Severity and Frequency of Proximal Tubule Injury Determines Renal Prognosis

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ABSTRACT

AKI increases the risk of developing CKD, but the mechanisms linking AKI to CKD remain unclear. Because proximal tubule injury is the mainstay of AKI, we postulated that proximal tubule injury triggers features of CKD. We generated a novel mouse model to induce proximal tubule–specific adjustable injury by inducing the expression of diphtheria toxin (DT) receptor with variable prevalence in proximal tubules. Administration of high-dose DT in mice expressing the DT receptor consistently caused severe proximal tubule–specific injury associated with interstitial fibrosis and reduction of erythropoietin production. Mild proximal tubule injury from a single injection of low-dose DT triggered reversible fibrosis, whereas repeated mild injuries caused sustained interstitial fibrosis, inflammation, glomerulosclerosis, and atubular glomeruli. DT–induced proximal tubule–specific injury also triggered distal tubule injury. Furthermore, injured tubular cells cocultured with fibroblasts stimulated induction of extracellular matrix and inflammatory genes. These results support the existence of proximal-distal tubule crosstalk and crosstalk between tubular cells and fibroblasts. Overall, our data provide evidence that proximal tubule injury triggers several features of CKD and that the severity and frequency of proximal tubule injury determines the progression to CKD.

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AKI is a common clinical complication associated with high mortality, longer hospital stay, and high medical cost.1–4 Although AKI had been previously regarded as a benign and reversible syndrome, it is now considered to be associated with the progression of CKD and ESRD.5–8 Recent studies further revealed that severity9–11 and frequency12 of AKI are related to poor outcome. Although the clinical effect of AKI to CKD progression has been established, the molecular mechanism of AKI to CKD progression remains unclear. Previously, we showed that renal anemia and fibrosis, hallmarks of CKD, are caused by the transition of resident fibroblasts to myofibroblasts,13–15 but the trigger of this transition remains unknown. Proximal tubule is the mainstay of injury during AKI, and we hypothesized that proximal

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Figure 1. DTR (hHBegf) is selectively expressed in proximal tubules of \(N\)drg1\(^{\text{CreERT2/}}\) mice after the administration of tamoxifen. (A) Schema of \(N\)drg1\(^{\text{CreERT2/}}\) mice and iDTR mice. (B) Real-time RT-PCR analysis confirmed the high expression of hHBegf in the kidney. The expression levels were normalized to those of GAPDH and expressed relative to the expression in the brain. (C–K) Immunostaining of hHBegf in the kidney. (D and E) hHBegf was expressed in aquaporin 1 (AQP1)-positive proximal tubules. hHBegf was


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tubule injury is a dominant trigger of this transition and other features of CKD. Pathologic findings that interstitial fibrosis emerges around the injured proximal tubules\textsuperscript{16,17} also support this hypothesis.

When analyzing the role of proximal tubule injury in AKI to CKD progression, there are shortcomings with traditional AKI models, because these models simultaneously damage various types of cells.\textsuperscript{18} Recently, Grgic et al.\textsuperscript{19} developed a mouse model of selective tubule injury by using Cre-LoxP technology combined with the toxin receptor–mediated cell knockout method.\textsuperscript{20,21} By using Six2-Cre mice and Cre–inducible diphertheria toxin receptor (iDTR) mice,\textsuperscript{21} they induced the expression of diphertheria toxin receptor (DTR) on all nephron epithelial cells derived from metanephric mesenchyme, including podocytes and proximal and distal tubules.\textsuperscript{22} With an adjustment of the dose and timing of diphertheria toxin (DT) injection, preferential proximal tubule–specific injury was induced, triggering interstitial fibrosis and glomerulosclerosis. However, injury to glomerular cells and distal tubules remains a distinct possibility in that model.

In this study, to specialize the site of injury and control the severity of injury, we used a novel mouse model and showed that selective proximal tubule injury causes several features of CKD. We also clarified that the severity and frequency of proximal tubule injury underlies the degree of AKI to CKD progression.\textsuperscript{9–12} The reversibility of fibrosis after the regeneration of proximal tubules was shown with mild proximal tubule injury, and the presence of proximal-distal tubule crosstalk was confirmed. Our data strongly support the importance of protecting proximal tubules to halt AKI to CKD progression.

RESULTS

Human Heparin–Binding EGF–Like Growth Factor (DTR) Is Abundantly Expressed in the Proximal Tubules of Ndrg1\textsuperscript{CreERT2/+}:iDTR Mice

Previously, we generated Ndrg1\textsuperscript{CreERT2/+} mice in which the inducible form of Cre protein (Cre\textsuperscript{ERT2}) is abundantly expressed in proximal tubules and activated after tamoxifen injection.\textsuperscript{23} To induce proximal tubule–specific injury, we crossed an Ndrg1\textsuperscript{CreERT2/+} mouse with an iDTR mouse,\textsuperscript{21} generating Ndrg1\textsuperscript{CreERT2/+}:iDTR mice (Figure 1A). In this strain, Cre recombinase is activated by tamoxifen injection, which induces the expression of DTR, human heparin–binding EGF–like growth factor (hHBegf). High expression of hHBegf was confirmed in the kidney (Figure 1B), especially in proximal tubules (Figure 1, C–E). The recombination efficiency was nearly 100% in the S1 and S2 segments (Figure 1D), but 53.5%±8.8% in the S3 segment (Figure 1E), reflecting the expression pattern of Ndrg1.\textsuperscript{23} In total, the recombination efficiency in proximal tubules is 85.8%±1.4% (Figure 1L). hHBegf was also expressed in 12.5%±3.0% of collecting ducts (Figure 1, H, I, and L). The expression of hHBegf was rarely observed in the distal tubule or glomerulus (<1%) (Figure 1, F, G, J, and L). Expression of hHBegf was not observed before tamoxifen injection (Figure 1K).

DT Administration Induces AKI in Ndrg1\textsuperscript{CreERT2/+}:iDTR Mice

After binding to the DTR, DT inactivates elongation factor 2 (eEF2) by preventing its reattachment to ribosomes.\textsuperscript{24} DT administration induces AKI in Ndrg1\textsuperscript{CreERT2/+}:iDTR mice because after tamoxifen injection,\textsuperscript{24} Ndrg1\textsuperscript{CreERT2/+}:iDTR mice did not show any toxicity in the kidney after tamoxifen injection (Supplemental Figure 1A).

Expression of kidney injury molecule-1 (Kim-1)\textsuperscript{26} was significantly elevated (Figure 2, E and J), whereas the expression of megalin, organic anion transporter 1 (OAT1), and organic anion transporter 3 (OAT3), markers of the differentiated proximal tubule, was decreased after DT injection (Figure 2, K–M). Expression of cleaved caspase-3 was observed in 14.9%±3.0% of proximal tubules, indicating the presence of apoptosis (Figure 2, F and G). Some proximal tubules were positive for Ki-67 (Figure 2H), indicating the regenerative process. Massive glucosuria (Figure 2N) in the absence of

expressed in all epithelial cells in (D) the S1 and S2 segments but not in (E) the S3 segment of proximal tubules. (F and G) hHBegf did not colocalize with Tamm–Horsfall protein (THP) or NaCl cotransporter (NCC), markers of distal tubules. (H and I) There are a very few hHBegf–positive cells in collecting ducts colocalizing with (H) AQP2 (a marker for principal cells) or (I) V-ATPase (a marker for intercalated cells). (J) hHBegf did not colocalize with nestin (a marker for podocytes). (K) The expression of hHBegf was not detected in the kidneys of Ndrg1\textsuperscript{CreERT2/+}:iDTR mice before the administration of tamoxifen. (L) Recombination efficiencies of hHBegf in proximal tubules (PTs), distal tubules (DTs), collecting ducts (CDs), and glomeruli (G) (n=3). Scale bars, 10 μm. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
hyperglycemia (Supplemental Figure 2) also supported the functional impairment of proximal tubules. Although very few collecting ducts expressed DTR, the injury of these cells was undetectable (Supplemental Figure 3). We also analyzed the expression of hHBegf after the administration of DT and found that hHBegf was expressed in proximal tubules and a part of collecting ducts and rarely expressed in the distal tubule and glomerulus (<1%) (Supplemental Figure 4, A–G). Expression of Ndrg1 in the kidney was not activated after injury (Supplemental Figure 4, H and I).

**Proximal Tubule Injury Triggers Interstitial Fibrosis**

We next analyzed the effect of proximal tubule injury on the interstitium. Collagen 1 expression was increased after DT injection (Figure 3, A and G). To visualize collagen-producing cells, we used collagen 1a1-GFP mice, generating Ndrg1CreERT2/+:iDTR:Col1a1-GFP mice. GFP-positive fibroblasts were increased after DT injection (Figure 3B). α-Smooth muscle actin (α-SMA)–positive myofibroblasts emerged in the interstitium, and both Acta2 mRNA and the α-SMA–positive area were increased (Figure 3, C, H, and I), whereas Epo mRNA was significantly decreased (Figure 3I), bars, 10 μm. (C) PAS-stained section revealed diffuse acute tubular injury after the administration of DT. (D) Electron microscopy revealed proximal tubule injuries with loss of brush border, vacuolization, and degeneration of mitochondria and lysosome. (E and F) DT induced the expression of (E) Kim-1 and (F) cleaved caspase-3 in (G) 14.9% ± 3.0% of proximal tubules. (H) Some of the proximal tubule epithelial cells were positive for Ki-67. (I) Scoring of tubular injury (n=3–4). (J–M) Real-time RT-PCR analysis revealed (J) the induction of Kim-1 mRNA and (K–M) the reduction of megalin, organic anion transporter 1 (OAT1), and OAT3 mRNA, respectively, after the administration of DT (n=3–4). (N) Glucosuria was detected after DT administration (n=3–4). Statistical analysis by (G and I) t test and (B and J–N) ANOVA followed by Bonferroni post hoc analysis. AQP1, aquaporin 1; Ctrl, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *P<0.05; **P<0.01.

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**Figure 2.** Administration of DT induces proximal tubule injuries in Ndrg1CreERT2/+:iDTR mice. (A) We injected high-dose DT (25 ng/g) to Ndrg1CreERT2/+:iDTR mice after the administration of tamoxifen and euthanized them at various time points. (B) Serum creatinine and BUN were elevated after DT administration (n=3–5). (C–H) Histologic analysis of the kidney of Ndrg1CreERT2/+:iDTR mice 3 days after the administration of DT. Scale
indicating the transition of fibroblasts to myofibroblasts.\textsuperscript{13} α-SMA-positive myofibroblasts emerged around Kim-1- or cleaved caspase-3-positive proximal tubules (Figure 3, D and E), supporting the hypothesis that damaged proximal tubules induce the transition of fibroblasts to myofibroblasts. The number of F4/80-positive cells and the expression of Emr1, a surface marker of macrophages, were both increased (Figure 3, F and K), indicating the possibility that the infiltrating inflammatory cells contribute to the progression of interstitial fibrosis.

Reversibility of Myofibroblasts to Fibroblasts after the Regeneration of Proximal Tubules

We next injected low-dose DT (0.25 ng/g) to Ndrg1\textsuperscript{CreERT2/+}:iDTR mice to evaluate the reversibility of proximal tubule injury and interstitial fibrosis (Figure 4A). Although selective proximal tubule injury and interstitial fibrosis were observed 3 days after DT injection (Figure 4B), these changes disappeared after 5 weeks, indicating the reversibility of myofibroblasts to fibroblasts when proximal tubular injury is mild (Figure 4C). Regeneration of proximal tubules and the reversibility of fibrosis were also confirmed by the scoring of tubular injury (Figure 4D) and the quantification of the α-SMA-positive area in the interstitium (Figure 4E). Real-time RT-PCR of Kim-1, Acta2, and fibronectin (Fn) mRNA also confirmed the complete recovery from AKI and fibrosis secondary to AKI (Figure 4F).

Repeated Mild Proximal Tubule Injuries Trigger Sustained Interstitial Fibrosis

In contrast, severe sustained interstitial fibrosis was observed when the same low-dose DT (0.25 ng/g) was injected repeatedly (Figure 4, G–K), and about 70% of the mice died within 2 weeks after the last DT injection. Periodic acid–Schiff (PAS) staining and immunostaining of the kidneys of survivors revealed severe fibrosis, and an increase of α-SMA-positive myofibroblasts accompanied the infiltration of macrophages (Figure 4, H and K). Acta2 and Fn mRNA was also increased after the repeated DT injections (Figure 4I), whereas the number of F4/80-positive cells and the expression of Emr1 were increased (Figure 3, F and K), indicating the possibility that the infiltrating inflammatory cells contribute to the progression of interstitial fibrosis.
expression of Kim-1 mRNA was not significantly changed. Although most proximal tubules were completely regenerated, some proximal tubules still showed mild signs of injury (Figure 4I). These results indicate that repeated mild proximal tubule injuries are sufficient to induce severe sustained interstitial fibrosis.

Scattered Proximal Tubule Injury Does Not Induce Interstitial Fibrosis

Next, we injected various doses of tamoxifen to label proximal tubules with various prevalence of hHBegf expression (Figure 5A). The prevalence of hHBegf–positive proximal tubules increased along with the dose and frequency of tamoxifen injection (Figure 5, B and C). We injected high–dose myofibroblasts. (C) Histologic analysis 35 days after low–dose DT administration revealed the complete recovery of pathologic findings. Neither a Kim–1–positive proximal tubule nor a α-SMA–positive myofibroblast was detected. (D and E) Scoring of tubular injury and quantification of α-SMA–positive area in the interstitium indicated the regeneration of proximal tubules and the reversibility of fibrosis (n=3–4). (F) Kim–1, Acta2, and Fn mRNA were elevated at day 3 and returned to the baseline at day 35 after low–dose DT administration (n=3–8). Statistical analysis by ANOVA followed by Bonferroni post hoc analysis. (G) Ndrg1CreERT2/+;DTR mice were repeatedly injected with low-dose DT (0.25 ng/g three times) after tamoxifen treatment and euthanized 3–4 weeks after DT administration. (H) Histologic analysis after the repeated DT injections revealed severe interstitial fibrosis. Immunostaining showed the presence of Kim–1–positive proximal tubules and α-SMA–positive myofibroblasts. Massively infiltration of F4/80-positive cells was also observed after the repeated DT injections. (I) Acta2 and Fn mRNA were elevated after the repeated DT injections, whereas the elevation of Kim–1 mRNA was not significant (n=3–8). (J) Scoring of tubular injury. Some of proximal tubules had not completely recovered (n=3–4). (K) Quantification of α-SMA–positive area in the interstitium. The α-SMA–positive area was increased after the repeated DT injections (n=3–4). Statistical analysis by t test. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPF, high power field; NT, non treat. Scale bars, 10 μm. *P<0.05; **P<0.01.
Serum creatinine levels, proximal tubule injuries, Kim-1 expression in the kidney, and urinary Kim-1 increased along with the dose and frequency of tamoxifen injection (Figure 5, D–H, Supplemental Figure 5). Interestingly, Kim-1 expression in the kidney treated with the highest dose of tamoxifen was lower than that treated with the lower doses of tamoxifen (Figure 5, F and G), possibly because the proximal tubules were too severely damaged to express Kim-1. However, a-SMA–positive myofibroblasts were undetectable in the kidney treated with the lowest dose of tamoxifen (0.025 × 1) and sparsely observed with low-dose tamoxifen (0.05 × 1), whereas they were abundantly observed with high-dose tamoxifen (0.15 × 1 and 0.15 × 5) (Figure 5, F and I). Real-time RT-PCR analysis also confirmed the similar results in the expression of Acta2 and Fn mRNA (Figure 5G). These results indicate that scattered proximal tubule injury does not induce interstitial fibrosis and identify the presence of a threshold level of proximal tubule injury sufficient to induce interstitial fibrosis.

Figure 5. Scattered proximal tubule injury does not induce interstitial fibrosis. (A) Ndrg1CreERT2/+;idTR mice were pretreated with various doses of tamoxifen (0.025 mg/g × 1 day, 0.05 mg/g × 1 day, 0.15 mg/g × 1 day, and 0.15 mg/g × 5 days [the protocol of previous experiments]) and injected with high-dose DT (25 ng/g). These mice were euthanized 3 days after the administration of DT. (B) Immunostaining revealed that the prevalence of hHBegf–positive proximal tubules increases along with dose and frequency of tamoxifen administration. (C) Real-time RT-PCR also revealed that hHBegf expression increases along with dose and frequency of tamoxifen administration (n=3). (D) Serum creatinine levels after the administration of high-dose DT increase along with dose and frequency of tamoxifen administration (n=3–4). (E and H) PAS–stained kidney sections of Ndrg1CreERT2/+;idTR mice treated with DT and scoring of tubular injury revealed that the severity of proximal tubule injury increases along with dose and frequency of tamoxifen administration. (F) Immunostaining revealed that Kim-1–positive proximal tubules emerge at lower doses of tamoxifen (0.025 × 1 and 0.05 × 1) than do a-SMA–positive myofibroblasts. (G) Although Kim-1 mRNA increased in the kidney treated with low-dose tamoxifen (0.05 × 1), Acta2 and Fn mRNA did not (n=3–4). (I) Quantification of a-SMA–positive area in the interstitium. a-SMA–positive myofibroblasts increased at higher doses of tamoxifen (0.15 × 1 and 0.15 × 5). Statistical analysis by ANOVA followed by Bonferroni post hoc analysis. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPF, high power field. Scale bars, 20 μm. *P<0.05; **P<0.01.
Surprisingly, Kim-1 gene expression was not a good correlate of subsequent interstitial fibrosis.

Injured Tubular Epithelial Cells Induce the Transition of Fibroblasts by Secretory Factors
Although our in vivo data strongly suggest that proximal tubular injury induces the transition of fibroblasts, it is still unclear whether injured tubules directly trigger this transition or inflammatory cells recruited by the injured tubules do so. It is also unknown whether the crosstalk between tubules and fibroblasts is mediated by physical contact or secretory factors. To answer these questions, we cocultured renal fibroblasts (normal rat kidney 49F [NRK49F]) and tubules (NRK52E) without physical contact (Figure 6A). Col1α1 and Acta2 mRNA expression of NRK49F cells was increased when cocultured with NRK52E cells pretreated with TGFβ1 (Figure 6B). Ccl2 and IL-6 mRNA expression was also increased when cocultured with NRK52E cells pretreated with TGFβ1 (Figure 6C), indicating the acquisition of an inflammatory phenotype.27,28 These results indicate that damaged tubules might secrete factors that either directly or indirectly (via enhanced inflammatory cell recruitment) promote fibroblast transition.

Proximal Tubule Injury Leads to Distal Tubule Injury
Next, we examined whether injured proximal tubules affect distal tubules. We first confirmed the upregulation of neutrophil gelatinase–associated lipocalin (NGal) mRNA after high–dose DT injection (Figure 7A). In the healthy kidney, circulating NGal is reabsorbed by the proximal tubule, whereas in distal tubule injury, de novo expression of NGal emerges in the distal tubules.29 Immunostaining revealed the expression of NGal in distal tubules after DT injection, indicating the presence of distal tubule injuries (Figure 7B).

Next, we collected distal tubules using uterine sensitization–associated gene-1+LacZ (USAG-1+/LacZ) mice,30 in which LacZ is specifically expressed in distal tubules. We generated Ndrg1CreERT2+/iDTR:USAG-1+/LacZ mice and subjected these kidneys to FACS analysis, in which galactosidase cleaves fluorescein di-b-D-galactopyranoside (FDG) and releases a fluorescence product FITC.31 Efficient sorting of distal tubules was confirmed by high expression of distal tubule markers (USAG-1 and uromodulin) and low expression of megalin, a proximal tubule marker, in LacZ-positive cells (P3) (Figure 7C). Using this system, we showed the upregulation of NGal and osteopontin in P3 after DT injection (Figure 7D), confirming the presence of distal tubule injury.

We also found that scattered proximal tubule injury did not cause distal tubule injury (Figure 7E: the samples analyzed in Figure 5G). NGal–positive distal tubules emerged only after the high–dose tamoxifen injection (0.15×1 and 0.15×5) (Figure 7F), indicating that severe proximal tubule injury is necessary to induce distal tubule injury.
Repeated Mild Proximal Tubule Injuries Cause Glomerulosclerosis and Atubular Glomeruli

We next examined glomerular changes. After the single injection of high-dose DT (25 ng/g), PAS staining and electron microscopy analysis revealed no overt abnormalities (Figure 8, A–E). The expression of nestin, nephrin (markers of podocytes), PECA (a marker of endothelial cells) protein, and podocin and nephrin mRNA was maintained (Supplemental Figure 6, A and B). PAS staining showed very few sclerotic glomeruli. Immunostaining of α-SMA, a marker of activated mesangial cells,32 revealed very few α-SMA-positive cells within the glomeruli, except for vascular smooth muscle cells of arterioles, and the ratio of the glomeruli positive for α-SMA was <1% (Figure 8E). These results indicate that glomerulosclerosis is rarely observed after acute proximal tubule injury.

However, sclerotic glomeruli were observed after repeated injections of low-dose DT (0.25 ng/g) (Figure 8, F and G). The ratio of the glomeruli positive for α-SMA was 14.2% ± 2.4% (Figure 8H), whereas the expression of podocin and nephrin mRNA was preserved (Supplemental Figure 6C). In addition, we noticed that 41.6% ± 2.5% of glomeruli were atubular, possibly because of severe injury at glomerulotubular junctions (Figure 8I, Supplemental Figure 7). Albuminuria was not observed in this model (8.7 ± 2.8 mg/ml in the repeated low-dose DT group compared with 8.1 ± 0.9 mg/ml in the control group).

DISCUSSION

We successfully generated a novel model of selective proximal tubule injury and showed that selective proximal tubule injury can trigger several features of CKD, including fibrosis, reduced Epo production, glomerulosclerosis, atubular glomeruli, and distal tubule injury.

Our finding that selective proximal tubule injury induces the transition of fibroblasts is not only in accordance with the previous reports showing the contribution of secretory factors,33 signaling pathways,34–36 and cellular status of tubules37

Figure 7. Proximal tubule injury triggers distal tubule injury. (A) Ngal mRNA was increased after the administration of high-dose DT (25 ng/g). (B) Ngal emerged in the distal tubule stained by Tamm–Horsfall protein (THP) and NaCl cotransporter (NCC) after DT administration. Ngal staining in proximal tubules indicates the reabsorption from the urine. (C) Analysis of LacZ-positive cells in Ndrg1CreERT2CreERT2; USAG-1+/LacZ mice. The expression of USAG-1 (Sostdc1) and uromodulin mRNA, distal tubule markers, was high, whereas the expression of megalin mRNA, a proximal tubule marker, was low in LacZ-positive cells (P3). (D) Analysis of LacZ-positive cells in Ndrg1CreERT2CreERT2; USAG-1+/LacZ mice after the administration of DT. Ngal and osteopontin mRNA, markers of tubule injury, was increased in LacZ-positive distal tubule cells after DT administration. (E) Expression of Ngal mRNA after the administration of various doses and frequencies of tamoxifen and high-dose DT (25 ng/g). Ngal mRNA was increased only when treated with high-dose tamoxifen (n=3–4). Samples used in Figure 5G were analyzed in this experiment. (F) Immunostaining revealed that Ngal–positive distal tubules emerged when treated with high-dose tamoxifen (arrows). Statistical analysis by ANOVA followed by Bonferroni post hoc analysis. Ctrl, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Scale bars, 10 μm. *P<0.05; **P<0.01.
but also, provides the direct evidence that selective proximal tubule injury causes fibrosis. Our in vitro coculture data indicate that secretory factors from damaged tubules directly induce the transition of fibroblasts, whereas the involvements of other factors, such as the infiltration of inflammatory cells, cannot be excluded in vivo.

We also showed that fibrosis is reversible when proximal tubule injury is mild. Although the reversibility of fibrosis has been shown by the administration of tamoxifen,\textsuperscript{13} and other renoprotective reagents, this is the first report showing the spontaneous reversal of fibrosis secondary to tubular injury without additional treatment. On the other hand, we showed that repeated mild proximal tubule injuries can cause sustained interstitial fibrosis. In the case of the repeated proximal tubule injuries, proximal tubules cannot completely recover and remain in a dedifferentiated state, which might release secretory factors inducing sustained fibrosis. These observations are supported by the clinical observation that the frequency of AKI is related to the progression to CKD.\textsuperscript{12}

We also revealed the existence of a threshold level of proximal tubule injury sufficient to induce interstitial fibrosis and distal tubule injury. This might explain the clinical findings that the magnitude of serum creatinine increase during AKI is associated with the progression to CKD.\textsuperscript{9,10} That is, the worse the AKI, the worse the prognosis will be for CKD progression, even after recovery.

We also showed that repeated proximal tubule injuries cause glomerulosclerosis. This is in accordance with the previous report using Six2-cre:iDTR mice.\textsuperscript{19} In Six2-cre:iDTR mice, DTR can be expressed on podocytes, because Six2 is expressed in the progenitor cells of the nephron.\textsuperscript{22} We confirmed the absence of DTR on podocytes in Ndrg1CreERT2/+:iDTR mice, thus completely excluding the possibility of subclinical podocyte injury. Therefore, we can safely conclude that selective proximal tubule injury causes glomerulosclerosis. There are several explanations for this phenomenon. First, recruited inflammatory cells might contribute to the glomerulosclerosis. Second, DTR is also expressed in parietal epithelial cells (PECs), reflecting a high expression of Ndrg1 in PECs,\textsuperscript{23} and the injury of PECs may affect the neighboring cells in the glomeruli. Third, the crosstalk between tubules and the glomerulus could be postulated.\textsuperscript{38,39} Glomerulosclerosis secondary to proximal tubule injuries is also observed in clinical situations. Mesoamerican nephropathy\textsuperscript{40} represents interstitial fibrosis and glomerulosclerosis after repeated episodes of AKI.

We also showed a high prevalence of atubular glomeruli after repeated proximal tubule injuries. Atubular glomeruli are commonly observed in various types of CKD,\textsuperscript{41–44} and also

![Figure 8](image-url)
reported in animal models of tubular injury.\textsuperscript{45,46} Our data indicate that selective proximal tubule injury is sufficient to cause atubular glomeruli.

The crosstalk between proximal tubules and distal tubules was also confirmed. Mice deficient of Tamm–Horsfall protein, a glycoprotein expressed in distal tubules, are reported to be sensitive to proximal tubule injury,\textsuperscript{47} indicating that the molecule from distal tubules protects proximal tubules. There are several possible mechanisms of this crosstalk, such as obstruction of the distal nephron by detached proximal tubule cells, molecules released by the injured proximal tubules, and decreased peritubular capillary flow after proximal tubule injury.

To analyze the cause of death after repeated DT injections, we analyzed multiple tissues of the mice 1 week after the last DT injection. We were unable to find any abnormalities in the vital tissues, such as the heart, lung, or liver, of these mice. Pleural fluid, ascites, abscess, or other signs of infection were not observed. However, severe demyelination in sciatic nerves (Supplemental Figure 8B) was observed together with the limping phenotype, because Ndrg1 and DTR were also expressed in peripheral nerves\textsuperscript{48} (Supplemental Figure 8A). Although it is hard to pinpoint the exact cause of death, we believe that malnutrition caused by leg demyelination could be a contributing factor and might affect the renal function.

In conclusion, we clarified that selective proximal tubule injuries cause several features of CKD. We have also shown that the severity and frequency of AKI are associated with the progression to CKD. Previously, we have proved the limitation of proximal tubule repair capacity by showing the shortening of the proximal tubule after repair.\textsuperscript{23} On the basis of the findings in this manuscript, it seems that severe and frequent proximal tubule injuries and the inadequate repair are likely the key cellular mechanisms of AKI to CKD progression.

Novel findings in this work raised the possibility that fibrosis after AKI is secondary to tubular injury and that the treatment of fibrosis itself is not sufficient to halt the AKI to CKD progression. In addition to the antifibrotic approach, there is an urgent need to develop therapeutic strategies to protect proximal tubules from repeated injury and restore healthy proximal tubular function.

\textbf{CONCISE METHODS}

\textbf{Animals} \textit{Ndrg1}\textsuperscript{floxRT2+/−} mice were generated in our laboratory.\textsuperscript{23} iDTR mice were purchased from The Jackson Laboratory\textsuperscript{21} (Bar Harbor, ME). \textit{Col1a1-GFP} mice,\textsuperscript{27} a transgenic mouse strain expressing enhanced GFP under the regulation of the \textit{col1a1} promoter and enhancers, were a gift from Prof. Jeremy Duffield. \textit{USAG-1\textsuperscript{17}LacZ} mice were described previously.\textsuperscript{30} Serum and urine creatinine, serum BUN, serum and urine glucose, and urinary albumin were measured by the creatininase-HMMPS method, the urase/glutamate dehydrogenase method, the hexokinase-glucose-6-phosphate dehydrogenase method, and ELISAs (Albuwell; Exocell Inc., Philadelphia, PA), respectively. Urinary Kim-1 was measured by the Quantikine Mouse TIM-1 Immunoassay (R&D Systems, Minneapolis, MN).

\textbf{Administration of Tamoxifen} To obtain maximum recombination in proximal tubules, we administered 0.15 mg/g body wt tamoxifen (Sigma-Aldrich, St. Louis, MO) to mice by intraperitoneal injection for 5 consecutive days as described previously.\textsuperscript{24} To obtain mosaic recombination, we administered 0.025, 0.05, and 0.15 mg/g body wt tamoxifen by intraperitoneal injection for 1 day.

\textbf{Administration of DT} DT was prepared as described previously.\textsuperscript{20} Appropriate aliquots of DT solution were administered to mice by intraperitoneal injection.

\textbf{Histologic Studies and Immunostaining} The histologic studies were performed as described previously.\textsuperscript{30,49,50} Primary antibodies against the following proteins were used for immunostaining: HbBegf (R&D Systems), aquaporin 1 (Abcam, Inc., Cambridge, MA), Tamm–Horsfall protein (Biomedical Technologies Inc., Stoughton, MA), thiazide-sensitive NaCl cotransporter (Chemicon, Temecula, CA), aquaporin 2 (Calbiochem, San Diego, CA), V-ATPase B1/2 (Santa Cruz Biotechnology, Santa Cruz, CA), nestin (Abcam, Inc.), Tim-1/Kim-1 (ebioscience, San Diego, CA), Ngal (R&D Systems), cleaved caspase-3 (Cell Signaling Technology, Danvers, MA), Ki-67 (Vision Biosystems, Norwell, MA), collagen type 1 (Rockland Immunocchemicals Inc., Gilbertsville, PA), PDGFRB (ebioscience), GFP (Abcam, Inc.), F4/80 (Molecular Probes, Eugene, OR), nephrin (R&D Systems), \textit{α–SMA} (Sigma-Aldrich), and PECAM (BD Biosciences, San Jose, CA). Alexa-conjugated phallolidin (Molecular Probes) was used to visualize the brush borders of the proximal tubules. Staining was analyzed with a confocal microscope (FV1000D; Olympus, Tokyo, Japan).

\textbf{Quantitative Assessment of Labeling Efficiency} The labeling efficiency (Figure 1L, Supplemental Figure 4, E–G) was assessed by counting the number of segment marker–positive cells and hHBegf marker/segment marker double–positive cells in four cortical fields and two corticomedullary fields randomly selected at \( \times 200 \) magnification in each kidney as described previously.\textsuperscript{23} Three mice were examined at each time point.

\textbf{Quantitative Assessment of Tubular Injury, Fibrosis, and Atubular Glomeruli} Tubular injury was scored semiquantitatively by a blinded pathologist who examined \( \geq 10 \) cortical fields (\( \times 200 \) magnification) of PAS-stained sections (\( n=3–4 \)) as previously described.\textsuperscript{51,52} Tubular injury was defined as tubular dilation, tubular atrophy, tubular cast formation, vacuolization, degeneration, and sloughing off of tubular epithelial cells or loss of the brush border and thickening of the tubular basement membrane. The tubules were evaluated according to the following scoring system: 0= no tubular injury; 1=10%–15% tubules injured; 2=11%–25% tubules injured; 3=26%–50% tubules injured; 4=51%–74% tubules injured; and 5=75% tubules injured.
Quantification of fibrosis was performed by measuring the α-SMA–positive area in the interstitium. Eight images of each kidney section at cortical fields (×200 magnification) were taken at random (n=3). All images were obtained using the same laser power and gain intensity with a confocal microscope (FV1000D; Olympus). α-SMA–positive areas, except for vascular smooth muscle cells, were automatically calculated by MetaMorph software (Universal Imaging Corporation, Downingtown, PA).34,35

The prevalence of atubular glomeruli in the kidneys of Ndrgt1CreERT2–DTIR mice after repeated injections of low-dose DT (n=3) and PBS (n=3) was evaluated as described previously.23,34–36 Paraffin-embedded kidneys were sectioned at 4-µm intervals for 27 slices for each sample, and the glomeruli in the 14th section were analyzed for continuity with proximal tubules by the consecutive sections. Percentages of atubular glomeruli, which lack any connection to proximal tubules, were determined in each sample. Samples were analyzed with a fluorescence microscope (BZ-X710; KEYENCE, Osaka, Japan).

**Flow Cytometric Analyses**

Flow cytometric analysis was performed using BD FACSaria. Mice were anesthetized and then, perfused with PBS. Kidneys were harvested, and the tissue was minced with a razor blade and digested by 1 mg/ml collagenase A and 0.1 mg/ml DNase type 1 as previously described.57 The cell suspensions were filtered through a cell strainer to remove debris. Fluorescein di-β-D-galactopyranoside (Sigma-Aldrich) was used to distinguish LacZ–positive cells as described previously.31

**Coculture of NRK52E and NRK49F Cells**

NRK-52E and NRK49F cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured with DMEM/5%FCS. Transwell (Corning, Corning, NY) was used for coculture experiments. NRK52E cells were seeded on the surface plates (day 0), and TGFβ1 (10 ng/ml; R&D Systems) was administrated (day 1). NRK49F cells were seeded on the Transwell inserts in a different well (day 2) and then, transferred to the well containing NRK52E cells (day 3) (Figure 6). Before coculture, the surface plates with NRK52E cells were vigorously washed with PBS three times to avoid the transfer of TGFβ1 to NRK49F cells. On day 4, the RNA of NRK49F cells was harvested and subjected to real–time RT-PCR.

**Quantification of mRNA by Real–Time RT-PCR**

Real–time RT-PCR was performed as described previously.50 Specific primers were designed using Primer Express software (Applied Biosystems, Foster City, CA). Serially diluted cDNA was used to generate the standard curves for each primer. The sequences of the primers used for RT-PCR are listed in Supplemental Table 1.

**Statistical Analyses**

Data were presented as means±SDs. To analyze the difference between two groups, t test was used. ANOVA followed by Bonferroni post hoc analysis was used to determine the differences between groups of three or more. Statistical tests were one sided. A P value <0.05 was considered statistically significant.

**REFERENCES**


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