insensitive measurement of the kidney filtration function, was also significant higher (Figure 4C-E). Taken together, FoxO3 deletion resulted in more severe tubular injury that triggered proliferation. However, there was more progressive injury and incomplete tubular repair manifested with worse renal morphology and function when tubular FoxO3 was deleted.

Tubular injury is known to cause interstitial inflammation and fibrosis (5). Tubular injury as a result of FoxO3 deletion was accompanied by more CD45<sup>+</sup> leukocyte infiltration and more fibrotic changes demonstrated by trichrome stain and deposition of collagen I and vimentin in the interstitial compartment. The difference in microvascular density did not reach statistical significance (Figure 4F-G). Additional method of evaluating fibrosis with picrosirius red stain also indicated more fibrosis under the polarized light (Supplemental Figure 8). Overall, the degree of renal structural injury and functional decline was much greater in mice with FoxO3 deletion compared to that of Hif-1<sub>α</sub> deletion (tubular injury score 229 ± 5.3 with FoxO3 deletion vs. 54.3 ± 6.8 with Hif-1<sub>α</sub> deletion), despite the similarity of the endothelial rarefaction in the injured kidneys. Our results suggest that FoxO3 may play a more important role in preventing AKI to CKD transition, hence a better potential pathway to target to ameliorate CKD development.
Unlike most primary glomerular disease, glomeruli at 4 w post-IRI showed no apparent increase in cellularity in kidneys with and without tubular FoxO3 deletion. Silver stain showed mild thickening of basement membrane in Bowman’s capsule but not the basement membrane of capillary loops in glomeruli trapped in the fibrotic region. Podocyte foot process effacement was rarely detected, although more mesangial deposits were observed in FoxO3\textsubscript{tub} kidneys (Figure 5A). Immunostaining of p57, a marker that identified podocytes (36, 37), showed no significant changes in the number of podocytes per glomerulus at 4 w post-IRI (9.1± 0.8 in normal, 7.5 ± 1.9 in FoxO3\textsubscript{tub} and 7.2 ± 0.7 in FoxO3\textsubscript{ctl}). In concordance with the electron microscopic findings, 52% of glomeruli of FoxO3\textsubscript{tub} kidneys showed increased vimentin deposition with focal areas of loss of capillary loops, whereas FoxO3\textsubscript{ctl} kidneys had milder glomerular changes with 15% of glomeruli affected (Figure 5B). Since Pax8-rtTA system is not active in podocytes (31), our results suggest a secondary glomerular response from the loss of FoxO3 protection in tubules, which could also contribute to increased urinary albumin excretion (shown in Figure 4). Similarly, analysis of glomeruli in Hif-1\alpha\textsubscript{tub} and Hif-1\alpha\textsubscript{ctl} kidneys with PAS staining, and immunostaining of p57 and vimentin also showed no differences 4 w post-IRI (Supplemental Figure 9).

**Loss of FoxO3 results in reduced autophagic adaptation**

To examine whether the renal protection in mice with intact FoxO3 was also associated with autophagy, we performed electron microscopic examination, which remained as the gold standard for detection of autophagic vesicles. Autophagosomes and autolysosomes were more frequently observed in tubules 4 w post-IRI (Figure 6A). In comparison, no autophagic vesicles were easily detectable in uninjured tubules (not shown). Next, we deleted FoxO3 from tubules during the AKI to CKD transition (days 8-21). As predicted, autophagic levels estimated by the ratio of LC3II/I were reduced in the FoxO3\textsubscript{tub} kidneys. Bnip3 protein, a known target of FoxO3 and a key component in the autophagic machinery was decreased as well, although ULK protein that was essential to the initial event of autophagic activation showed no significant changes (Figure
To complement the in vivo findings, we exposed primary cultures isolated from uninjured kidney to 1% O\textsubscript{2} for 30 min. While FoxO3\textsuperscript{ctl} cells showed a significant increase in LC3\textsubscript{II}/I ratio from that of cells exposure to 21% O\textsubscript{2}, FoxO3\textsuperscript{tub} cells had insignificant increase in LC3\textsubscript{II}/I ratio (Supplemental Figure 10). When the autophagy reporter CREL mice (12) were bred into the FoxO3\textsuperscript{tub} and FoxO3\textsuperscript{ctl} lines and subjected to IRI, fewer RFP dots were present in renal tubules when FoxO3 was deleted. These tubules with decreased autophagic dots appeared atrophic. In contrast, tubules of FoxO3\textsuperscript{ctl} mice contained more abundant RFP dots and renal tubules appeared less atrophic (Figure 6C), suggesting that FoxO3 stimulates autophagy to maintain tubular homeostasis. Quantification of RFP dots in proximal tubules indicated that the majority of autphagic cells in FoxO3\textsuperscript{tub} kidneys contained ≤ 10 dots per cell. In comparison, more than 70% of autphagic cells in FoxO3\textsuperscript{ctl} kidneys contained ≥ 10 dots per cell (Figure 6D). These results are in a good agreement with our previous finding that FoxO3 activation increases autophagic capacity in tubules injured by urinary tract obstruction (11).

**Loss of FoxO3 results in more oxidative injury**

FoxO3 is a stress responsive gene and its targets also include molecules responsible for the control of oxidative stress (38). Renal hypoxia has been shown to increase production of reactive oxygen species (ROS) resulting in oxidative injury (39-41). We measured superoxide dismutase 2 (SOD2), a mitochondrial scavenging enzyme that is inducible by FoxO3 and plays a critical role in ROS clearance (refs). We detected a significantly lower level of SOD2 protein in FoxO3\textsuperscript{tub} kidneys 4 w post-IRI (relative protein density 0.65 ± 0.04 vs. 1.13 ± 0.02 level in FoxO3\textsuperscript{ctl} mice) (Figure 7A). Mitochondrial superoxide levels in live cells of primary cultures isolated from FoxO3\textsuperscript{tub} kidneys showed higher intensity of the fluorogenic dye MitoSOX Red compared to that of FoxO3 undeleted cells after exposing to 1% O\textsubscript{2} for 1h. Similarly, incubating cells with dihydroethidium (DHE), a substance that could be oxidized by intracellular ROS and emit red fluorescent signals in the nuclei showed that nearly 100% of cells isolated from of FoxO3\textsuperscript{tub}
kidneys emitted red nuclear signals compared to 49.6% of that in cells isolated from FoxO3<sup>ctl</sup> kidneys. DHE staining of kidneys 4w post-IRI also indicated stronger nuclear oxidative stress in FoxO3<sup>tub</sup> kidneys (Figure 7B). Furthermore, EM studies showed dramatic mitochondrial swelling with a loss of cristae. In FoxO3<sup>tub</sup> cells, frequent ruptures of mitochondrial inner and outer membrane were detected (Figure 7C), which could lead to leakage of ROS to the cytoplasm and cause oxidative injury. Taken together, our results suggest an anti-oxidant effect of FoxO3 in preventing tubular damage. Future in-depth studies designed to effectively reduce or remove ROS in renal tubules will delineate the functional significance of FoxO3-ROS pathway in CKD development.

**FOXO3 is activated in injured tubules of human kidneys**

To investigate a possible involvement of hypoxia-induced FOXO3 activation in human AKI (2 patients) or AKI on CKD (10 patients), we examined renal microvasculature and nuclear FOXO3 expression in all 12 kidney samples biopsied for renal ischemia or hypoperfusion due to renal atheroembolization, volume depletion, congestive heart failure, respiratory failure, or hepatorenal syndrome. Five controls that had no specific pathological alterations were obtained from biopsies for microscopic hematuria or sub-nephrotic range proteinuria (Table 1). Representative images showed diminished expression of endothelial marker CD31 in injured kidneys where acute tubular damage, chronic tubular changes with atrophy and cast formation were apparent. Nuclear FOXO3 was mainly localized to the distal nephron in controls, but had increased expression in the injured proximal tubules that exhibited flat epithelia and denuded basement membrane. Furthermore, EM examination showed mitochondrial swelling with a loss of cristae as well as the presence of autophagosomes and autolysosomes in injured kidneys (Figure 8). These results suggest the correlation of the loss of renal microvasculature with FOXO3 activation in human AKI or AKI on CKD, and further support the significance of FOXO3 activation in tubular stress response.
In summary, our study indicates that chronic hypoxia, which is an important pathogenic factor for CKD development, can activate FoxO3 via inhibition of its prolyl hydroxylation and degradation in a similar fashion to Hif regulation by PHD enzymes. Hif-1α contributes to hypoxia-mediated activation of FoxO3, which plays an important role to counteract the detrimental effects of hypoxia by increasing autophagy and decreasing oxidative stress (Figure 9). Further research and discoveries to stimulate FoxO3-mediated responses that ameliorate hypoxia-induced stress and injury may provide basis for therapies to prevent or slow down the development of CKD.

Discussion

We demonstrate that the transcription factor FoxO3 regulates adaptive responses that counteract adverse effects of chronic hypoxia in the kidney through increased autophagy and reduced oxidative stress. Unlike extensive cell death and cell proliferation after abrupt cessation of blood flow and oxygen supply, chronic hypoxia does not lead to a dramatic increase in tubular proliferation as a repair mechanism 4 w post-IRI. On the other hand, autophagy, which is an evolutionally conserved mechanism for cell survival, can be activated during acute renal IRI (12), or with chronic hypoxia and metabolic perturbations (11). Signals for autophagy induction can be transduced to the nutrient and energy sensors that include AMPK, mTORC1, and NADH-dependent deacetylases of the sirtuin family, as well as other molecular pathways that involve stress response (42, 43). FoxO3 is one of the four mammalian forkhead box O (FoxO) transcription factors that include FoxO1, FoxO3, FoxO4 and FoxO6 (44-47). FoxO3 has been studied most extensively, and has been shown to modulate stress response, metabolism, autophagy, apoptosis, and cell differentiation (48, 49). FoxO3 activity is largely regulated by post-translational mechanisms that include phosphorylation, acetylation, and ubiquitination (49-51). In the presence of survival factors, such as growth factors and nutrients, FoxO3 is phosphorylated, predominantly by Akt, and translocated to the cytoplasm where it is degraded by the ubiquitin proteasomal system. In contrast, under stress conditions, FoxO3 accumulates in the nucleus and
functions as either a transcription activator or repressor, in a context-dependent fashion, to regulate adaptive responses (48, 49).

Recent identification of FoxO3 as a novel substrate for PHD1 enzyme (18) has led to our current understanding of the mechanism of hypoxia-induced FoxO3 activation in the kidney. Of the three PHD enzymes that function as oxygen sensors, PHD2 is the primary prolyl hydroxylase for the \( \alpha \) subunit of the Hif proteins (52, 53), while PHD1 and PHD3 may play additional role beyond regulating Hif proteins (23, 54). Using decarboxylation assays to screen 1,000 proteins that could potentially be hydroxylated by PHD1 in vitro, Zheng et al. demonstrated that FoxO3 was hydroxylated at Pro426 and Pro437, which disrupted the binding with USP9x deubiquitinase, thereby promoting FoxO3 degradation via ubiquitin proteasomal system (UPS). The prolyl hydroxylation was confirmed in a breast cancer cell line and in 293 embryonic kidney cells (18). The recent development of an antibody raised specifically to hydroxyl Pro437 in FoxO3 has allowed us to detect endogenous prolyl hydroxylated FoxO3 in renal tubular epithelial cells. Furthermore, UPS inhibition with MG132 increases but hypoxia reduces OH-FoxO3 (P437) protein levels, confirming that prolyl hydroxylation and proteasomal degradation is one of the mechanisms regulating FoxO3 protein abundance. Interestingly, we also show that levels of p-FoxO3 at S253 increased when primary cultures of renal epithelia cells were exposed to hypoxic condition. The same culture condition did not increase Akt and pAkt proteins significantly, suggesting that higher levels of p-FoxO3 are more likely a result of decreased degradation rather than an increase in formation. At the present time, it is unclear whether p-FoxO3 can also be prolyl hydroxylated or FoxO3 prolyl hydroxylation facilitates the degradation. Future biochemical studies will address the relationship among post-translational modifications.

It is interesting to note that the hydroxylase activity shared by Phd isoforms is not unique to FoxO3 because all three isoforms have also been shown to hydroxylate synthetic Hif-1\( \alpha \) peptides in vitro (55-57) and Hif-1 \( \alpha \) proteins in cells expressing high levels of isoforms (58).
Indeed, in vitro and animal studies have suggested that Phd2 is the main hydroxylase for Hif (52, 53, 58). It is possible that the level and pattern of the enzyme expression as well as conditions affecting the enzymatic activity could influence substrate preference and product formation in a cell-type and context dependent manner. At this time, the selectivity and relative contribution of the isozyme to Hif and FoxO3 regulation during CKD development is not known. Future studies selectively inhibiting or deleting the individual isoform in renal tubules will help address this important question.

We have previously shown that persistent obstructive injury to the kidney leads to FoxO3 activation, which increases the formation of autophagosomes. FoxO3 activation increases the expression of core Atg proteins including Ulk1, Beclin-1, Atg9A, Atg4B and Bnip3 in kidneys to sustain autophagic response in the obstructed kidneys (11). This study shows that following IRI, FoxO3 is progressively activated in the hypoxic tubular cells to stimulate autophagy. Using an antibody to LC3B, we demonstrated increased conversion of LC3I to LC3II, which is a commonly used method to evaluate autophagy. It is important to note that LC3 proteins, such as microtubule-associated protein 1 light chain 3 alpha (LC3A) plays a crucial role in LC3-associated phagocytosis (LAP) (59-61). The CREL mice have not been entirely investigated for its value in assessing LAP, which may occur in the kidneys with hypoxia and inflammation.

FoxO3 activation reduces oxidative stress and injury by increasing mitochondrial SOD2 expression. In HeLa cells, FoxO3 has been shown to be activated by hypoxia to represses a set of nuclear-encoded mitochondrial genes to reduce oxygen consumption (21). It is conceivable that metabolic adaptation combined with reduction in ROS formation promote cell survival under hypoxic conditions. However, prolonged and escalating stress input may exceed compensatory mechanism by FoxO3 activation leading to eventual CKD. This is supported by that fact that ROS-induced stress signals were reduced but not absent in FoxO3 competent renal tubular cells, as
oxidative injury and cell death that has recently been referred to as ferroptosis (62-64) could also be induced by ROS generated from organelles other than the mitochondria (65).

In this study, we found that tubular deletion of FoxO3 leads to much more profound renal injury than tubular deletion of Hif-1α (tubular injury score 229 ± 5.3 vs. 54.3 ± 6.8). In addition, FoxO3 is expressed in hypoxic kidneys, as well as in normal kidneys when Hif-1α protein is not easily detectable. These results suggest a pleiotropic effect of FoxO3 in renal hemostasis and stress adaptation. Further investigations of FoxO3 effects on other CKD pathogenic factors such as cell death, immune response, interstitial inflammation and fibrosis will provide biological basis for targeting FoxO3 as a potential therapeutic approach for CKD. Our current study cannot rule out possible contributions to renal protection by other FoxO members that may have overlapping functions. For example, FoxO1, highly expressed in insulin responding tissues, can orchestrate the transcriptional networks thus regulating glucose and lipid metabolism (66). However, FoxO1 is not consistently elevated in response to hypoxia (18, 21, 67). Future studies manipulating FoxO1 alone or in combination with FoxO3 may shed light on a possible role of FoxO1 in CKD.

Molecular oxygen is essential to mitochondrial energy metabolism and numerous chemical reactions and activities. The VHL/PHD/HIF is the best characterized O2-sensing system to date. This pathway regulates a large molecular network required for adaptation to low oxygen by increasing the expression of hundreds of genes involved in diverse cellular functions. Hif-1 protein is critical for rapid as well as long-term adaptations to hypoxia (68). Like MEFs, endothelial cells and some other cell lines (21, 22), FoxO3 activation in renal tubular cells is partially dependent on Hif-1α, as deletion of Hif-1α blunted FoxO3 accumulation. Hif-1α can interact with a large number of proteins to regulate protein stability, but it is not known to interact with FoxO3 at the protein-protein level (69). In the promoter regions of human and mouse FoxO3 gene, there are nine conserved hypoxia-response elements (HREs). This suggests that Hif-1 may activate FoxO3 promoter to increase its transcription, as shown in our studies and in the cases of MEFs.
and NIH3T3 cells. However, FoxO3 protein is present under normoxic conditions when Hif-1α protein is barely detectable. Furthermore, Hif-1α deletion prevented hypoxia-induced increase in FoxO3 mRNA significantly but did not prevent the increase in FoxO3 protein abundance completely, confirming that Hif-1α could function as a transcriptional regulator for FoxO3, and that hypoxia stabilizes existing FoxO3 protein at the post-translational level.

Hif-1α protein levels increase in the hypoxic kidneys 1-4 weeks post-IRI. Using the inducible cre system, we selectively deleted Hif-1α in all tubular cells between 8-21 days starting 1 week after the ischemic insult. This model retains the known beneficial effect of Hif-1α during the acute phase and allows the examination of Hif-1α effects during the AKI to CKD transition. We show that Hif-1α deleted kidneys had more tubular injury and interstitial fibrosis. Tubular and interstitial interdependence has been well-demonstrated by elegant studies, in which direct ablation and injury to tubular cells leads to interstitial fibrosis. At this time, the role of Hif-1α in CKD-associated fibrosis is not fully understood. Studies using different models of CKD and various pharmacological or genetic approaches to modulate Hif proteins have generated conflicting results. In the kidney, Hif-1α is predominantly expressed by the tubules, but a low level of expression is also detected in the Foxd1-derived cells in the interstitium. In contrast, Hif-2α is expressed in the interstitial cells to regulate erythropoietin production. While treating mice with a PHD enzyme inhibitor before ischemic injury ameliorated renal fibrosis and anemia, pharmacological inhibition of PHDs in the early phase following injury had no effect. PHD inhibitor treatment reduces degradation of Hif-1α, Hif-2α and likely other PHD substrates in all kidney cells and extra-renal organs, thus preventing accurate assessment of Hif1 function in the kidney. Another study shows that selective ablation of Hif-1α in proximal tubules before obstructive injury reduces epithelial to mesenchymal transition (EMT) in mice with UUO. The profibrotic effect of Hif1 was attributed to increased migration and fibrogenic gene expression in tubular epithelia. Since the contribution of EMT to interstitial fibrosis may not
be as significant as previously thought (73-75), progressively atrophic tubules in the model of UUO could express profibrotic genes that signal interstitial cells to undergo fibrotic changes rather than provide a direct cellular source for myofibroblasts. In comparison, a 35 min of IRI produces a mild CKD phenotype with focal and mild tubular atrophy when epithelial stress responses are intact. However, tubular deletion of FoxO3 or Hif-1α removes survival and adaptive mechanisms leading to more severe tubular injury, which could crosstalk with interstitium and aggravate interstitial pathology. Therefore, our study supports the interdependency of tubules, renal microvessels and interstitia in maintaining kidney health and confirms the importance of taking an integrated approach to prevent CKD.

Methods

Animal studies. Methods for renal ischemia-reperfusion injury (IRI) in mice have been described previously (12). Briefly, 6-8 weeks old females of various strains were anesthetized with isoflurane and the left renal pedicle was clamped for 35 or 45 minutes before clamp release to allow reperfusion to the kidney. The right kidney was left intact or removed at the same time. To delete FoxO3 or Hif-1α, Pax8-rtTA;tetO-cre;FoxO3f/f or Pax8-rtTA;tetO-cre;Hif1αf/f mice were given 2 mg/ml of doxycycline in drinking water between days 8-21 (2 weeks) staring 1 week post-IRI. Vehicle treated mice were used as controls.

Tubular injury score and quantification of renal fibrosis and tubular dilation. Random images (10 photos at OSOM for each kidney) of PAS stained sections were obtained at 20x magnification using Zeiss Observer Z1 microscope and the Image J software was used to obtain tubular injury scores. Briefly, a graticule grid was randomly placed on each picture to generate 100 grids. Histology of tubular profiles of each grid was carefully examined and a score 0 or 1 was given per grid: 0 = normal histology; 1 = tubular cell swelling, brush border loss, nuclear condensation, or tubular atrophy. Injury score was calculated by adding all 100 grids per picture.
and all 10 pictures per kidney. Using this grid point count method, renal fibrosis and tubular dilatation was quantified on kidney sections stained with Masson’s trichrome.

**Quantification of autophagic capacity.** Images (630× magnification) of proximal tubules identified by LTA staining were randomly selected. Cells that contained ≥ 3 RFP dots were counted as autophagic cells and at least 100 cells were counted in each kidney. Autophagic cells were further divided into subgroups that contained 3-5, 6-10, 11-15, 16-20, 21-30, 31-40, 41-50, or >50 RFP dots per cell. Quantification was performed in duplicates.

**Renal epithelial cell culture and analysis.** Kidneys were collected from 3-5 weeks old mice and tubular cells were isolated for primary culture using the established methods (11). For adenoviral infection, cells grown in chamber slides at 70% confluence were infected with Ad-GFP (Vector Biolabs, 1060) or Ad-GFP-FOXO3a (Vector Biolabs, 1026) MOI of 5 for 48 h, then exposed to 1% O₂ in the hypoxic chamber (Biospherix Ltd., C21) for 15 min to 2h. Media for the hypoxia study were equilibrated in the 1% O₂ atmosphere overnight before use. Infected cells grown in 21% O₂ were used as controls. To quantify nuclear FoxO3 expression, cells were fixed with cold 4% PFA (Affymetrix, 19943 1LT) for 10 min, washed in PBS and cover slipped in DAPA before imaging at 400× magnification. To starve cells, cells were incubated in Earle’s balanced salt solution (EBSS) (Sigma, E2888) for 2h to deprive them of glucose and amino acid with and without 5 mM of dimethyl ketoglutarate (DMKG) (Sigma, 34631-5G). Cells under normal culture conditions were used as controls. To delete Hif-1α, Pax8-rtTA;tetO-cre;;Hif1α°/° mice were treated with doxycycline in drinking water (2 mg/ml) for 2 w before cultures and analysis.

**Immunostaining and image analysis.** Immunostaining was performed using established methods and conducted in duplicates (12). Detailed information on staining procedures and antibodies used is provided in Supplemental Methods. Sections were visualized with a Zeiss AxioObserver Z1 fluorescence microscope, photographed with a digital camera, and analyzed with Axiovision software.
**Immunoblot and immunoprecipitation analysis.** Tissue preparation and immunoblot analyses were performed as previously described (76). Detailed procedures and antibody information is provided in Supplemental Methods. Immunoprecipitation of FoxO3 followed by immunoblot analysis with a pan-hydroxyl proline antibody was performed with total cellular proteins from primary cultures using Catch and Release V2.0 Reversible Immunoprecipitation System (Millipore, 17-500A). Briefly, 500 µg of cellular proteins extracted with RIPA buffer were mixed with 0.1 µg of FoxO3 antibody (Cell Signaling, 2497), 10 µl affinity ligand, and washing buffer to 500 µl onto each column, incubated at room temperature for 1h, and diluted with 70µl elution buffer denatured with 5% 2-Mercaptoethanol (Sigma, M-6250). The elution was boiled at 95°C for 10 min and loaded as 10µl per lane with immunoblot to detect the hydroxylation of FoxO3.

**Detection of OH-FoxO3 (Pro437).** The specificity of the antibody to OH-FoxO3 at Pro 437 (Millipore, ABE1848) was first tested by spotting various amount of N-terminal biotinylated FoxO3 peptides (420-444) on the nitrocellulose membrane and detected by the antibody. An anti-biotin antibody was used to confirm the detection of FoxO3 peptides. Peptide sequences are as follows: WT FOXO3 420-444: GSGLGSPTSSFNSTVFGPSSLNSLR; FOXO3 P426-OH: GSGLGSPTSSFNSTVFGPSSLNSLR; and FOXO3 P437-OH: GSGLGSPTSSFNSTVFGP(OH)SSLNSLR. Next, renal tubular cells grown in normal culture conditions were treated with a proteasome inhibitor MG132 (10 µM, Sigma) in the presence or absence of a prolyl hydroxylase (PHD) inhibitor Dimethyloxalylglycine (DMOG, 1 mM, Sigma) for 2.5h, or with no inhibitor. Another group of cells were exposed to 1% O2 for 1h. Proteins were subjected to immunoblot analysis of OH-FoxO3 at Pro437 using the above antibody.

**Superoxide detection with MitoSOX Red and DHE.** Renal epithelial cells grow in chamber slides were exposed to 1% O2 for 1h followed by incubation with MitoSOX Red fluorescent probe (Sigma, M36008) at 5 µM or DHE (Thermo Fisher, D11347) at 0.5 µM for additional 30 min in 1% O2. Cells were rinsed with PBS, cover slipped in PBS and examined. To measure kidney oxidative
stress, freshly prepared snap frozen sections (10 µm) were incubated with DHE at 1 µM in a humidified chamber at 37°C for 10 min in dark before rinsing in PBS and mounting with Vectashield (Vector, H-1200-10). Sections were examined immediately, photographed at a magnification of ×200, and analyzed.

**siRNA Transfection.** Primary cultures of renal epithelial cells grown to 60-80% confluence were transfected with scramble siRNA, or siRNA to Phd1, Phd2 or Phd3 (20 nM) using Lipofectamine RNAiMax reagents (Invitrogen, 13778-150) for 72 h before cell harvesting for Western blot analysis of Phd isoforms and OH-FoxO3 proteins. Transfection procedure and siRNA sequences are provided in the Supplements.

**Urine and blood analyses.** Urine albumin was measured with the mouse albumin ELISA kit (Crystal Chem, 80630), and urine and serum creatinine was measure with the mouse creatinine kit (Crystal Chem, 80350) by following manufactures’ instructions. Urinary N-gal was analyzed using published methods (77, 78).

**Human kidney biopsy studies.** All human kidney biopsy samples were obtained from Columbia University Medical Center during 2012-2018. Five patients biopsied for microscopic hematuria or sub-nephrotic range proteinuria but had no specific pathologic alterations were designated as control group. Twelve patients biopsied for AKI or AKI on CKD due to renal ischemia and/or hypoperfusion were designated as injury group. Paraffin sections (3 µm thickness) were used for immunohistochemistry of CD31 (1A10) (Leica, PA0250) followed by the refined DAB detection method using Leica HistoCore SPECTRA system at the Anatomical Pathology and Cell Biology Core at Columbia University Medical Center, or FOXO3 (75D8) (Cell Signaling Technology, 2497, 1:50) with a DAB detection method. Transmission electron microscopic examinations were performed using the standard method and images were acquired with JEOL JEM-1011 electron microscope equipped with a Gatan digital camera.

**Study approval.** All procedures involving mice were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by Columbia
University Institutional Animal Care and Use Committee (IACUC). Human kidney biopsy samples were obtained under an approved IRB protocol at Columbia University Medical Center and de-identified before further study.

Statistics. The number of animals or cell cultures used is indicated in each experiment. All cell cultures were performed in duplicates. Data were presented as mean ± SE and compared using two-tailed Student's t tests to determine the statistical significance between the two groups. Renal injury scores, tubular dilatation and renal fibrosis (Masson’s trichrome stain) between wild type and mutant mice were analyzed with non-parametric t test (Wilcoxon-Mann-Whitney). One-Way ANOVA followed by Dunnett's post hoc test for multiple comparisons was used to analyze study results when more than 2 groups were involved. A p value of <0.05 was considered to be statistically significant.

Author contributions

LL helped design and performed the majority of experiments, analyzed data, and assisted with manuscript preparation. HK performed the experiments, acquired and analyzed data. QZ validated antibody to OH-FoxO3 at Pro437 and provided helpful discussions. QA advised on study design and data interpretation, and edited manuscript. VDD provided expertise and insightful discussions in pathology of human kidney biopsy samples and reviewed mouse kidney histopathology. FL designed and directed the study, performed experiments, and wrote the manuscript.

Acknowledgements

This work was supported by the NIH grant R01DK107653 to FL. We thank Ms. Julia Liu and Ms. Catherine Ha for technical assistance, and Dr. Pamela Good for helpful suggestions and professional proofreading. We thank Millipore for providing the anti-OH-FoxO3 (P437) as a non-
catalogue antibody for the study and Dr. Lianxin Hu for characterization of the antibody. We thank Dr. Zhimin Yu for expert assistance in electron microscopic preparation and image acquisition.

References


Figure 1. Severe renal IRI leads to CKD, hypoxia and activation of autophagy and FoxO3. A. Mice were subjected to a 45 min of unilateral left renal IRI and kidneys examined up to 4 w post-IRI. A. Representative PAS staining indicates partial repair and CKD development after initial IRI. Arrows denote tubular atrophy and arrowheads indicate cast formation in the lumen of tubules. B. Reduction of the density of renal microvessels identified by the endothelial marker endomucin (Endo, green) and renal hypoxia indicated by the intensity of the hypoxia-dependent pimonidazole protein adducts (Pim, red). n=4 for normal controls and n=7 for 1, 2 and 4 w post-IRI *p<0.05 compared with normal. C. Increased renal autophagy with higher ratio of LC3II/I during CKD development (n=5, *p<0.05 compared with normal). Gapdh served as loading controls. D. Appearance of autophagic dots (RFP dots, arrows) in tubules surrounding low density of capillaries that labeled with endomucin (Endo, white). E. FoxO3 activation with nuclear expression (red) in renal tubules labeled with E-cadherin (green, E-cdh), n=4 for normal control and n=5 for IRI at 1, 2 and 4 w, *p<0.05 compared with normal. Nuclei were counter-stained with DAPI (blue) in D and E. Scale bar, 100 µm in A, 50 µm in B and 20 µm in D and E. One-way ANOVA followed by Dunnett’s post hoc test for multiple comparisons was performed in B, C and E.
Figure 2. Hypoxia inhibits FoxO3 prolyl hydroxylation and its degradation. A-B. Exposing primary cultures of renal epithelial cells infected with adeno-FoxO3-GFP to 1% O₂ induced nuclear accumulation of FoxO3. C. Immunostaining of endogenous FoxO3 (without overexpression) showed abundant nuclear FoxO3 (red) in cells exposure to 1% O₂ for 30 min. D-E. Endogenous FoxO3 and p-FoxO3 (S253) protein levels increased in primary cultures exposure to 1% O₂, while no significant differences in Akt and p-Akt were detected (n=6 with duplicates, *p˂0.05 and **p˂0.01 compared to 21% O₂). F. Proximal tubules of the kidney (labeled by LTA in green at the brush border) had increased FoxO3 expression in the nucleus (red), but undetectable p-FoxO3 in the cytoplasm (red) 4w post-IRI. n=4 for normal controls and n=5 for IRI 4w. G. Immunoprecipitation (IP) of proteins isolated from primary cultures with an antibody to FoxO3 followed by immunoblotting (IB) with a pan-hydroxyl proline antibody showed reduced prolyl hydroxylated FoxO3 but increased FoxO3 proteins in cells exposure to 1% O₂, or cells grown in 21% O₂ and treated with a hypoxia-mimetic, DMOG that inhibited PHD enzymes. Rabbit IgG served as a control. n=4, *p˂0.05 comparing starved with starved + DMKG. H. An antibody raised against prolyl hydroxylated FoxO3 at Pro437 was used to detect OH-FoxO3 in primary cultures. OH-FoxO3 was present under 21% O₂, increased when MG132 was used to block its degradation via ubiquitin degradation pathway, and decreased when treated with the combination of MG132 and a PHD enzyme inhibitor DMOG, or exposure to 1% O₂ (n=3 for rabbit IgG controls and n=5 with duplicates for experimental conditions, *p˂0.05 comparing 21% O₂). J. Knocking down Phd1 (>80%), Phd2 (>80%) or Phd3 (>60%) with siRNA resulted in significant reduction of OH-FoxO3 proteins in primary renal epithelial cells cultured under 21% O₂, n=4 with duplicates, *p˂0.05 compared to cells transfected with scramble siRNA (SC). Gapdh served as loading controls in D, E and H; and actin served as loading controls in J. Nuclei were counter-stained with DAPI (blue) in A, C and F. Scale bar, 20 µm in A and C, and 50 µm in F. A two-tailed Student’s t test was performed in E, G and J. One-way ANOVA followed by Dunnett’s post hoc test for multiple comparisons was performed in D, H and I.
Figure 3. Hif-1α contributes to FoxO3 activation and CKD protection. A. Hif-1α protein levels increased during CKD development. n=4. B-C. Hif-1α was deleted using the Pax8-rtTA and Tet-O-Cre system by giving mice with doxycycline for 2 w prior to primary cultures (Hif-1αtub). Vehicle-treated mice were used as controls (Hif-1αctl). B. Hypoxia-induced increase in FoxO3 protein was partially dependent on Hif-1α in primary cultures. n=5 with duplicates. ††p<0.01 and *p<0.05 comparing Hif-1αtub with Hif-1αctl at 0 and 1h, respectively. C. Hif-1α deletion abolished the increase in FoxO3 mRNA in response to hypoxia with 1% O2. n=3-8 with duplicates. ** p< 0.01 and *** p<0.001 comparing to cells prior to exposure to 1% O2 (0 min) within Hif-1αctl or Hif-1αtub group. D. Higher nuclear FoxO3 expression (red) in proximal tubules (labeled with LTA in green) of Hif-1αctl kidneys 4 w-post IRI. n=4, ** p< 0.01. E-I, Tubular Hif-1α was deleted at days 8-21 following a 35 min left IRI and right nephrectomy, and kidneys were analyzed at 4 w post-IRI. Tubular deletion of Hif-1α led to more severe tubular atrophy (arrowhead) and cast formation (arrow) (E, PAS staining, n=5), higher urinary albumin/creatinine (F, n=3 or 5 for normal controls and n=5 or 6 for IRI 4w), higher urinary N-gal excretion (G, n=3 or 5 for normal controls and n=5 or 6 for IRI 4w), and more interstitial inflammation and fibrosis (H). In H, top panel shows more infiltrating CD45-expressing leukocytes (red, n=5), middle panel shows more fibrosis with collagen I (Col I) deposition (green, n=5) and bottom panel shows no significant differences in the density of capillaries labeled by endomucin (Endo, green, n=4) compared to that of Hif-1αctl kidneys. I. Lower levels of FoxO3 protein in Hif-1αtub kidneys compared with that of Hif-1αctl kidneys, but no significant difference in LC3II/I ration was detected (n=6). *p<0.05 and **p<0.01, comparing Hif-1αtub with Hif-1αctl. Scale bar, 100 µm in E, 50 µm in top and bottom panels of H, and 20 µm in D and the middle panel of H. A two-tailed Student's t test was performed in B, D, F, and H-I. Wilcoxon-Mann-Whitney test was performed in E. One-way ANOVA followed by Dunnett’s post hoc test for multiple comparisons was performed in C.
Figure 4. Tubular FoxO3 deletion during the AKI to CKD transition accelerates CKD development. Tubular FoxO3 was deleted at days 8-21 following a 35 min left kidney IRI and right nephrectomy. Kidneys were analyzed 4 w post-IRI. FoxO3\textsuperscript{ctl} indicates FoxO3 deletion with doxycycline and FoxO3\textsuperscript{ctl} indicates control with vehicle treatment. A. Pax8-rtTA and Tet-O-Cre system led to a dramatic reduction of FoxO3 protein in the kidney lysates, n=4. B. More severe tubular injury in FoxO3\textsuperscript{ctl} mice compared to FoxO3\textsuperscript{ctl} mice (PAS staining, n=5). Arrows denote tubular atrophy and arrowheads indicate cast formation. No increase of proliferation (Ki67 expression, red) in tubules (labeled with laminin in green) was detected 4 w post-IRI in mice without FoxO3 deletion (FoxO3\textsuperscript{ctl}) comparing to normal kidneys. However, tubular Ki-67 expression increased in FoxO3 deleted (FoxO3\textsuperscript{ctl}) mice compared to that of FoxO3 undeleted mice (FoxO3\textsuperscript{ctl}, n=4). Kim 1 expression (green) also indicated more severe proximal tubular injury in FoxO3\textsuperscript{ctl} mice, n=6. C-E. Increased urinary N-gal (C) and albumin excretion (D), and increase in serum creatinine (E) with FoxO3 deletion (FoxO3\textsuperscript{ctl}) (n=8). F. Trichrome stain (n=6) indicated more interstitial fibrosis with FoxO3 deletion (FoxO3\textsuperscript{ctl}). G. Deletion of FoxO3 (labeled with laminin in green) led to more severe interstitial inflammation with infiltration of CD45-expressing cells (red, top panel, n=5), interstitial fibrosis with vimentin (Vim) expression (red, middle panel, n=4) and collagen I (Col I) deposition (green, bottom panel, n=5). The differences in the density of microvessels labeled with endomucin (Endo, green, middle panel) did not reach statistical significance. *p<0.05 comparing FoxO3\textsuperscript{ctl} with FoxO3\textsuperscript{ctl}. Scale bar, 50 µm in all except the bottom panel in G where scale bar represents 20 µm. A two-tailed Student’s t test was performed in A, B (Ki67 and Kim1), D-E and G. Wilcoxon-Mann-Whitney test was performed in injury scores in B and F.
Figure 5. Tubular deletion of FoxO3 (FoxO3\textsuperscript{tub}) during the AKI to CKD transition resulted in focal glomerular sclerosis. Tubular FoxO3 was deleted at days 8-21 following a 35 min left kidney IRI and right nephrectomy, and kidneys were analyzed 4 w post-IRI. A. PAS staining indicated no increases in glomerular cellularity and Silver stain showed mild thickening of basement membrane in Bowman’s capsule but no changes in the basement membrane of capillary loops in the glomeruli of FoxO3\textsuperscript{tub} or FoxO3\textsuperscript{ctl} mice. EM showed rare podocyte foot process effacement, although more mesangial deposits were observed in FoxO3\textsuperscript{tub} kidneys. B. Top panel, immunostaining of podocyte with P57 (red) showed that in both FoxO3\textsuperscript{tub} or FoxO3\textsuperscript{ctl} mice, there were no significant changes in the number of podocytes per glomerulus after IRI. Endomucin (Endo, green) labeled glomerular capillary loops. Bottom panel, vimentin (Vim, red) and endomucin (Endo, green) staining showed focal glomerular sclerosis with vimentin deposition and loss of capillary loops in FoxO3\textsuperscript{tub} mice. n=4. Scale bar, 50 µm in A (top and middle panels), 10 µm in B, and 5 µm in A (bottom panel).
Figure 6. Loss of FoxO3 results in reduced autophagic adaptation. Tubular FoxO3 was deleted at days 8-21 following a 35 min left kidney IRI and right nephrectomy. Kidneys were analyzed at 4 w post-IRI. A. Representative EM images of autophagosomes and autolysosomes (white arrow) in tubules of FoxO3 control mice (FoxO3ctl) 4 w post-IRI. B. Lower levels of LC3II/I and Bnip3 proteins but no significant changes in ULK1 protein in FoxO3tub kidneys (n=4). C. The autophagy reporter CREL mice was bred into the FoxO3tub and FoxO3ctl mice and subjected to the above IRI. Fewer FRP dots were presented in renal tubules labeled with E-cadherin (E-cdh, green) that appeared atrophic in FoxO3tub mice. Tubules in FoxO3ctl mice contained more abundant RFP dots. D. Quantification of RFP dots in proximal tubules (labeled with LTA in cyan) indicated that the majority of autophagic cells in FoxO3tub kidneys contained ≤ 10 dots per cell, whereas the majority of autophagic cells in FoxO3ctl kidneys contained ≥ 10 dots per cell. Nuclei were counter-stained with DAPI in blue in C-D. *p<0.05 comparing FoxO3tub with FoxO3ctl. Scale bar, 20 µm in C-D. A two-tailed Student’s t test was performed in B.
Figure 7. Loss of FoxO3 results in more severe oxidative injury. A. Tubular FoxO3 was deleted at days 8-21 following a 35 min left kidney IRI and right nephrectomy. Kidneys were analyzed 4 w post-IRI. Lower levels of SOD2 protein were detected in FoxO3\textsubscript{tub} comparing to FoxO3\textsubscript{ctl} mouse kidneys. n=4, *p<0.05 comparing FoxO3\textsubscript{tub} with FoxO3\textsubscript{ctl}. B. Left and middle panels, primary cultures were isolated from normal kidneys of mice treated with doxycycline or vehicle for 2 w (FoxO3\textsubscript{tub} or FoxO3\textsubscript{ctl}) and exposed to 1% O\textsubscript{2} for 1h prior to incubation with MitoSOX Red or dihydroethidium (DHE). Mitochondrial superoxide levels indicated by MitoSOX Red (left panel) showed higher intensity in FoxO3\textsubscript{tub} cells. DHE (middle panel) showed that nearly 100% of FoxO3\textsubscript{tub} cells emitted red nuclear signals comparing to 49.6% in FoxO3\textsubscript{ctl} cells. Right panel, DHE staining of the kidney showed stronger nuclear oxidative stress in FoxO3\textsubscript{tub} kidneys 4 w post-IRI. C. EM showed dramatic mitochondrial swelling with a loss of cristae in epithelial cells 4 w post-IRI. In FoxO3\textsubscript{tub} cells, frequent ruptures of mitochondrial inner and outer membrane were detected (white arrow). Scale bar, 10 \mu m in left, 50 \mu m in middle, and 100 \mu m in right panels in B. A two-tailed Student’s t test was performed in A.
Figure 8. FOXO3 is activated in human kidneys with ischemic injury. Human kidney samples were obtained from biopsy for AKI or AKI on CKD. Control samples were obtained from biopsy for microscopic hematuria or proteinuria with no specific pathologic alterations. **A.** Endothelial marker CD31 (dark brown) in top panel (200x magnification) and middle panel (400x magnification) indicated abundant peritubular capillaries in controls, but diminished microvessels surrounding tubules with acute injury, atrophy or cast formation. Bottom panel, nuclear FOXO3 expression (dark brown) was predominantly localized to the distal tubules (dt) in controls, but increased expression (black arrows) in the injured proximal tubules (pt) with flat epithelia and denuded basement membrane. **B-C.** EM images showed normal mitochondria in controls but diffused mitochondrial swelling with loss of cristae in injured epithelia. Note the same magnification in B-C with a scale bar of 2 µm. **D-E.** EM detected autophagosomes and autolysosomes (white arrow) in epithelia of injured kidneys. Scale bar, 50 µm in A.

Figure 9. Proposed mechanism of hypoxia-induced FoxO3 activation leading to renal protection.
Table 1. Characteristics of human kidney biopsies

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls, n=5</th>
<th>Kidney Injuries, n=12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age range (year)</td>
<td>25-74 ( 51.0 ± 19.4)</td>
<td>50-81 ( 68.2 ± 8.5)</td>
</tr>
<tr>
<td>Baseline Scr (mg/dl)</td>
<td>N/A</td>
<td>0.7-2.1 ( 1.7 ± 0.5)</td>
</tr>
<tr>
<td>Scr at biopsy (mg/dl)</td>
<td>0.81-1.0 ( 0.9 ± 0.1 )</td>
<td>1.6-9.4 ( 4.4 ± 2.1)</td>
</tr>
<tr>
<td>Biopsy indications</td>
<td>Microscopic hematuria</td>
<td>Kidney ischemia or hypoperfusion^A</td>
</tr>
<tr>
<td></td>
<td>Sub-nephrotic proteinuria</td>
<td></td>
</tr>
<tr>
<td>Biopsy findings (number of patients)</td>
<td>No specific pathologic alterations (5/5)</td>
<td>Acute tubular injury (12/12) Chronic renal disease (10) with focal (8/10) or diffuse (2/10) tubular atrophy and interstitial fibrosis*</td>
</tr>
</tbody>
</table>

^A, a total of 12 renal biopsy samples were obtained from patients with kidney ischemia or hypoperfusion due to renal atheroembolization (5), volume depletion (2), congestive heart failure (2), respiratory failure (2), or hepatorenal syndrome (1). B, focal <50% and diffuse, ≥50% of tubular atrophy and interstitial fibrosis. Values are expressed as mean ± SD. Scr, serum creatinine