Induction of Retinol Dehydrogenase 9 Expression in Podocytes Attenuates Kidney Injury

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ABSTRACT

The intracellular concentration of retinoic acid is determined by two sequential oxidation reactions that convert retinol to retinoic acid. We recently demonstrated that retinoic acid synthesis is significantly impaired in glomeruli of HIV-1 transgenic mice (Tg26), a murine model of HIV-associated nephropathy. This impaired retinoic acid synthesis correlates with reduced renal expression of retinol dehydrogenase 9, which catalyzes the rate-limiting step of retinoic acid synthesis by converting retinol to retinal. Because retinoic acid has renal protective effects and can induce podocyte differentiation, we hypothesized that restoration of retinoic acid synthesis could slow the progression of renal disease. Herein, we demonstrate that overexpression of retinol dehydrogenase 9 in cultured podocytes induces the expression of podocyte differentiation markers. Furthermore, we confirm that podocyte-specific overexpression of retinol dehydrogenase 9 in mice with established kidney disease due to either HIV-associated nephropathy or adriamycin-induced nephropathy decreases proteinuria, attenuates kidney injury, and restores podocyte differentiation markers. Our data suggest that restoration of retinoic acid synthesis could be a new approach to treat kidney disease.

Retinoic acids (RAs) are derivatives of vitamin A and have multiple cellular functions, including inhibition of proliferation, induction of cell differentiation, and inhibition of inflammation.1 In addition to their established benefits in the treatment of a variety of cancers, RA has also been shown to protect against renal injury in multiple experimental models of kidney disease,2 including HIV-associated nephropathy (HIVAN).3 Both in vitro and in vivo studies suggest that RA restores the expression of podocyte differentiation markers, including nephrin, podocin, and synaptopodin.3,4 These studies provide strong evidence supporting the therapeutic benefit of RA in kidney diseases with podocyte injury. In fact, a phase II clinical trial examining the efficacy of RA for treatment of podocyte diseases, including minimal change disease, FSGS, or collapsing glomerulopathy, is ongoing (ClinicalTrials.gov identifier NCT00098020). However, clinical use of RA is challenging because of its side effects.5

After cellular uptake, retinol is converted to RA by two sequential oxidation reactions. Retinol dehydrogenases (RHDs) oxidize retinol to retinal,6 which is further metabolized to retinoic acid by retinaldehyde dehydrogenases (ALDHs).7 The expression of these enzymes varies greatly among different cell types and at different stages of cell differentiation.7 Both the synthesis and metabolism of the bioactive metabolites of retinol are impaired in cancer cells.8 The kidney is a major organ for retinoid metabolism. However, not much is known regarding how retinoid metabolism is altered in renal disease.

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Our previous work showed that although the expression of retinoic acid receptor-α—target genes is suppressed in kidneys of a murine model of HIVAN (Tg26) and of patients with HIVAN, the expression of retinoic acid receptor-α is not different between normal and diseased kidneys. The concentration of RA, however, is significantly reduced in the kidney cortex and isolated glomeruli of Tg26. We also found that the glomerular concentration of RA is >10-fold higher than the concentration in the kidney cortex. Examination of enzymes involved in RA metabolism reveal that two key enzymes in the RA synthetic pathway, retinol dehydrogenase type 1 and type 9 (RDH1 and RDH9), were significantly downregulated in Tg26 glomeruli. RDH9 is a rate-limiting enzyme in RA synthesis. Because it is known that RA has renal protective effects and is able to induce podocyte differentiation, we hypothesize that overexpression of RDH9 to restore endogenous RA synthesis could slow the progression of renal disease.

Consistent with our findings, recent studies also showed that endogenous RA synthesis is impaired in kidneys of diabetic db/db mice and TGF-β transgenic mice. These data together with ours suggest that endogenous RA synthesis is impaired in diseased kidneys. Thus, a better understanding of RA metabolism in kidney disease could help us to identify potential new therapy for kidney diseases.

RESULTS

Overexpression of RDH9 Induced the Expression of Differentiation Markers in Cultured Podocytes

We observed that overexpression of RDH9 in cultured mouse podocytes significantly induced neprhin and synaptopodin expression compared with podocytes without RDH9 overexpression after 7 days of incubation at 37°C (Figure 1A and B). The effect of RDH9 overexpression on nephrin expression was similar to that observed in cells treated with all-trans-retinoic acid (Figure 1C). Supplementation of retinol in the culture media further increased nephrin expression in podocytes with RDH9 overexpression (Figure 1C). These effects on nephrin expression were also confirmed by Western blot (Figure 1D). Taken together, these in vitro data suggest that improvement of RA synthesis induces podocyte differentiation.

An Inducible Podocyte-Specific RDH9 Overexpression Mouse Model

We obtained 14 mice positive for the pTRE2-FLAG-Rdh9 transgene, which encodes a fusion FLAG-tagged Rdh9 under the transcriptional control of a tetracycline-responsive promoter element (TRE), from two rounds of pronuclear injection of fertilized FVB-N embryos. Germ-line transmission of the transgene was confirmed in eight founders. Mice derived from the F1 generation were bred with FVB-N mice homozygous for podocin-rtTA (gift from Dr. J. Kopp, Bethesda, MD) expressing the reverse tetracycline transactivator (rtTA) specifically in podocytes to generate double transgenic mice, podocin-rtTA; TRE2-FLAG-Rdh9, which are on a pure FVB-N background (hereafter referred to as PODRDH9). First-generation offspring carrying both transgenes were studied for doxycycline-inducible transgene expression. Five-week-old adult PODRDH9 mice were administered with either doxycycline or vehicle for 6 weeks. Both sexes showed a similar phenotype and were analyzed together. By Western blotting, we confirmed that RDH9 was expressed highly in kidney cortex, highly in liver, and weakly in heart (Supplemental Figure 1A). PODRDH9 mice that were exposed to doxycycline showed significantly (P<0.05) increased RDH9 levels in isolated glomeruli compared with PODRDH9 mice without doxycycline feeding (Supplemental Figure 1B). We also confirmed that mRNA levels of RDH9 were increased in glomeruli of PODRDH9 mice fed with doxycycline compared with PODRDH9 mice without doxycycline feeding (Supplemental Figure 1C).

Doxycycline feeding of PODRDH9 mice had no effect on the body weight and serum levels of urea nitrogen and creatinine (Table 1). Induction of RDH9 expression in podocytes did not affect expression of podocyte differentiation markers at baseline (Supplemental Figure 2).

Induction of RDH9 in Podocytes Improved Renal Function and Reduced Proteinuria in Tg26 Mice

To determine whether podocyte-specific overexpression of RDH9 is sufficient to slow the progression of kidney disease, Tg26 mice were crossed with PODRDH9 mice and triple transgenic mice (Tg26;PODrdh9) harboring the Tg26, TRE2-FLAG-RDH9, and podocin-rtTA transgenes, and were fed with doxycycline at age 5 weeks for a total of 6 weeks. Our preliminary studies showed that doxycycline was able to induce RDH9 expression in podocytes after feeding of doxycycline for 1 week (data not shown) and Tg26 mice are known to develop proteinuria starting at 4 weeks of age. Urine was collected biweekly to assess for proteinuria and mice were euthanized at the end of experiments for assessment of renal function and histology.

After 6 weeks of doxycycline feeding (11 weeks of age), we found that the body weight was not significantly different between Tg26;PODRDH9 mice fed with doxycycline and vehicle. The levels of serum BUN and creatinine were significantly lower in Tg26;PODRDH9 mice fed with doxycycline compared with mice fed with vehicle (Table 1; P<0.05). No significant difference in BUN and creatinine were observed between the Tg26;PODrdh9 group fed with doxycycline and the PODRDH9 fed with either doxycycline or vehicle (data not shown).

Urinary albumin excretion measured by the urinary albumin/creatinine ratio (UACR) in Tg26;PODRDH9 mice fed with either doxycycline or vehicle was significantly higher than that of PODRDH9 mice fed with either doxycycline or vehicle at 2, 4, and 6 weeks (Figure 2A; P<0.05). However, the UACR of Tg26;PODrdh9 mice fed with doxycycline was significantly lower than Tg26;PODrdh9 mice fed with vehicle only after 6 weeks of doxycycline feeding (P<0.05). Coomassie blue staining confirmed that urinary proteins were predominately albumin, which was reduced in two doxycycline-fed
Induction of RDH9 Expression in Podocytes Improved Kidney Injury in Tg26 Mice

Tg26;POD mice developed significant glomerulosclerosis, interstitial nephritis, and tubular microcystic dilation. However, induction of RDH9 expression in podocytes of mice fed with doxycycline from age 5 weeks to 11 weeks significantly reduced glomerulosclerosis and tubular injuries compared with mice fed with vehicle (Figure 3, Table 2).

Induction of RDH9 Expression in Podocytes Increased Expression of Podocyte Differentiation Markers and Inhibited Glomerular Cell Proliferation in Tg26 Mice

We assessed the expression of nephrin, podocin, and synaptopodin in the glomeruli of these mice by both real-time PCR and immunostaining. We found that induction of RDH9 expression in podocytes increased nephrin and synaptopodin expression at both mRNA and protein levels in Tg26;POD mice fed with doxycycline, whereas expression of these markers remained suppressed in Tg26;POD mice fed with vehicle (Figure 4, A and B; P<0.05). Similarly, the mRNA level of podocin was also increased in Tg26;POD mice fed with doxycycline compared with those fed with vehicle (Figure 4B; P<0.05). Because glomerular cell proliferation is considered as a hallmark of collapsing FSGS in HIVAN,12 we examined the expression of Ki67 in kidneys of Tg26;POD mice and found that Ki67 was markedly reduced in Tg26;POD mice fed with doxycycline compared with Tg26;POD mice fed with vehicle (Figure 4C; P<0.01).

Induction of RDH9 Expression in Podocytes Modulated RA Target Gene Expression in Tg26

We also determined whether induction of RDH9 expression in podocytes affected the expression of RA target genes that are involved in cell differentiation, cell proliferation, anti-inflammatory processes, and reactive oxygen species detoxification as we previously described.13 We first confirmed that expression of RDH9 was significantly increased in glomeruli of Tg26;POD mice fed with vehicle (Figure 5A). Expression of Kruppel-like factor 15 (KLF15), a transcription factor involved in podocyte differentiation,13 was significantly increased in Tg26;POD mice fed with doxycycline compared with Tg26;POD mice fed with vehicle (Figure 5B). Glutathione S-transferase-α-4 (GSTA4), which is involved in the inhibition of reactive oxygen species generation, was also increased in Tg26;POD mice fed with doxycycline (Figure 5B). Consistent with our previous

Table 1. Body weight, serum urea nitrogen, and creatinine

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Mice (n)</th>
<th>Body Weight (g)</th>
<th>Serum Urea Nitrogen (mg/dl)</th>
<th>Serum Creatinine (mg/dl)</th>
</tr>
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<tbody>
<tr>
<td>POD+vehicle</td>
<td>4</td>
<td>27.25±1.01</td>
<td>22.87±4.75</td>
<td>0.10±0.02</td>
</tr>
<tr>
<td>POD+doxycycline</td>
<td>4</td>
<td>28.18±1.09</td>
<td>22.22±4.01</td>
<td>0.09±0.03</td>
</tr>
<tr>
<td>Tg26;POD+vehicle</td>
<td>8</td>
<td>24.82±4.73</td>
<td>31.24±9.19</td>
<td>0.17±0.04</td>
</tr>
<tr>
<td>Tg26;POD+doxycycline</td>
<td>10</td>
<td>24.23±4.31</td>
<td>24.09±3.79</td>
<td>0.13±0.04</td>
</tr>
</tbody>
</table>

Body weight was recorded and serum samples were obtained at the end of experiments after feeding of doxycycline or vehicle for 6 weeks. Serum creatinine was measured by HPLC-based assay.

aP<0.05 compared with all other groups.
bP<0.05 compared with the Tg26;POD+vehicle group.

Figure 1. RDH9 overexpression increases expression of markers of differentiated podocytes. (A and B) Mouse conditionally immortalized podocytes infected with control vector or RDH9 expression vector are cultured at 37°C for 3, 5, and 7 days to induce differentiation. RNA is isolated from these cells for real-time PCR analysis of nephrin and synaptopodin. (C) Podocytes are treated with all-trans retinoic acid (atRA), or overexpressed with RDH9 with or without retinol (1.25 μM) at 37°C for 7 days. Real-time PCR is performed in these cells for nephrin. *P<0.01 compared with control cells; **P<0.01 compared with RDH9 without retinol treatment (n=4). (D) Mouse podocytes are cultured at 37°C for 7 days with atRA (1 μM) in serum-free medium or infected with a RDH9 expression vector, and Western blots are performed for RDH9, nephrin, and β-actin. The representative blots of three independent experiments are shown. CL, control cell.
findings, Cyclin D2 (Ccnd2) and WNT1-inducible signaling pathway protein 1 (Wisp1) were significantly suppressed in Tg26;PODRDH9 mice fed with doxycycline (Figure 5B). Glial cell line–derived neurotrophic factor (GDNF), a growth factor that is critical for neural and renal development, was recently found to function as a survival factor for injured podocytes in puromycin nephropathy nephrotic rats. IFN-related developmental regulator 1 (IFRD1) is the human homolog of the rat PC4 gene. The rat PC4 gene was initially isolated as a nerve growth factor–inducible sequence in PC12 cells and it was recently shown that PC4 is necessary to muscle differentiation (Figure 5B). These findings illustrate potential mechanisms to explain how restoration of RA synthesis by induction of RDH9 expression in podocytes restored cell differentiation and inhibited cell proliferation in our model.

Induction of RDH9 Expression in Podocytes Protected Mice from Adriamycin-Induced Nephropathy

To further confirm whether overexpression of RDH9 in podocyte could improve kidney disease and podocyte injury, we induced nephropathy by injection of adriamycin (18.8 mg/kg intravenously) in PODRDH9 mice. Mice were fed with doxycycline or vehicle foods at day 7 postinjection when they developed kidney injury. Urine was collected at days 14 and 28 postinjection to assess the UACR. Mice were euthanized at day 28 postinjection to assess renal function and kidney histology.

We found that PODRDH9 mice treated with doxycycline exhibited significantly reduced proteinuria compared with mice in the vehicle group at day 28 postinjection (Figure 6A; \( P < 0.05 \)). Coomassie staining revealed that the change in proteinuria is mainly albuminuria. The representative gel shows two mice in each group (lanes 1 and 2, vehicle Tg26;POD; lanes 3 and 4, doxycycline-treated Tg26;POD; lanes 5 and 6, vehicle Tg26;POD\(^{RDH9}\), and lanes 7 and 8, doxycycline-treated Tg26;POD\(^{RDH9}\)). DOX, doxycycline.

Table 2. Summary of kidney histology

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Glomerulosclerosis Index</th>
<th>Podocyte Hypertrophy</th>
<th>Tubular Casts/Cysts</th>
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<tbody>
<tr>
<td>Tg26;POD(^{RDH9})+vehicle</td>
<td>0</td>
<td>0</td>
<td>0.59±0.13</td>
</tr>
<tr>
<td>Tg26;POD(^{RDH9})+doxycycline</td>
<td>32±19.64*</td>
<td>2.47±0.84*</td>
<td>7.81±6.43*</td>
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</tbody>
</table>

Kidney histologic analysis was performed in Tg26;POD\(^{RDH9}\) mice treated with doxycycline or vehicle. The glomerulosclerosis index, podocyte hypertrophy, and tubular casts/cysts were quantified as described in the Concise Methods.

* \( P < 0.05 \) compared with the vehicle group.
group (Table 3; P < 0.05), but was not different between the other groups. In addition, the expression of synaptopodin and nephrin was significantly increased in PODRDH9 mice fed with doxycycline compared with the mice fed with vehicle, as determined by immunofluorescence staining of kidney sections from mice at day 28 postinjection (Figure 8).

**Figure 4.** Podocyte differentiation and cell proliferation markers. (A) Immunofluorescence labeling for nephrin and synaptopodin. Nuclei are labeled by DAPI in blue. (B) Quantitative real-time PCR for nephrin, podocin, and synaptopodin mRNA expression is performed (n=4 in vehicle group and n=6 in doxycycline group). (C) Quantitative real-time PCR for Ki67 mRNA expression is performed (n=4 in vehicle group and n=6 in doxycycline group). *P<0.05 compared with vehicle group. DAPI, 4’6-diamidino-2-phenylindole; DOX, doxycycline.

**Figure 5.** Quantitative real-time PCR for RA synthesis and RA targeting gene mRNA expression is performed (n=4 in vehicle group and n=6 in doxycycline group). *P<0.05 compared with the vehicle group; #P<0.01 compared with vehicle group. DOX, doxycycline.

**DISCUSSION**

It is known that the kidney is one of the major organs for RA metabolism. However, it remains unclear whether RA metabolism is altered in the diseased kidney and how RA metabolism contributes to the progression of kidney disease. Our studies suggest RA synthesis is impaired in the diseased kidney and contributes to the progression of kidney disease. We previously found that endogenous RA synthesis is impaired in kidneys of Tg26 mice due to downregulation of key enzymes—RDH9 and RDH1—involving in RA synthesis. A similar deficiency of RA synthesis was also reported in diabetic kidney and in kidneys of TGF-β transgenic mice. In addition, recent studies suggest that albumin in the urine could sequester endogenous RA synthesized locally by kidney cells, leading to the progression of kidney disease. Previous studies also suggest that local RA concentration is critical for kidney development. These studies together with ours support a critical role of endogenous RA in the progression of kidney disease.

Previous studies strongly support a role of RA in inducing podocyte differentiation. Recent studies suggest that RA also induces regeneration of podocytes from parietal epithelial cells. Here, we showed that overexpression of RDH9 in podocytes induced expression of differentiation markers. In addition, induction of RDH9 expression in podocytes in vivo improved proteinuria and podocyte differentiation markers in two animal models with podocyte injury. Our studies provided additional evidence to support the critical role of RA in podocyte differentiation.

In addition, our studies showed that induction of RDH9 in podocytes regulated several RA target genes in Tg26 mice, including KLF15, GSTA4, Ccnd2, Wisp1, GDNF, and IFRD1. Our recent studies confirmed that RA could induce KLF15 expression both in vivo and in vitro and that KLF15 is a critical transcriptional factor mediating RA-induced podocyte differentiation. Our unpublished data suggest that GSTA4 mediates the antioxidative effect of RA in podocytes. It is known that HIV infection activates Ccnd2 leading to podocyte proliferation and RA suppresses podocyte proliferation in Tg26 mice through inhibition of Ccnd2. Wisp1, GDNF, and IFRD1 are RA target genes that mediate cell proliferation and differentiation. These data...
suggest that induction of RDH9 expression in podocytes activates these RA target genes to improve podocyte differentiation and attenuate kidney injury. Many studies have shown that exogenous RA is able to improve kidney diseases in various experimental models, including the HIV-1 transgenic mouse (Tg26),3 anti-Thy-1 nephritis,24 puromycin aminonucleoside-induced nephrosis,25 lupus nephritis,26 diabetic nephropathy,27 and anti-glomerular basement membrane nephritis.4 However, clinical application of RA is limited by its side effects. Our studies suggest that restoration of endogenous RA synthesis could be a new approach to treat patients with kidney disease. In addition, we showed that induction of RDH9 in mice with established kidney disease was able to attenuate kidney injury and reduce proteinuria, indicating that restoration of RA synthesis could be developed to slow the progression of kidney disease.

Vitamin A is absorbed from animal food and derived from beta-carotene of plants.28 In the blood, retinol binds to serum retinol-binding protein 4 and is then taken up by cells via a cell membrane receptor for retinol-binding protein 4.29 The intracellular concentrations of various retinoids are regulated by the activities of several key metabolic enzymes.30 RHDs oxidize retinol to retinal, which is further metabolized to retinoic acid by ALDHs.7 RA is further oxidized to more polar metabolites by cytochrome P450 family members such as CYP26A1.31 RDH9 converts 9-cis-retinol into 9-cis-retinal, has the highest efficiency (V_m/K_m) among RHDs. Interestingly, when we induced RDH9 expression in kidney of Tg26 mice, RDH1 expression was also significantly increased. Consistent with this finding, RHD1 was downregulated 3- to 8-fold in RDH9-null mice.32 However, there were no changes in the expression of other enzymes in the RA synthetic pathway such as Aldh1a1,2. There are different RDH isoforms between human and mouse. We previously showed that RDH1 was also reduced in human diseased kidney.9 Further studies are required to determine how we could restore RA synthesis in humans to treat kidney disease.

In conclusion, our studies suggest that restoration of endogenous RA synthesis induces podocyte differentiation in vitro and improves kidney injury in vivo. Therefore, restoration of RA synthesis could be considered as another approach to treat patients with kidney disease.

**CONCISE METHODS**

**Generation of Transgenic Animals**

Construct generation (TRE, construct) was as follows. Mouse RDH9 cDNA corresponding to NM_153133.2 was purchased from Thermo Fisher Scientific. A fusion N-terminal FLAG-tagged RDH9 construct (FLAG-RDH9) flanked by 5′ BamH1 and 3′ Xhol restriction
endonuclease sequences was generated by PCR and then cloned into the pDNR-1r plasmid (Clontech Laboratories). The FLAG-RDH9 fusion construct was subcloned into the pLP-TRE2 acceptor vector (Creator Vector; Clontech Laboratories) by Cre-mediated recombineering to generate the pLP-TRE2-FLAG-RDH9 expression construct. The sequence and orientation of FLAG-RDH9 insert were confirmed by restriction endonuclease digestion and DNA sequencing. Doxycycline-inducible expression of FLAG-RDH9 was confirmed in the Tet-ON U2OS cell line (Clontech Laboratories). The 4.6-kb ApaLI-ApaLI DNA fragment of pLP-TRE2-FLAG-RDH9 was used for microinjection to generate the TRE2-FLAG-RDH9 transgenic mice in FVB/N background.

Animal Genotyping
TRE2-FLAG-RDH9 transgenic mice were genotyped by PCR using the following set of primers: 5′-ACG CCATCC ACG CTG TTT TG-3′ and 5′-AAG TCC TGT TTC TTC ATC CAC TCG-3′ (amplicon size 557 bp). The genotyping for the podocin-rtTA mice was performed as previously described.33 All animal studies were performed in accordance with the guidelines of and approved by the Institutional Animal Care and Use Committee at the Icahn School of Medicine at Mount Sinai.

Podocyte Cell Culture
A conditionally immortalized murine podocyte cell line was cultured as described.34 Cells were cultured on rat tail collagen I–coated plates and maintained at 33°C (5% CO2, 90% humidity). For induction of differentiation, cells were cultured at 37°C up to 7 days.

Antibodies
Antibodies to RHD9 were custom made by GenoScript. Rabbit antibody to nephrin was a gift from Dr. Lawrence Holzman (University of Pennsylvania). Mouse antibody to synaptopodin was a gift from Dr. Peter Mundel (Massachusetts General Hospital).

Measurements of Serum Urea Nitrogen And Creatinine
Serum BUN content was measured using a commercially available kit (BioAssay Systems). Serum creatinine was measured by a HPLC-based assay. Pregroups are before the injection of adriamycin, whereas the postgroups are 28 days after injection of adriamycin. AD, adriamycin.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Body Weight (g)</th>
<th>Serum Urea Nitrogen (mg/dl)</th>
<th>Serum Creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD;POD&lt;sup&gt;RDH9&lt;/sup&gt;+vehicle</td>
<td>25.15±2.17</td>
<td>26.22±4.86</td>
<td>0.11±0.04</td>
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<tr>
<td>AD;POD&lt;sup&gt;RDH9&lt;/sup&gt;+doxycycline</td>
<td>23.94±1.2</td>
<td>26.41±3.17</td>
<td>0.10±0.02</td>
</tr>
</tbody>
</table>

*P<0.05 compared with mice in the pregroups.

Western blot analyses
Western blotting was performed using specific antibodies for proteins of interest as previously described.36

Histopathology and Immunohistochemistry Staining of Kidney Tissue
Kidney samples were fixed in 10% formalin, embedded in paraffin, and sectioned to 4-µm thickness. Periodic acid Schiff–stained sections were used for assessment of kidney histology.

Table 3. Body weight, serum urea, and creatinine in the adriamycin-induced nephropathy model

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Body Weight (g)</th>
<th>Serum Urea Nitrogen (mg/dl)</th>
<th>Serum Creatinine (mg/dl)</th>
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<td>23.94±1.2</td>
<td>26.41±3.17</td>
<td>0.10±0.02</td>
</tr>
</tbody>
</table>

Body weight was recorded and serum samples were obtained in mice before and after injection of adriamycin for 28 days in both the doxycycline feeding and control groups. Serum creatinine was measured by HPLC-based assay. Pregroups are before the injection of adriamycin, whereas the postgroups are 28 days after injection of adriamycin. AD, adriamycin.
Renal histologic abnormalities were scored as previously described.9 The three parameters scored included the percentage of glomeruli exhibiting collapse and segmental or global sclerosis, the percentage of total renal parenchyma occupied by tubular casts and/or microcysts, and the severity of podocyte hypertrophy and hyperplasia (graded on a semiquantitative scale from 0 to +3). For the latter parameter, podocyte hypertrophy and hyperplasia was graded as 0 (absent), 1+ (involving 1%–25% of all glomeruli), 2+ (involving 26%–50% of glomeruli), and 3+ (involving >50% of glomeruli) as described. Frozen sections were used for immunofluorescence staining for nephrin and synaptopodin and images were taken by using a Zeiss Axioplan 2 IE microscope as previously described.37

**Urinary Albumin and Creatinine**

Urinary creatinine was quantified using commercial kits from BioAssay Systems. Urine albumin was determined using a commercial assay from Bethyl Laboratory, Inc. Urine albumin excretion was expressed as the UACR.

**Coomassie Blue Staining of Urinary Proteins**

Five microliters of urine was denatured with Laemmli sample buffer and then resolved on a 10% SDS-PAGE. Proteins on the gel were detected using Coomassie blue staining.

**Isolation of Glomeruli**

Glomeruli were isolated by Fe3O4 perfusion as previously described.38 Mice were perfused with sterile HBSS containing 2.5 mg/ml Fe3O4 (Sigma-Aldrich) and 1% BSA. At the end of perfusion, kidneys were digested with 100 μg/ml of collagenase A and 1 μl/ml of DNase I in HBSS at 37°C for 30 minutes. Digested samples were washed and passed through a 100-μm cell strainer. After centrifugation, the pellets were resuspended in HBSS and glomeruli were collected using a magnet (Invitrogen). The purity of the glomeruli was verified under light microscopy and by Western blot analysis for podocyte-specific markers as described.13,39

**Quantitative Real-Time PCR**

Primers for RT-PCR were designed by using the National Center for Biotechnology Information Primer-BLAST tool (see Table 4). Gene expression was normalized to Gapdh and fold change in expression relative to the control group was calculated using the 2-ΔΔCt method. Two technical replicates per gene were used.

**Statistical Analyses**

Data are expressed as the mean±SEM. For comparison of means between three or more groups, two-way ANOVA with the Bonferroni post-test was applied. For comparisons of means between two groups, two-tailed, unpaired t tests were performed. GraphPad Prism 5 software was used for statistical analyses.

**REFERENCES**


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**DISCLOSURES**

None.


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