Review

Regulation of kidney development by the Mdm2/Mdm4–p53 axis

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While p53 activity is required for tumour suppression, unconstrained p53 activity on the other hand is detrimental to the organism, resulting in inappropriate cellular death or proliferation defects. Unimpeded p53 activity is lethal in the developing embryo, underlining the need for maintaining a tight control on p53 activity during this period. The critical role of the negative regulators of p53, Mdm2 and Mdm4, in vertebrate development came to light by fatal disruption of embryogenesis that was observed with Mdm2 and Mdm4 gene deletions in mice. Embryonic lethality was rescued only by superimposing p53 removal. Here we summarize the contribution of the Mdm2/Mdm4–p53 axis that occurs at multiple steps of kidney development. Conditional, cell type-specific deletions reveal distinct functions of these proteins in renal morphogenesis. The severe impact on the renal phenotype from targeted gene deletions underscores the critical role played by the Mdm2/Mdm4–p53 nexus on nephrogenesis, and emphasizes the need to monitor patients with aberrations in this pathway for kidney function defects and associated cardiovascular dysfunction.

Keywords: progenitor, nephrogenesis, embryonic, metanephric, Mdm2/MdmX/p53

Introduction

The process of morphogenesis/organogenesis involves a complex interplay of growth stimulatory and differentiation signals that direct the formation of functional organs during development. Although initially concealed on some genetic backgrounds, the role of p53 in vertebrate development is now indisputable. Mice with germline p53 deletion exhibit defects in kidney development, neural tube closure, spermatogenesis, and ocular abnormalities among other defects (Armstrong et al., 1995; Sah et al., 1995; Saifudeen et al., 2009; Molchadsky et al., 2010). Besides controlling genes in cell cycle and apoptosis pathways, p53 also regulates cell migration, energy metabolism, differentiation, and renewal (Matoba et al., 2006; Gadea et al., 2007; Lebedeva et al., 2009; Liu et al., 2009; Molchadsky et al., 2010; Sch oppy et al., 2010; Hwang et al., 2011). Stimulation of apoptosis and cell cycle arrest by p53 emphasizes the need for tight control of p53 activity during development, highlighting the critical role of Mdm2 and Mdm4 (also known as MdmX) in the negative regulation of p53 function. This was revealed by mouse models of either Mdm2 or Mdm4 germline deletion that were embryonic lethal in a p53-dependent manner (Montes de Oca Luna et al., 1995; Parant et al., 2001). The oncogenic nature of Mdm2/Mdm4 was recognized from their overexpression observed in several human cancers, thereby subduing wild-type p53 activity and consequently transforming cells (Buesoramos et al., 1993; Reifenberger et al., 1993; Toledo and Wahl, 2007; Li and Lozano, 2013). Increased p53 expression also impacts organogenesis, as evidenced in transgenic mice overexpressing p53 in the ureteric bud (UB) lineage of the developing kidney (Godley et al., 1996). These mice have nephron deficiency, renal hypoplasia, and altered differentiation of the ureteric epithelium. Unabated p53 activity from conditional Mdm2 and/or Mdm4 deletion results in defects in lens and skeletal morphogenesis (Lengner et al., 2006; Zhang et al., 2014), and impaired nephrogenesis as discussed in this review.

Kidney development

The mammalian kidney develops via inductive signalling between three cell lineages that derive from the intermediate mesoderm at embryonic day E8.5 in the mouse and gestational weeks 4–5 in humans. The nephric or Wollfian duct gives rise to the UB that via reiterative branching and elongation arborizes to form the collecting duct system (Figure 1A); the metanephric mesenchyme (MM) gives rise to all segments of the nephron; and the stroma, surrounding the UB and cap mesenchyme (CM), gives rise to the vasculature, glomerular mesangial cells, and
renin-forming cells (Figure 1A) (Costantini and Kopan, 2010; Little and McMahon, 2012). The cells of the MM condense around the UB tip to form the CM that houses the nephron progenitor cells (NPC) (Figure 1A). NPC availability by self-renewal during embryonic development is a critical driver of nephron endowment at birth. Kidney function is directly proportional to functional nephron number. Low nephron endowment at birth is strongly associated with the development of cardiovascular diseases in adult life. The NPC express Six2, which maintains the NPC in progenitor state. Loss of Six2 results in precocious differentiation and depletion of the NPC pool, resulting in sub-optimal nephron endowment (Self et al., 2006). Therefore, maintenance of Six2 expression by the NPC is critical for their self-renewal to adequately populate the kidney with nephrons.

Wnt9b from the UB maintains the Cited1+/Six2+ NPC in progenitor state, while inducing the Cited1−/Six2+ NPC to undergo differentiation (Carroll et al., 2005; Karner et al., 2011). The differentiating NPC are lateral to the UB tip and will form the pretubular aggregate (PA, Figure 1A) before undergoing mesenchyme-to-epithelial transition (MET) that results in the formation of the renal

**Figure 1** Developmental expression of Mdm2, Mdm4, and p53 in the murine kidney. (A) The nephric duct gives rise to the UB that arborizes to form the collecting duct system; the MM gives rise to all segments of the nephron; and the stroma surrounding the UB and CM gives rise to the vasculature, glomerular mesangial cells, and renin-forming cells. The cells of the MM will condense around the UB tips along the periphery to form the CM that houses the NPC. The CM will self-renew, and a subset of the NPC will be induced and undergo differentiation to form nascent nephrons including the PA, RV, and CS, which will mature to functional nephrons. (B) Temporal regulation is observed for Mdm2 and p53, with high mRNA expression levels during kidney organogenesis declining 3- and 4-fold, respectively, in neonate kidney and further still by postnatal day P20. This is modified from Saifudeen et al. (2009) and Hilliard et al. (2011). (C) Schematic showing spatial distribution of differentially modified p53. P-p53S392 is localized in differentiated proximal tubule cells but not in proliferating cells, whereas acetylated p53K373/K382/K386 shows predominant localization in the nephrogenic niche that houses UB tips, NPC, and cortical stroma, and also in the distal tubule and the collecting duct. This is modified from Aboudehen et al. (2012).
vesicle (RV). The PA expresses Wnt4, Fgf8, Pax8 while decreasing levels of Six2; these cells have exited the progenitor pool (El-Dahr et al., 2008; Georgas et al., 2009). The RV expresses epithelial markers ZO1 and E-Cadherin and will progress through nascent nephron structures (CS, comma-shaped body) that gradually mature to functional nephrons (Georgas et al., 2009).

Mdm2, Mdm4, and p53 expression in the developing kidney

Mdm2 and Mdm4 mRNA expression expectedly follow the spatial expression pattern of p53, ubiquitous in all the cell lineages of the developing mouse kidney (Saifudeen et al., 2009; Hilliard et al., 2011). Temporal regulation is observed for Mdm2/Mdm4 and p53, with high mRNA expression levels declining 3- and 4-fold, respectively, in neonate kidney and further still by postnatal day P20 (Figure 1B). Protein levels follow this temporal pattern of transcript expression, with a more pronounced decrease in p53 protein levels in adult kidneys (Aboudehen et al., 2012). In addition to protein stability, functional aspects of p53 activity are regulated by stimuli-specific post-translational modifications (Meek and Anderson, 2009). Interestingly, differentially modified p53 shows spatial distribution, with P-p53\(^{3582}\) localized in differentiated proximal tubule cells but not in proliferating cells and acetylated P-p53\(^{3733/382/386}\) showing predominant localization in the nephrogenic niche that houses UB tips, NPC, and cortical stroma (Figure 1C) (Aboudehen et al., 2012). This spatio-temporal expression pattern is suggestive of differential context-driven activities for the Mdm2/Mdm4–p53 pathway during nephrogenesis.

Lineage-specific Mdm2 deletion from the mammalian embryonic kidney

Mdm2 deletion from the ureteric epithelia

We generated and characterized mice with conditional deletion of Mdm2 from the ureteric epithelium (UB\(^{Mdm2−/−}\)); these mice display severe renal hypoplasia and die soon after birth (Hilliard et al., 2011). The kidneys show defective UB branching and a marked decrease in UB tips. Although UB\(^{Mdm2−/−}\) cells showed reduced proliferation rate and 3-fold higher apoptosis, two markers of UB tip identity, c-Ret and Wnt11, continue to be expressed. The nephrogenic zone is underdeveloped with decrease in Wnt signalling from the ureteric epithelium, as a result of decreased Wnt9b expression. Wnt signalling is essential for all aspects of kidney development. Accordingly, the disruption of UB–CM crosstalk secondary to the branching morphogenesis defect results in a disorganized CM and impaired nephron formation. Elimination of both p53 alleles restored normal branching of the UB as well as the nephron deficit and postnatal survival, whereas one-allele p53 deletion produced only a partial rescue of the phenotype. Mdm4 deletion from the ureteric epithelium also results in UB branching defects, renal hypoplasia, and nephron deficit, but does not result in perinatal death, and the extent of apoptosis and proliferation defects is much less severe than that from Mdm2 gene deletion (our unpublished data). Double deletion of Mdm2 and Mdm4 from the UB, however, resulted in severe renal hypoplasia (Figure 2A). Most double mutants die perinatally. Hematoxylin and eosin (H&E) staining shows severely dysmorphic kidneys (our unpublished data).

Figure 2 Lineage-specific Mdm2, Mdm4, or p53 deletion from the mammalian embryonic kidney. (A) Severe renal hypoplasia with double deletion of Mdm2 and Mdm4 from the UB. Most double mutants die perinatally. H&E staining shows severely dysmorphic kidneys (our unpublished data). P3, postnatal day 3. (B) E14.5 NPC\(^{Mdm2−/−}\) mutant kidneys are hypoplastic with a markedly thinner CM and fewer Six2+ cells (red). E-cadherin (green) marks the epithelial structures. This is modified from Hilliard et al. (2014). (C) Immunofluorescent staining with CM marker Six2 (red) demonstrates a diminished CM in NPC\(^{p53−/−}\) mutant kidneys at E13.5. BrdU-labelling of proliferating cells in the CM shows decreased BrdU incorporation (green) in the p53-mutant CM. This is modified from Li et al. (2015).
Mdm2 deletion from NPC

Our laboratory generated and characterized mice with conditional deletion of Mdm2 in the Six2+ NPC (NPC^Mdm2^−/−), which also results in perinatal lethality (Hilliard et al., 2014). The mutant kidneys are hypoplastic. The CM is markedly thinner with fewer self-renewing Cited1+/Six2+ cells (Figure 2B) (Hilliard et al., 2014) and greatly reduced or lost progenitor markers such as Amphiphysin, Sal1, Pax2, Eya1, and Bmp7, whereas expression of differentiation markers Wnt4, Lhx1, and Pax8 is unchanged. Increased p53 levels drive increased apoptosis and reduced proliferation observed in the NPC^Mdm2^−/−, along with reduced renal parenchyma and increased stroma. As with the germline and UB^Mdm2^−/− mutants, p53 deletion rescued the progenitor cell depletion and hypoplasia and restored normal renal development and postnatal survival of mice (Hilliard et al., 2014). These results demonstrate a critical and cell autonomous role for Mdm2 in the UB and MM lineages. Mdm2-mediated inhibition of p53 activity is a prerequisite for renal organogenesis and plays an essential role in the renewal and survival of NPC. Loss of functional Mdm2 results in stable, constitutively active p53 that is responsible for elevated apoptosis and premature deletion of the Six2+ cap cells.

Rescue of the kidney phenotype from Mdm2 deletion by co-deletion of p53 indicates that the renal phenotype is largely p53-dependent, and that the effects of p53 are dominant (Hilliard et al., 2011). However, this does not mean that Mdm2/Mdm4 do not have p53-independent actions. Elevated Mdm2/Mdm4 levels are associated with genomic instability independent of p53 (Bouska et al., 2008; Carrillo et al., 2015). Recent studies demonstrate that Mdm2 interacts with enhancer of zeste homologue 2 (EZH2), a member of the Polycomb Repressive Complex 2 (PRC2), to promote trimethylation at histone H3 lysine 27 (H3K27me3) and ubiquitination at histone 2A lysine 119 (H2AK119). This interaction promotes stemness in mesenchymal stem cells by supporting PRC2-driven repression of lineage-specific genes (Wienken et al., 2016). Whether any of the above-mentioned p53-independent actions of Mdm2/Mdm4 occurs during kidney development is under investigation.

p53 deletion from the mammalian embryonic kidney

A variety of developmental defects of the kidney and urinary tract are observed with germline p53 deletion in mice on a C57BL6 background, most frequently double ureters and renal hypoplasia (Table 1) (Saifudeen et al., 2002, 2009). The p53^−/− embryo at E11.5 has a smaller MM, observed by whole mount immunofluorescence with Pax2 antibody (our unpublished data). All mouse models of p53 gene deletion on B6SJL and FVB mixed backgrounds showed hypoplasia, whereas duplex ureters were only observed when p53 function was deleted or impaired in the nephric duct/UB lineage (Table 1) (Saifudeen et al., 2009; Li et al., 2015).

p53 loss in the nephric duct lineage

That p53 antagonizes the glial cell line-derived neurotrophic factor (GDNF) → c-Ret → phosphatidylinositol-3 kinase (PI3K) pathway was demonstrated by siRNA-mediated p53 inactivation in UB cells. The enhanced PI3K activation by GDNF was measured by the increase in phospho-Akt levels (Saifudeen et al., 2009). As p53-null nephric ducts (ND^p53^−/−) do not exhibit increased cell proliferation, other Akt-mediated pathways such as cell migration may contribute to the duplex phenotype. The underlying mechanisms for ND^p53^−/− hypersensitivity to GDNF remain to be elucidated. Whether this increased sensitivity also extends to other growth factors such as fibroblast growth factors (FGFs) is not known. While other models of UB ectopia exhibit increased receptor tyrosine kinase (RTK) signalling resulting either from decreased levels of the RTK signalling antagonist Sprouty-1 (Spry1) or an expanded GDNF domain, these were unchanged in p53-null models (Saifudeen et al., 2009). Chimera experiments with GFP-labelled p53-null cells in the UB lineage revealed neither a preponderance nor exclusion of mutant cells at the leading edge of the UB or tips (our unpublished data). An attractive possibility that remains to be tested is that p53 and phospho-Smad cooperate to inhibit ectopic budding in the nephric duct by activating a cytostatic pathway, given that heterozygous deletion of Bmp4 results in duplex ureters (Miyazaki et al., 2000). Alternatively, modulation of regulators of heparin sulphate such as heparanase and extracellular heparin sulphate 6-O-endosulfatases, which are known p53 targets, may deregulate heparin sulphate proteoglycans that control effective growth factor (GDNF, FGFs, BMPs) concentrations in a microenvironment, thereby contributing to ectopic signalling and bud formation (Baraz et al., 2006; Chau et al., 2009; Shah et al., 2011). Also, the involvement of other factors such as modifier genes (Nadeau, 2001) cannot be discounted in regulating UB induction.

Table 1 Comparison of phenotypes after p53, Mdm2, or Mdm4 deletion.

| Model          | p53                        | Mdm2                        | Mdm4                        |
|----------------|-----------------------------|-----------------------------|-----------------------------|
| Germline       | Viable                      | Embryonic lethal            | Embryonic lethal           |
|                | Duplex, nephron deficit, hypoplasia | (Montes de Oca Luna et al., 1999) | (Parant et al., 2001) |
| Deletion in UB | Viable                      | Neonatal lethal             | Die postnatally around weaning age |
|                | Duplex, hypoplasia          | Branching defects, nephron deficit, high apoptosis in UB | Branching defects, hypoplasia, nephron deficit, high apoptosis in UB (our unpublished data) |
| Deletion in CM | NPC loss, apoptosis not increased, longer cell cycle, hypoplasia, nephron deficit, stroma expansion | Rescued by p53 gene deletion (Hilliard et al., 2011) | Not done |

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p53 loss in the MM

We recently reported that conditional deletion of p53 from the MM using a Six2Cre driver results in kidney hypoplasia with a sparse, less compact, and disorganized E13.5 CM (Figure 2C) (Li et al., 2015). A clear deficit of differentiating nephrons is observed in P0 kidney sections. Glomerular dysfunction is indicated in PAS-stained kidneys displaying proteinaceous or hyaline material in Bowman’s space. As Cited1 marks the self-renewing population, the progressive loss of Cited1+/Six2+ suggests a decreased capacity for self-renewal. Quantitatively, Six2p53−/− kidneys have 30% less Six2+ cells. Neural cell adhesion molecule (NCAM1), present at intercellular domains of CM and nascent nephrons, is greatly decreased in the mutant CM (Li et al., 2015). NCAM1 is required for cell–cell and cell–matrix interactions during development and differentiation. Reduced Pax2 staining was reported in both germline and conditional Six2p53−/− kidneys. In vivo and in vitro reporter analyses demonstrated that Pax2 is a p53 target gene (Saifudeen et al., 2012), implicating p53 in regulating the expression of a key kidney development protein. Although the remaining Six2+(p53-null) cells are capable of differentiating into nascent nephrons, this does not alleviate the pronounced nephron defect observed in mutant kidneys in embryonic and P0 kidney sections. Induction of the Wnt/β-catenin pathway to induce MET in an in vitro assay on isolated Six2+ CM cells revealed a differentiation defect in p53-null cells (Li et al., 2015). The mutant cells were unable to form complex structures observed in wild-type cells, and showed decreased conversion to epithilia as determined by E-cadherin staining. Fgf8 expression (typically observed in the PA/RV, see Figure 1) is greatly diminished in the Six2p53−/− kidneys. However, expression of differentiation genes Pax8 and Wnt4 did not show a marked change. Treatment of Six2p53−/− kidneys ex vivo with Fgf8 beads did not demonstrate any localized rescue of MET, indicative of additional epithelialization defects in the p53-null CM. Thus, the nephron deficit in Six2p53−/− kidneys is a result of at least two developmental defects: (i) self-renewal defect of Cited1+/Six2+ NPC resulting in reduced availability of progenitors for nephrogenesis, and (ii) improper differentiation from an inefficient response to Wnt/β-catenin signalling, or defective MET, or both in Six2+(p53-null) cells (Figure 2C) (Li et al., 2015).

p53 regulates energy metabolism in nephron progenitors

No increase in either apoptosis or senescence was observed in Six2p53−/− cells, examined by PARP or active caspase-3 and senescence marker SA-βgal staining, respectively (Li et al., 2015). Moreover, γH2Ax staining—a gauge of DNA damage—was also not increased, indicating that p53 is not exclusively involved in maintenance of genomic stability in the developing kidney. Unexpectedly, after p53 loss, the proliferation index of the CM is significantly lower compared to wild-type CM at E15.5. Cell cycle analysis revealed that more Six2p53−/− cells are in GO/G1, and a significantly smaller fraction are in S and G2/M. Contrary to expectations, transcriptome profiling by RNA-seq of Six2p53−/− NPC surprisingly showed decreased, rather than increased, levels of transcripts of cell cycle inhibitors p21 (Cdkn1a) and p57 (Cdkn1c), indicative of an alternate explanation for decreased proliferation (Li et al., 2015).

Our studies using ChiP-seq showed that p53 chromatin occupancy was detected on diverse genes in the developing kidney beyond the canonical p53 targets (Li et al., 2013). Transcription analysis showed dysregulation of genes beyond the classical pathways associated with p53 regulation, including development and morphology, cell adhesion and migration, cell survival, metabolism, and ion transport (Li et al., 2015). Expression of multiple genes in cell adhesion and migration pathways is decreased, phenotypically observed by the loosely organized CM in Six2p53−/− kidneys. Disruption of biophysical and biomechanical cues from the extracellular matrix to cells within the niche can be envisioned to influence renewal and differentiation programmes (Cox and Erler, 2011).

We found that genes involved in glucose metabolism, the pentose phosphate pathway, and the electron transport chain or genes required to maintain a proton gradient for adenosine triphosphate (ATP) production during oxidative respiration are dysregulated in Six2p53−/− cells (Li et al., 2015). These are among the most regulated genes and include phosphoenol pyruvate carboxykinase 1 (Pck1, −14.3-fold), cytochrome P450 2D26 (Cyp2d26, −13.0-fold), fructose bisphosphatase 1 (Fbp1, −12.0-fold), and aldolase b (Aldob, −12.1-fold). Moreover, functional analyses showed decreased reactive oxygen species (ROS) and ATP levels in Six2p53−/− cells, indicative of mitochondrial dysfunction. Thus, it appears that the p53-deficient NPC undergo deregulation of metabolic homeoestasis that compromises the metabolic fitness of the Cited1+/Six2+ NPC, thereby impeding their self-renewal. It needs to be emphasized, however, that decreases in ATP (and ROS) are not the only outcomes of deregulated energy metabolism that impact cell fate. Alterations in energy pathways result in changes in metabolites as well (e.g. Acetyl-CoA), which are key regulators of epigenetic modifications that control gene expression (Katada et al., 2012; Moussaieff et al., 2015).

Surprisingly, a consistent observation in all the models studied has been the decrease in cell proliferation with p53 loss (Saifudeen et al., 2009, 2012; Li et al., 2015), suggesting that p53 mediates more divergent pathways in kidney development than in cancer. This idea is further supported upon comparison of p53 occupancy on chromatin from embryonic kidneys and cancer-derived cell lines (Li et al., 2013). Not only is the pattern of chromatin occupancy different between embryonic kidney and cancer cells (promoter versus non-promoter/intergenic regions, respectively), but also the number and variety of genes that are enriched vary. p53 gene occupancy in embryonic kidneys extends beyond cell cycle and apoptosis genes—expected in cells with genotoxic stress-induced p53—in development and morphology, chromatin modifying enzymes, and genes in metabolic pathways (Li et al., 2013).

Distinct mechanisms of UB branching and nephrogenesis defects in kidneys with Mdm2/Mdm4 or p53 loss

UB-specific Mdm2 deletion results in a hypoplastic UB and subsequent defects in branching and nephrogenesis, whereas
deletion of p53 from the UB results in duplex ureters in a third of the animals, as well as UB hypoplasia and branching defects (depicted in Figure 3A) (Saifudeen et al., 2009; Hilliard et al., 2011). Loss of Mdm2 or p53 resulted in NPC depletion. Thus, while the final outcome from both mutant models is nephron deficit and renal hypoplasia (depicted in Figure 3B), the mechanisms leading to this phenotype are quite different in each case as outlined in Figure 3C. Whereas p53 loss decreases NPC self-renewal possibly from disrupted cellular metabolism and adhesion, Mdm2 deletion causes NPC depletion by decreasing proliferation and increasing apoptosis. 

Figure 3 Schematic comparison of kidney phenotypes after Mdm2 or p53 conditional deletion from UB or CM lineage. (A) UB-specific Mdm2 deletion results in a hypoplastic UB and subsequent defects in branching and nephrogenesis. Deletion of p53 from the UB results in duplex ureters in a third of the animals, as well as UB hypoplasia and branching defects. Final outcome from both mutant models is nephron deficit and renal hypoplasia. This is adapted from Saifudeen et al. (2009) and Hilliard et al. (2011). (B) Conditional deletion of Mdm2 or p53 from the MM using a Six2Cre driver results in kidney hypoplasia with a sparse, disorganized CM. A marked paucity of differentiating nephrons is observed in P0 kidneys. Cortical stroma (dark grey) is expanded in both cases. This is adapted from Hilliard et al. (2014) and Li et al. (2015). (C) Distinct mechanisms of nephron deficit in kidneys with Mdm2 or p53 loss. While loss of either protein results in NPC depletion, the mechanisms leading to this phenotype are quite different in each case. Whereas p53 loss decreases NPC self-renewal possibly from disrupted cellular metabolism and adhesion, Mdm2 deletion causes NPC depletion by decreasing proliferation and increasing apoptosis.
Future perspectives

Conditional loss of either p53, Mdm2, or Mdm4 hinders mouse kidney development, with Mdm2/Mdm4 loss resulting in a fatal outcome from severe renal dysgenesis (Saifudeen et al., 2009; Hilliard et al., 2011; Hilliard et al., 2014; Li et al., 2015). Thus, control of the Mdm2/Mdm4–p53 nexus is crucial for proper kidney development and to achieve a full complement of nephrons. While mutations in Mdm2/Mdm4/p53 genes have yet to be identified in infants and children with congenital renal dysgenesis, it is also clear that adverse intra-uterine environmental conditions upregulate p53 activity in the developing kidney and are associated with impaired nephrogenesis (Pham et al., 2003). Accordingly, future studies should examine how gene–environment interactions shape nephron endowment via the Mdm2/Mdm4–p53 axis. Another area of fruitful investigation will be investigating non-canonical roles of p53 and Mdm2/Mdm4, as this will provide a more comprehensive view of their developmental functions that may be applicable to their roles in cancer.

Funding

This work was supported by the National Institutes of Health (R01DK62550 to S.E.-D.; R56DK104779 and U24DK076169 to Z.S.) and the Tulane Hypertension and Renal Center of Excellence.

Conflict of interest: none declared.

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