

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 severity^{9–11} 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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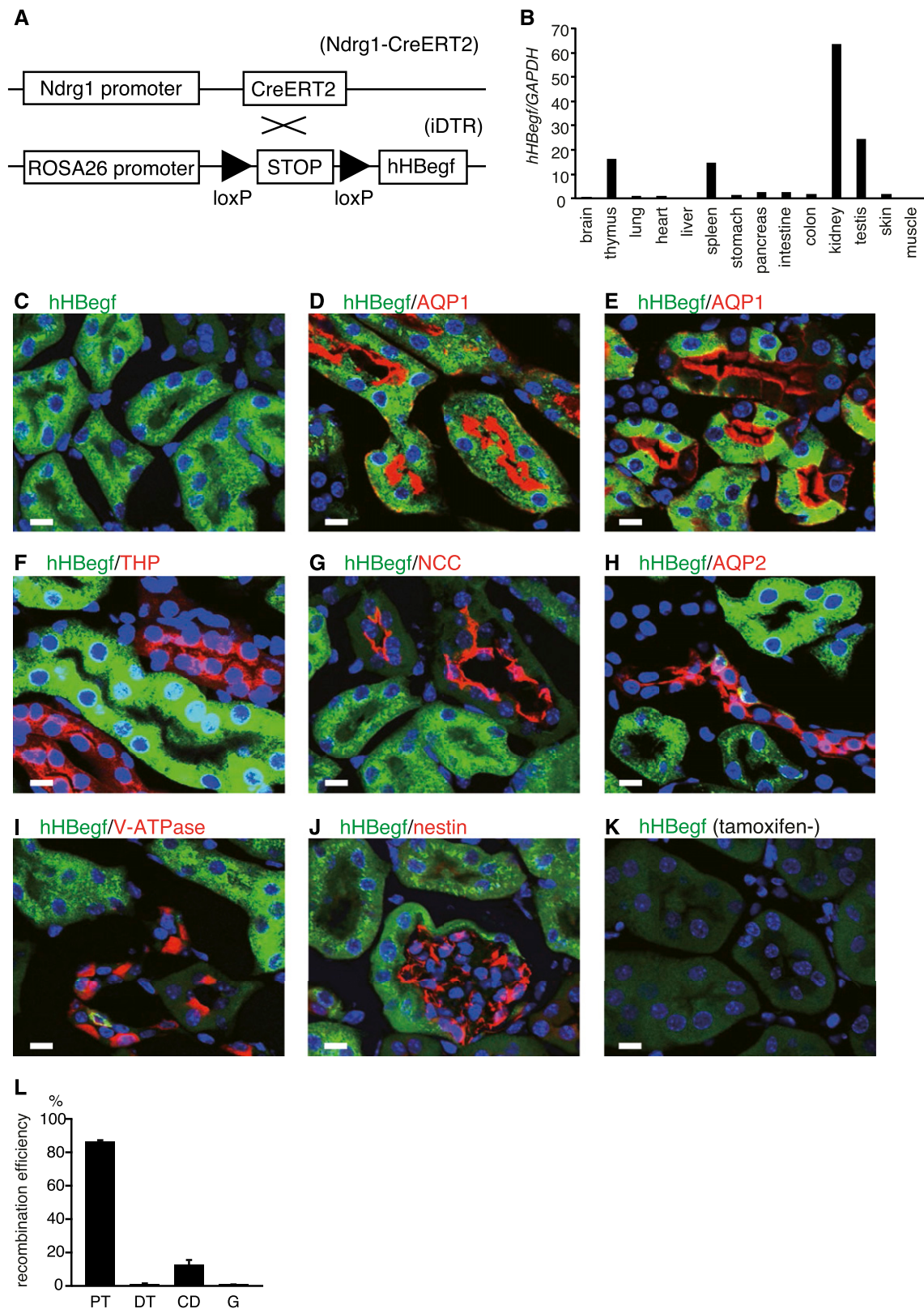


Figure 1. DTR (hHBegf) is selectively expressed in proximal tubules of *Ndr1*^{CreERT2/+};iDTR mice after the administration of tamoxifen. (A) Schema of *Ndr1*^{CreERT2/+} mice and iDTR mice. (B) Real-time RT-PCR analysis confirmed the high expression of *hHBegf* in the kidney. The expression levels 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

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^{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.¹⁸ Recently, Grgic *et al.*¹⁹ developed a mouse model of selective tubule injury by using Cre-LoxP technology combined with the toxin receptor-mediated cell knockout method.^{20,21} By using *Six2-Cre* mice and Cre-inducible diphtheria toxin receptor (iDTR) mice,²¹ they induced the expression of diphtheria toxin receptor (DTR) on all nephron epithelial cells derived from metanephric mesenchyme, including podocytes and proximal and distal tubules.²² With an adjustment of the dose and timing of diphtheria 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.^{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 *Ndr1^{CreERT2/+};iDTR* Mice

Previously, we generated *Ndr1^{CreERT2/+}* mice in which the inducible form of Cre protein (Cre^{ERT2}) is abundantly expressed in proximal tubules and activated after tamoxifen injection.²³ To induce proximal tubule-specific injury, we crossed an *Ndr1^{CreERT2/+}* mouse with an iDTR mouse,²¹ generating *Ndr1^{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 $53.5\% \pm 8.8\%$ in the S3 segment (Figure 1E), reflecting the expression pattern of *Ndr1*.²³ In total, the recombination efficiency in proximal tubules is $85.8\% \pm 1.4\%$ (Figure 1L). hHBegf was also expressed in $12.5\% \pm 3.0\%$ 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). Although we previously reported the hematologic toxicity in *R26^{CreERT2/+}* mice after tamoxifen injection,²⁴ *Ndr1^{CreERT2/+};iDTR* mice did not show any toxicity in the kidney after tamoxifen injection (Supplemental Figure 1A).

DT Administration Induces AKI in *Ndr1^{CreERT2/+};iDTR* Mice

After binding to the DTR, DT inactivates elongation factor 2 and causes the termination of protein synthesis of the target cell.^{20,25} To exclude the interference of the renoprotective effects of tamoxifen,¹³ DT injection was delayed until 2 weeks after the last tamoxifen injection (Figure 2A). We injected various doses of DT to *Ndr1^{CreERT2/+};iDTR* mice and chose 25 ng/g DT to induce severe injury. *Ndr1^{CreERT2/+};iDTR* mice exposed to DT showed AKI within 1 week after the injection (Figure 2B). Proximal tubules were detached from the basement membrane, and cellular debris was accumulated in the lumen (Figure 2, C and I). Electron microscopy showed severe injury of the proximal tubules with vacuolization, swelling of the cytoplasm, and loss of brush border (Figure 2D). AKI was not observed when *Ndr1^{CreERT2/+};iDTR* mice were not pretreated with tamoxifen (Supplemental Figure 1B), indicating that DT is not toxic unless it binds to its receptor.

Expression of kidney injury molecule-1 (Kim-1)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 $14.9\% \pm 3.0\%$ 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 *Ndr1^{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 μ m. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

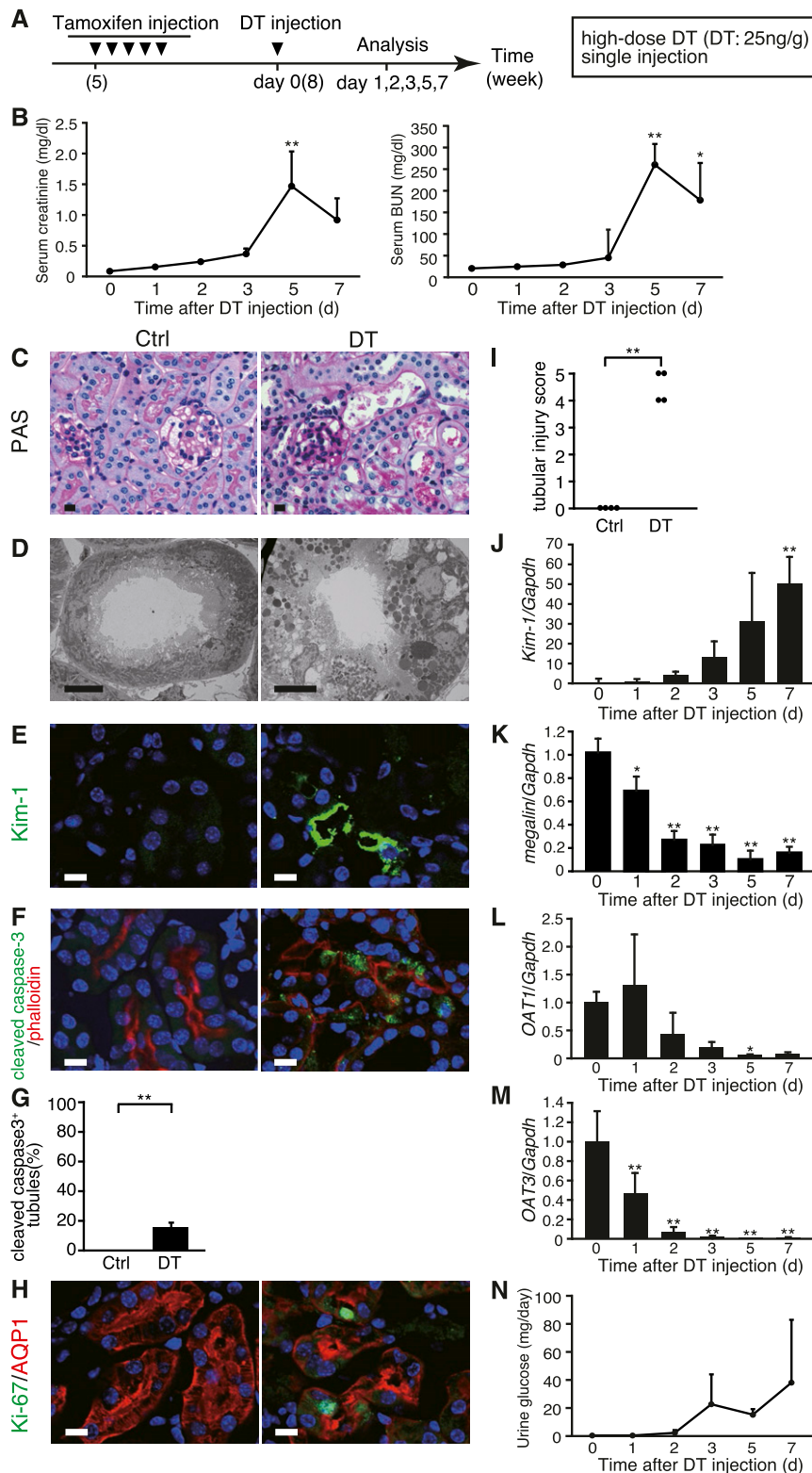


Figure 2. Administration of DT induces proximal tubule injuries in *Ndr1^{CreERT2/+};iDTR* mice. (A) We injected high-dose DT (25 ng/g) to *Ndr1^{CreERT2/+};iDTR* mice after the administration of tamoxifen and euthanized them at various time points. (B) Serum creatinine and BUN were elevated after DT administration ($n=3-5$). (C-H) Histologic analysis of the kidney of *Ndr1^{CreERT2/+};iDTR* mice 3 days after the administration of DT. Scale

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 *Ndr1* 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,²⁷ generating *Ndr1^{CreERT2/+};iDTR:Colla1-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 3J),

bars, 10 μ m. (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% $\pm$ 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 *megalyn*, *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. * $P<0.05$; ** $P<0.01$.

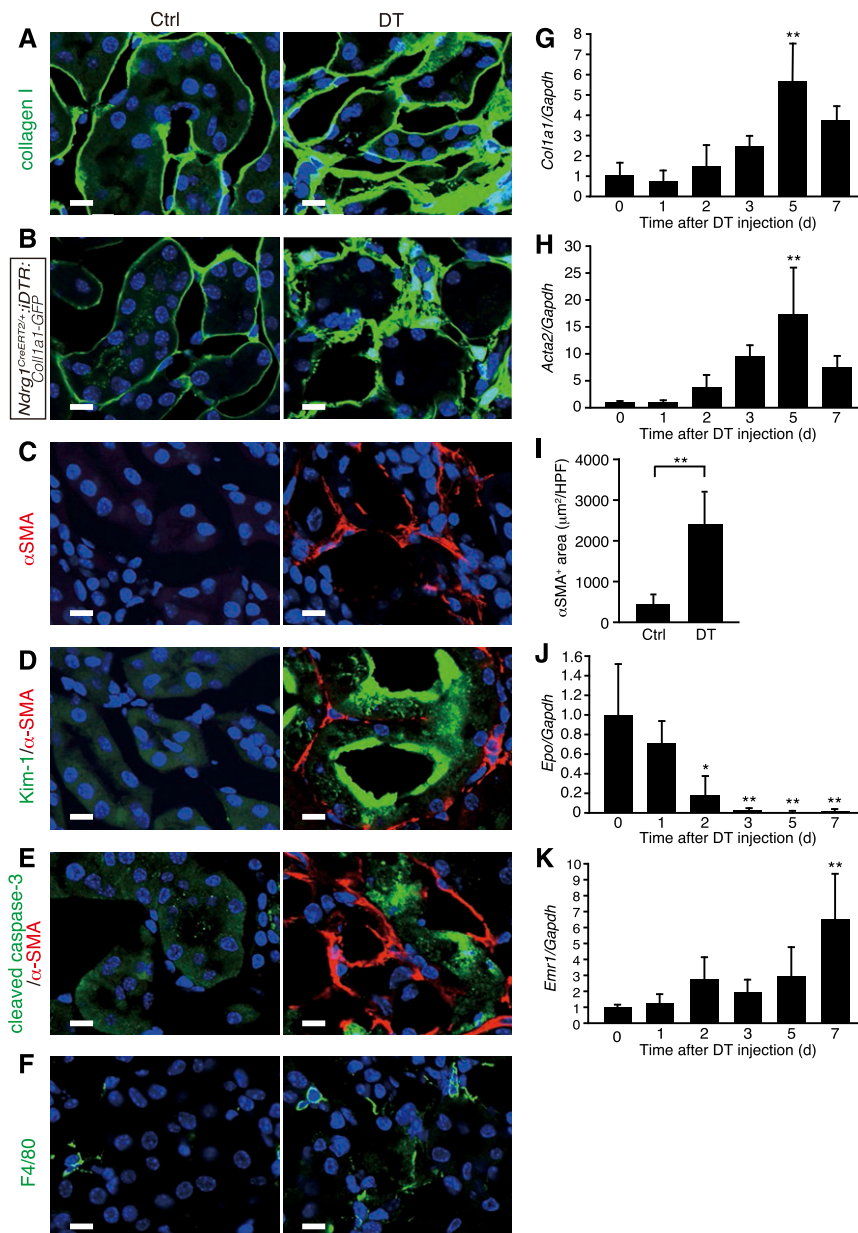


Figure 3. Administration of DT induces interstitial fibrosis in *Ndr1^{CreERT2/+};iDTR* mice. (A–F) Immunostaining of the kidney of *Ndr1^{CreERT2/+};iDTR* mice 3 days after the administration of high-dose DT. (A) Immunostaining revealed the increased expression of collagen 1 after the administration of DT. (B) GFP-positive collagen-producing fibroblasts were increased in the interstitium of *Ndr1^{CreERT2/+};iDTR:Coll1a1-GFP* mice. (C) DT administration increased the expression of α -SMA in the interstitium. (D and E) α -SMA-positive myofibroblasts emerged around (D) Kim-1 or (E) cleaved caspase-3-positive proximal tubules. (F) DT administration increased the number of F4/80-positive cells. Scale bars, 10 μ m. (G, H, J, and K) Real-time RT-PCR analysis of the kidney after the administration of DT. (G) *Col1a1* and (H) *Acta2* mRNA were elevated after DT administration, whereas (J) *Epo* mRNA was significantly decreased. (K) Expression of *Emr1* mRNA was increased after DT administration ($n=3-4$). (L) Quantification of α -SMA-positive area in the interstitium ($n=3-4$). Statistical analysis by (L) *t* test and (G, H, J, and K) ANOVA followed by Bonferroni *post hoc* analysis. Ctrl, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPF, high power field. * $P<0.05$; ** $P<0.01$.

indicating the transition of fibroblasts to myofibroblasts.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 *Ndr1^{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

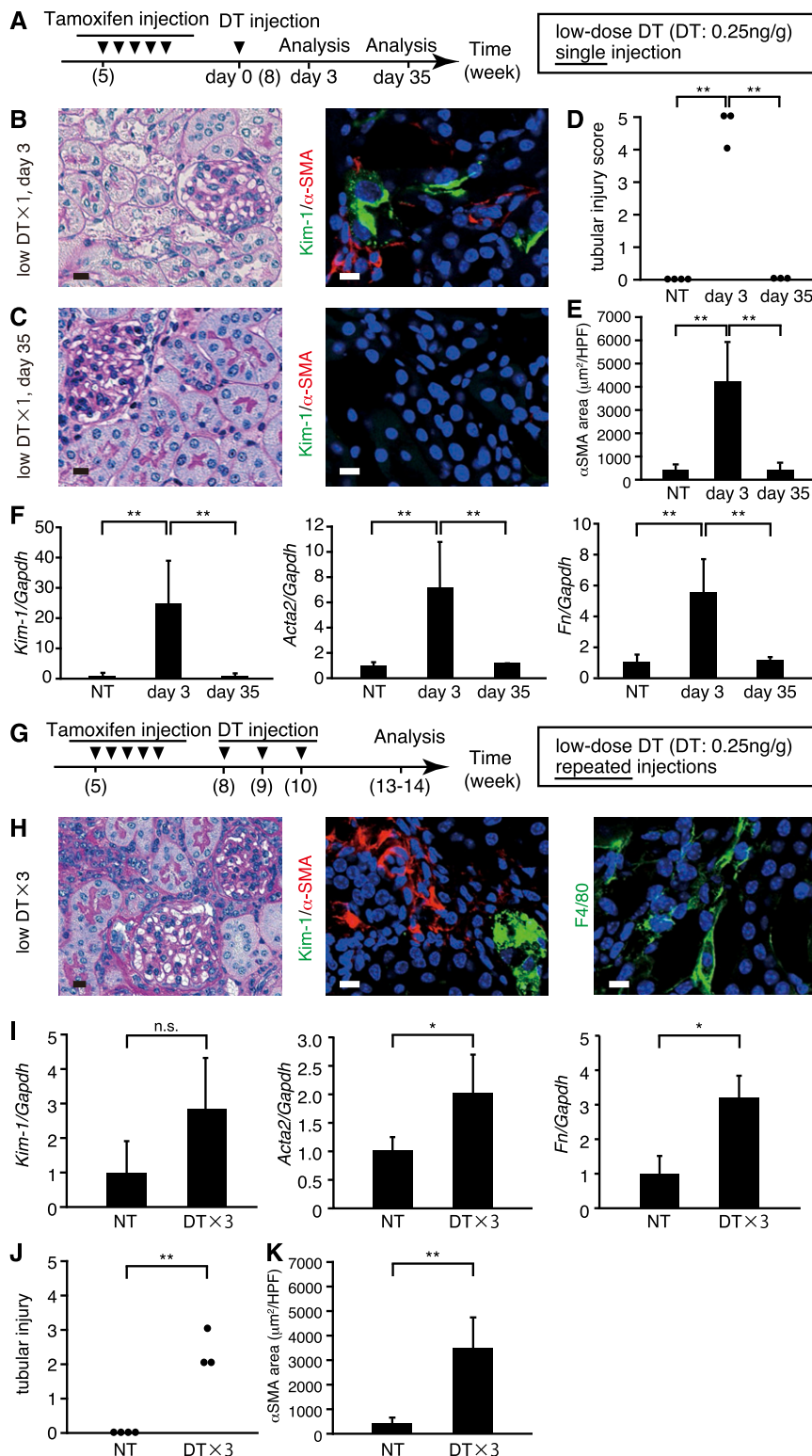


Figure 4. Mild proximal tubule injury triggers reversible fibrosis, whereas repeated mild injuries cause sustained interstitial fibrosis. (A) *Ndrp1^{CreERT2/+};iDTR* mice were injected with low-dose DT (0.25 ng/g) after tamoxifen treatment and euthanized 3 and 35 days after the administration. (B) Histologic analysis of *Ndrp1^{CreERT2/+};iDTR* mice 3 days after the administration of low-dose DT. Low-dose DT induced acute proximal tubular injury and the emergence of Kim-1-positive proximal tubules and α-SMA-positive

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 4J). 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) *Ndrp1^{CreERT2/+};iDTR* 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. Massive 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. * $P<0.05$; ** $P<0.01$.

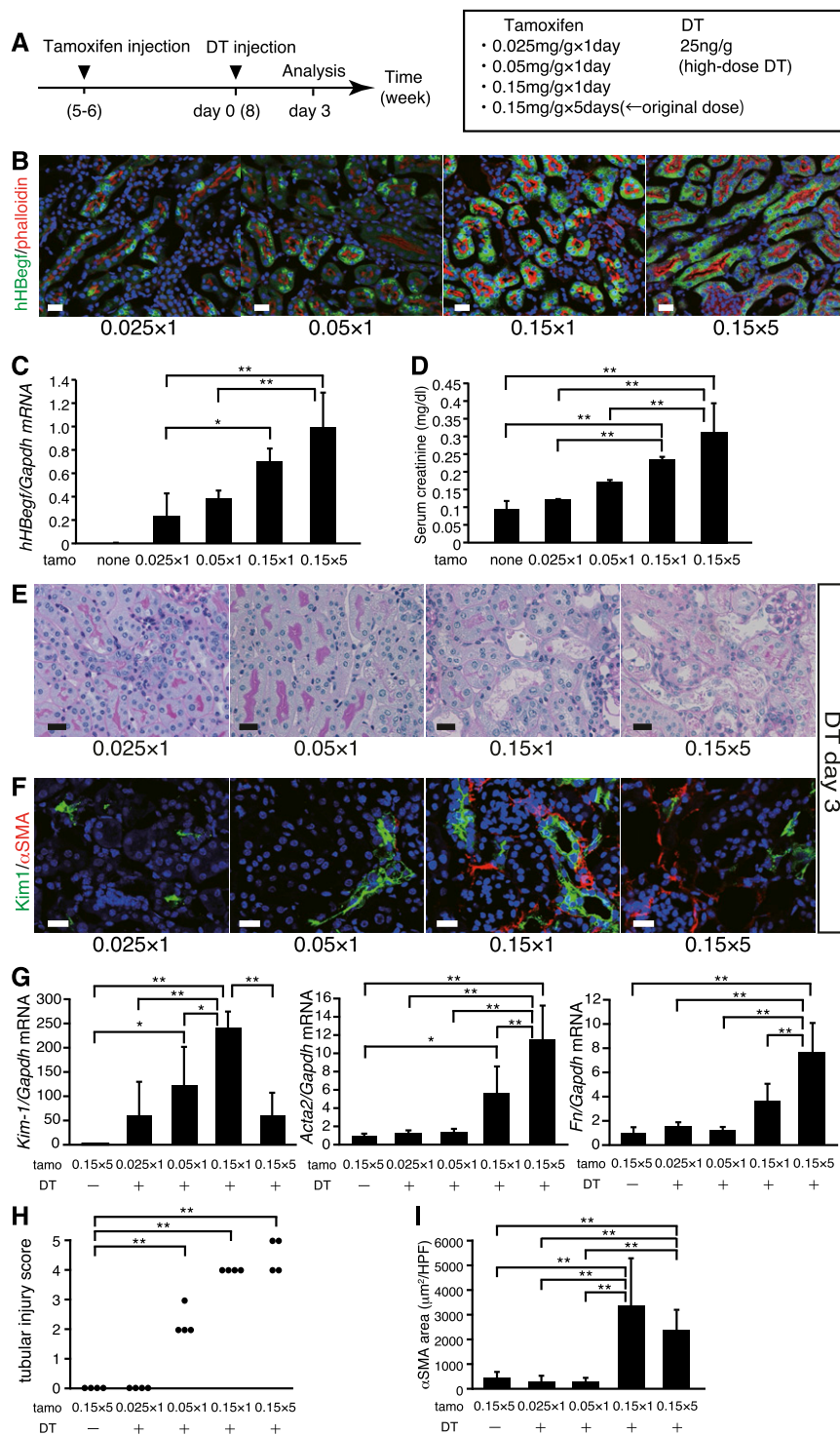


Figure 5. Scattered proximal tubule injury does not induce interstitial fibrosis. (A) *Ndr1^{CreERT2/+};iDTR* mice were pretreated with various doses of tamoxifen (0.025 mg/g × 1 day, 0.05 mg/g × 1 day, 0.15 mg/g × 1 day, and 0.15 mg/g × 5 days [the protocol of previous experiments]) and injected with 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

25 ng/g DT (the dose shown in Figures 1–3) to *Ndr1^{CreERT2/+};iDTR* mice to induce severe injury in the proximal tubules with DTR.

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, α -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.

DT increase along with dose and frequency of tamoxifen administration ($n=3-4$). (E and H) PAS-stained kidney sections of *Ndr1^{CreERT2/+};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 α -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 α -SMA-positive area in the interstitium. α -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 μ m. * $P<0.05$; ** $P<0.01$.

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). *Col1a1* and *Acta2* mRNA expression of NRK49F cells was increased when cocultured with NRK52E cells pretreated with TGF β 1 (Figure 6B). *Ccl2* and *IL-6* mRNA expression was also increased when cocultured with NRK52E cells pretreated with TGF β 1 (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.

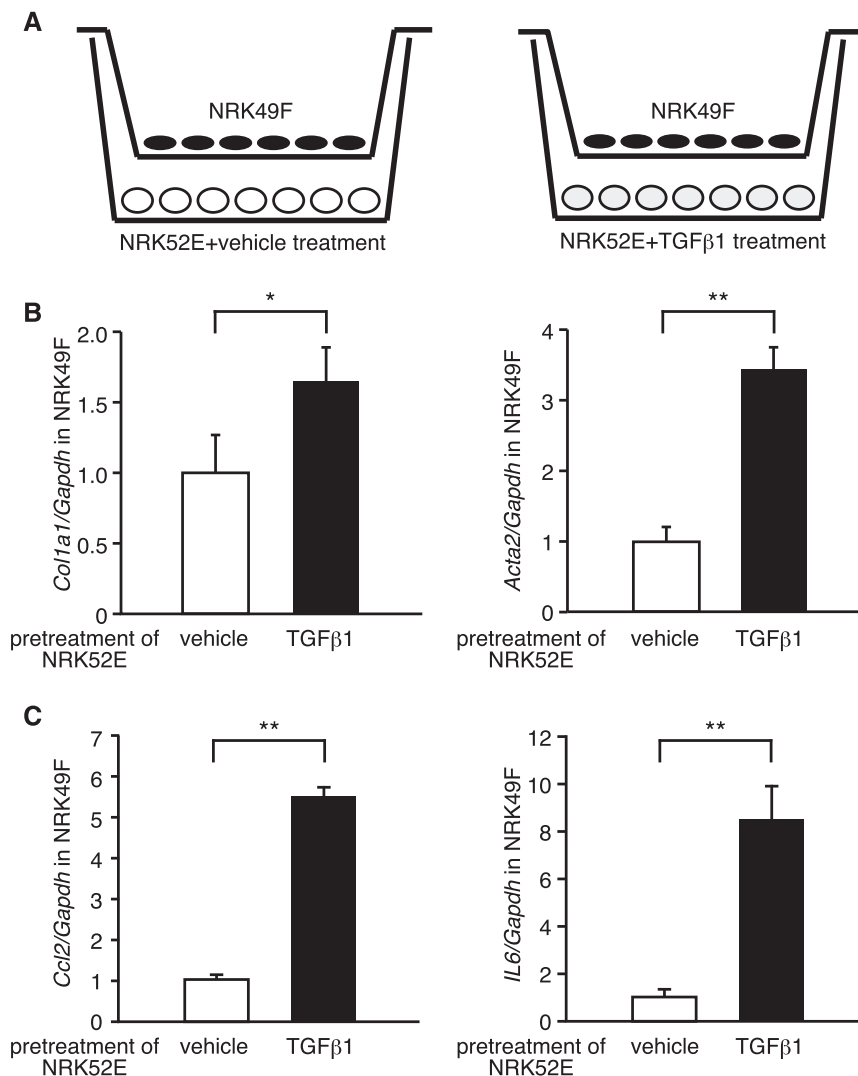


Figure 6. Fibroblasts cocultured with injured tubular epithelial cells express higher levels of collagen, α -SMA, and inflammatory genes. (A) Schema of coculture experiment. Renal fibroblasts (NRK49F) were cocultured with tubular epithelial cells (NRK52E) pretreated with vehicle or TGF β 1 (10 ng/ml). NRK52E cells were vigorously rinsed three times before coculture. (B) *Col1a1* and *Acta2* mRNA of NRK49F was increased when cocultured with NRK52E cells pretreated with TGF β 1. (C) *Ccl2* and *IL-6* mRNA of NRK49F was increased when cocultured with NRK52E cells pretreated with TGF β 1 ($n=4$). Statistical analysis by *t* test. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. * $P<0.05$; ** $P<0.01$.

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 *Ndr1*^{CreERT2/+}; *iDTR:USAG-1*^{+/LacZ} mice and subjected these kidneys to FACS analysis, in which galactosidase cleaves fluorescein *di-β-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.

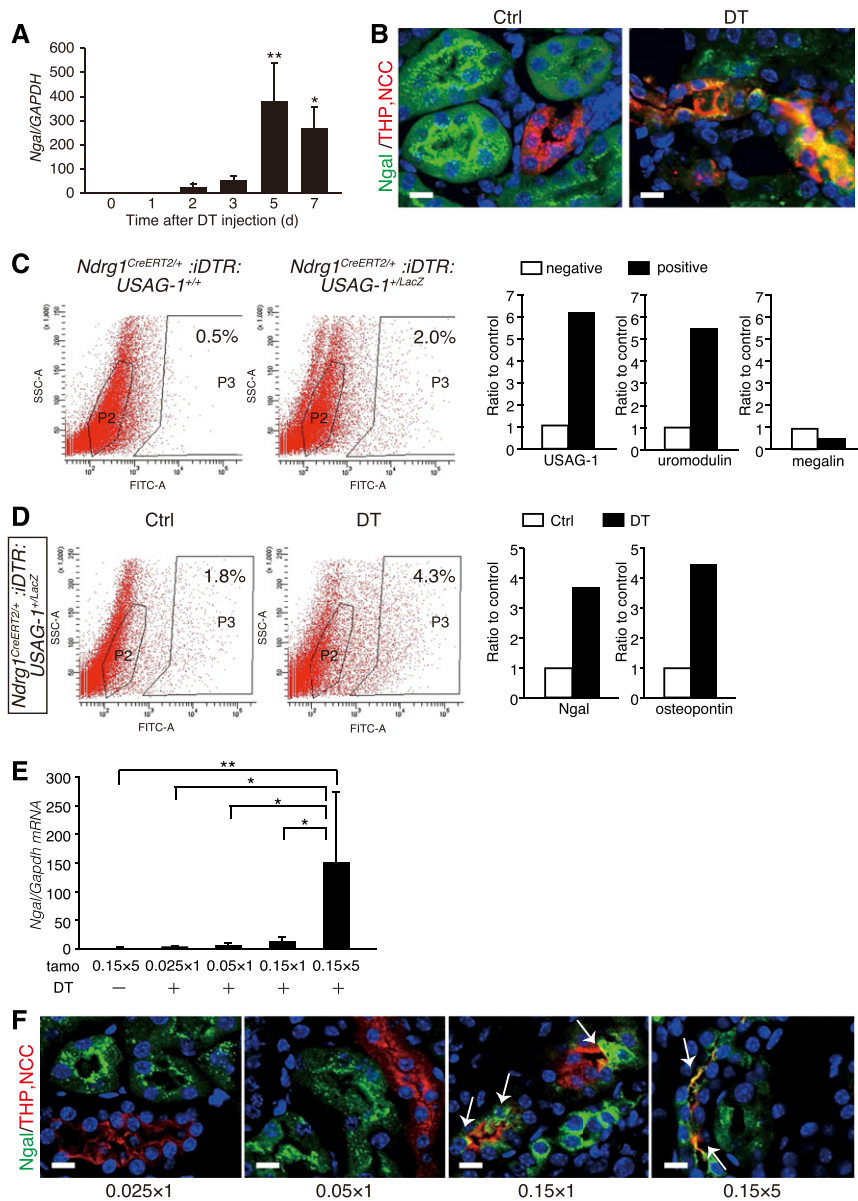


Figure 7. Proximal tubule injury triggers distal tubule injury. (A) *Ngal* mRNA was increased after the administration of high-dose DT (25 ng/g). (B) *Ngal* emerged in the distal tubule stained by Tamm–Horsfall protein (THP) and NaCl cotransporter (NCC) after DT administration. *Ngal* staining in proximal tubules indicates the reabsorption from the urine. (C) Analysis of LacZ-positive cells in *Ndr1^{CreERT2/+};iDTR:USAG-1^{+/LacZ}* mice. The expression of *USAG-1* (*Sostdc1*) and *uromodulin* mRNA, distal tubule markers, was high, whereas the expression of *megalyn* mRNA, a proximal tubule marker, was low in LacZ-positive cells (P3). (D) Analysis of LacZ-positive cells in *Ndr1^{CreERT2/+};iDTR: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 μ m. * $P<0.05$; ** $P<0.01$.

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), PECAM (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 $14.2\% \pm 2.4\%$ (Figure 8H), whereas the expression of *podocin* and *nephrin* mRNA was preserved (Supplemental Figure 6C). In addition, we noticed that $41.6\% \pm 2.5\%$ of glomeruli were atubular, possibly because of severe injury at glomerulotubular junctions (Figure 8I, Supplemental Figure 7). Albuminuria was not observed in this model ($8.7 \pm 2.8 \mu\text{g/ml}$ in the repeated low-dose DT group compared with $8.1 \pm 0.9 \mu\text{g/ml}$ 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

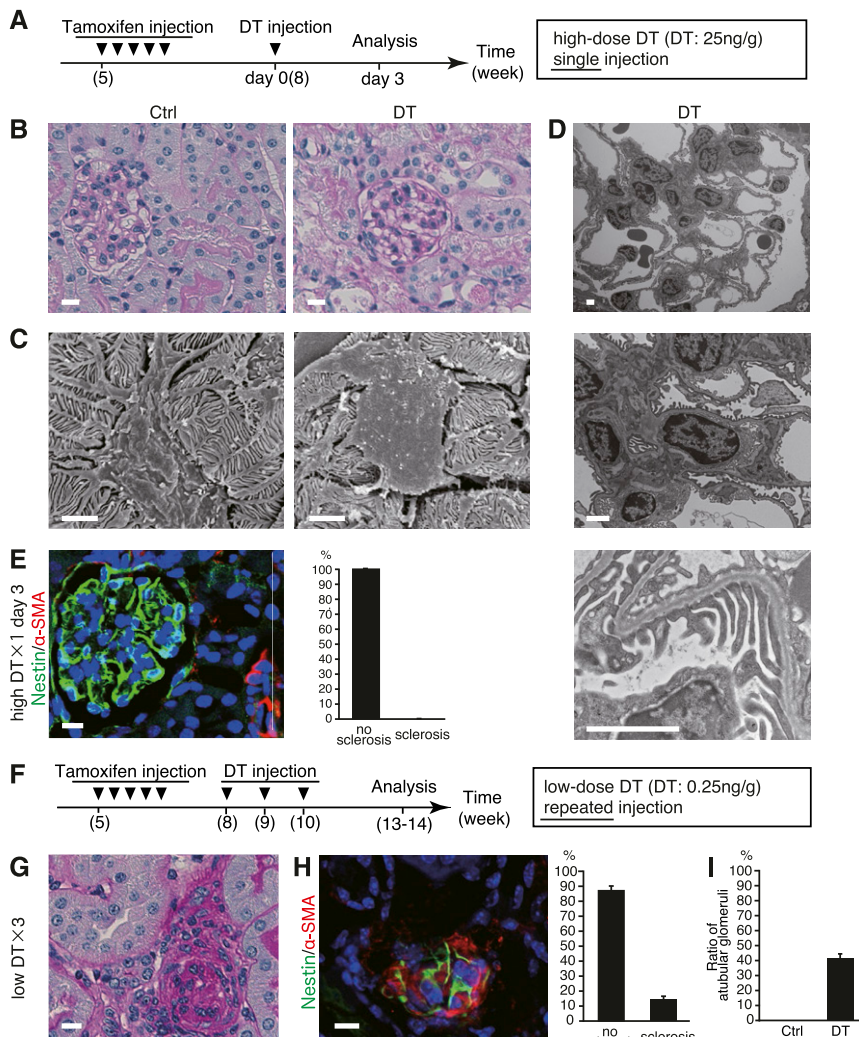


Figure 8. Repeated mild tubular injuries cause glomerulosclerosis and atubular glomeruli. (A) *Ndr* $g1^{CreERT2/+};iDTR$ mice were injected with high-dose DT (25 ng/g) after the administration of tamoxifen and euthanized 3 days after DT administration. (B) PAS-stained kidney sections of *Ndr* $g1^{CreERT2/+};iDTR$ mice revealed that the glomeruli were structurally maintained. (C and D) Analysis by (C) scanning electron microscopy and (D) transmission electron microscopy showed that podocytes maintained their structure. (E) Almost all glomeruli were negative for α -SMA. (F) Next, *Ndr* $g1^{CreERT2/+};iDTR$ mice were repeatedly injected with low-dose DT (0.25 ng/g) after the administration of tamoxifen and euthanized 3–4 weeks after the last DT administration. (G) Sclerotic glomeruli were observed after repeated DT administration. (H) About 14% of glomeruli were positive for α -SMA, and (I) approximately 42% of glomeruli were atubular ($n=3$). Ctrl, control. Scale bars, 10 μ m in B, E, G, and H; 2 μ m in C and D.

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,¹³

dexamethasone,^{13,28} 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.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.^{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 caused glomerulosclerosis. This is in accordance with the previous report using *Six2-cre;iDTR* mice.¹⁹ In *Six2-cre;iDTR* mice, DTR can be expressed on podocytes, because *Six2* is expressed in the progenitor cells of the nephron.²² We confirmed the absence of DTR on podocytes in *Ndr* $g1^{CreERT2/+};iDTR$ 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 *Ndr* $g1$ in PECs,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.^{38,39} Glomerulosclerosis secondary to proximal tubule injuries is also observed in clinical situations. Mesoamerican nephropathy⁴⁰ 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,^{41–44} and also

reported in animal models of tubular injury.^{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,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 *Ndr1* and *DTR* were also expressed in peripheral nerves⁴⁸ (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.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.

CONCISE METHODS

Animals

Ndr1^{CreERT2/+} mice were generated in our laboratory.²³ *iDTR* mice were purchased from The Jackson Laboratory²¹ (Bar Harbor, ME). *Coll1a1-GFP* mice,²⁷ a transgenic mouse strain expressing enhanced GFP under the regulation of the *coll1a1* promoter and enhancers, were a gift from Prof. Jeremy Duffield. *USAG-1*^{+/LacZ} mice were described previously.³⁰ Serum and urine creatinine, serum BUN, serum and urine glucose, and urinary albumin were measured by the creatininase-HMPS method, the urease/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).

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.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.

Administration of DT

DT was prepared as described previously.²⁰ Appropriate aliquots of DT solution were administered to mice by intraperitoneal injection.

Histologic Studies and Immunostaining

The histologic studies were performed as described previously.^{30,49,50} Primary antibodies against the following proteins were used for immunostaining: hHBegf (R&D Systems), aquaporin 1 (Abcam, Inc., Cambridge, MA), Tamm–Horsfall protein (Biomedical Technologies Inc., Stoughton, MA), thiazide-sensitive NaCl cotransporter (Chemicon, Temacula, 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 Immunochemicals Inc., Gilbertsville, PA), PDGFR β (eBioscience), GFP (Abcam, Inc.), F4/80 (Molecular Probes, Eugene, OR), nephrin (R&D Systems), α -SMA (Sigma-Aldrich), and PECAM (BD Biosciences, San Jose, CA). Alexa-conjugated phalloidin (Molecular Probes) was used to visualize the brush borders of the proximal tubules. Staining was analyzed with a confocal microscope (FV1000D; Olympus, Tokyo, Japan).

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.²³ Three mice were examined at each time point.

Quantitative Assessment of Tubular Injury, Fibrosis, and Atubular Glomeruli

Tubular injury was scored semiquantitatively by a blinded pathologist who examined ≥ 10 cortical fields ($\times 200$ magnification) of PAS-stained sections ($n=3-4$) as previously described.^{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 $\leq$ 10% tubules injured; 2=11%–25% tubules injured; 3=26%–50% tubules injured; 4=51%–74% tubules injured; and 5 $\geq$ 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 ($\times 200$ 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).^{34,53}

The prevalence of atubular glomeruli in the kidneys of *Ndrp1^{CreERT2/+};iDTR* mice after repeated injections of low-dose DT ($n=3$) and PBS ($n=3$) was evaluated as described previously.^{23,54–56} Paraffin-embedded kidneys were sectioned at 4- μ m intervals for 27 slices for each sample, and the glomeruli in the 14th section were analyzed for continuity with proximal tubules using 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 TGF β 1 (10 ng/ml; R&D Systems) was administered (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 TGF β 1 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 $\pm$ 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.

Study Approval

All animal studies were approved by the Animal Research Committee, Kyoto University Graduate School of Medicine and performed in accordance with the guidelines of Kyoto University as well as the National Institutes of Health guidelines.

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DISCLOSURES

The following authors report no conflicts of interest: K.T., J.N., S.Y., H.N., Y.S., M.N., T.Y., K.K., H.H., and K.S. M.T. was a graduate student when he conducted experiments in this paper and was employed by Mitsubishi Tanabe Pharma Corporation after his graduation. A.N.E. is an employee of Regeneron Pharmaceuticals Inc. and holds stock in the company. M.Y. is on the advisory board of Astellas and receives research grants from Astellas, Chugai Pharmaceutical Co (Tokyo, Japan), Daiichi Sankyo, Fujiyaku, Kyowa Hakko Kirin, Mitsubishi Tanabe Pharma Corporation, MSD, Nippon Boehringer Ingelheim, and Torii.

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