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ORIGINAL PAPER

Exploring the origin and limitations of kidney regeneration

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Abstract

Epidemiological findings indicate that acute kidney injury (AKI) increases the risk for chronic kidney disease (CKD), although the molecular mechanism remains unclear. Genetic fate mapping demonstrated that nephrons, functional units in the kidney, are repaired by surviving nephrons after AKI. However, the cell population that repairs damaged nephrons and their repair capacity limitations remain controversial. To answer these questions, we generated a new transgenic mouse strain in which mature proximal tubules, the segment predominantly damaged during AKI, could be genetically labelled at desired time points. Using this strain, massive proliferation of mature proximal tubules is observed during repair, with no dilution of the genetic label after the repair process, demonstrating that proximal tubules are repaired mainly by their own proliferation. Furthermore, acute tubular injury caused significant shortening of proximal tubules associated with interstitial fibrosis, suggesting that proximal tubules have a limited capacity to repair. Understanding the mechanism of this limitation might clarify the mechanism of the AKI-to-CKD continuum.

Keywords: acute kidney injury; regeneration; chronic kidney disease; fibrosis; tubule; nephron

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Introduction

Acute kidney injury (AKI) is a clinical syndrome characterized by the rapid decline of renal function and is often caused by an ischaemic, toxic, or obstructive insult [1]. AKI is a common complication in hospitalized patients and is associated with increased mortality, cost, and longer hospital stays [2–5]. AKI patients supported with appropriate treatment often recover, attesting to the intrinsic regenerative capacity of the kidney. A rapid proliferative response is a sign of the recovering phase of AKI, which leads to the restoration of nephron structure and function. However, the origin of proliferating cells remains controversial [6–9].

Although pathological analysis supported the traditional idea that surviving dedifferentiated tubules that are poised in G1 proliferate in response to injury to restore damaged tubules [10,11], the idea has been challenged by numerous studies suggesting the contribution of intrinsic or extrinsic stem/progenitor cells. Some groups have implicated the presence of progenitor cells in the kidney, utilizing various methods, including lineage tracing analysis, side population, label retention, and the expression of stem/progenitor cell markers [12–19].

Recently, lineage tracing utilizing *six2-Cre* mice [20], in which Cre recombinase was expressed in the progenitor population of the developing nephron and almost all epithelial cells of the resulting nephron – Bowman's capsule, podocytes, proximal tubules, loop of Henle, thick ascending limb, and distal tubules – were genetically labelled [21], demonstrated no dilution of the genetic label within nephrons after repair, implicating the exclusive contribution of $Six2^+$ progenitor-derived nephron constituents in the repair process. However, the relative contribution of the various types of cells in a nephron to kidney repair remains unknown.

Proximal tubules are vulnerable to ischaemic and toxic insult, and are the most severely affected nephron segment in many types of kidney disease. The loss of proximal tubular epithelial cells and a denuded tubular basement membrane are the pathological hallmarks of AKI. We hypothesized that terminally differentiated proximal tubule cells can proliferate and restore the proximal tubules by themselves, and developed a model to estimate the regenerative capacity of terminally differentiated proximal tubule cells. In this model, *N-myc downstream-regulated gene-1* (*Ndrg1*), which is abundantly expressed in mature proximal tubules [22,23], drives tamoxifen-inducible Cre recombinase (Cre^{ERT2}) [24]. In $Ndrg1^{CreERT2/+}$ mice, the Cre protein is expressed in all mature proximal tubules and is only activated after the administration of tamoxifen. When $Ndrg1^{CreERT2/+}$ mice are crossed with reporter strains, the Cre-dependent removal of the stop sequence in the progeny leads to the heritable expression of a marker gene in mature proximal tubule cells. Utilizing this system, we provided direct evidence for the proliferation of mature proximal tubule cells after acute tubular injury and showed that proximal tubules are mainly repaired by their own proliferation. Very recently in 2014, Kusaba *et al* reported similar results utilizing a different proximal tubule-specific Cre^{ERT2} mouse strain (*SLC34a1-*$Cre^{GCE/+}$ mice), demonstrating a clonal expansion of differentiated proximal tubules after injury [25]. In addition, Berger *et al* utilized another inducible transgenic mouse strain (*PEC-rtTA* mice) which labels scattered proximal tubules and parietal epithelial cells, and demonstrated the proliferation of these cells after injury [26]. Our data add a new model to faithfully demonstrate that mature proximal tubules are regenerated by their proliferation.

We further demonstrated the limitation of the repair capacity of kidney tubules for the first time. Traditionally, AKI has been believed to be a reversible and benign syndrome. However, over the past several years, epidemiological studies have shown that AKI increases the risk of CKD [27–34]. Various underlying mechanisms for the progression of AKI to CKD have been proposed, including maladaptive repair due to impaired angiogenesis, sustained inflammation, interstitial fibrosis, and cell cycle arrest; however, there has been no clear evidence showing the limitation of the regenerative capacity of the damaged kidney.

In this study, we showed for the first time that the repair capacity of proximal tubules is not sufficient and that the length of the proximal tubules becomes shorter after injury. The limitation of the repair capacity of proximal tubules might explain, at least in part, the mechanism of the AKI-to-CKD continuum. Because the volume of proximal tubules accounts for more than 80% of the kidney cortex [35], the shortening of proximal tubules may also contribute to cortical atrophy, a hallmark of many types of kidney disease.

Materials and methods

Generation of $Ndrg1^{CreERT2/+}$ mice

A genomic fragment containing the mouse *Ndrg1* gene and the first exon was isolated [36]. We inserted a Cre^{ERT2} cassette (Artemis Pharmaceuticals, Cologne, Germany), polyA tail, and *loxP*-flanked *PGK-Neo* cassette in the opposite transcriptional orientation to the first exon of the *Ndrg1* gene (Figure 2A). ES cells derived from C57BL/6 J mice were electroporated with a targeting vector and selected by the G418-containing medium. G418-resistant ES colonies were selected, and correctly targeted clones (#96 and #111) were identified by Southern blotting (data not shown) and genomic PCR (Figure 2B). These ES cells were injected into blastocysts to obtain mouse chimeras, which were crossed with wild-type C57BL/6 J mice for germline transmission. The sequence of the primers used was as follows: primer A, CTAGAATCTGCTCATAGCA CCAGGTGGC; primer B, TCACCTCAGCGAGGTC CACGTCATGTAG; primer C, TGGCAGCTCTCATGT CTCCAGCAGAATC.

Administration of tamoxifen

We administered 3 mg/20 g BW tamoxifen (Sigma-Aldrich, St. Louis, MO, USA) to mice by intraperitoneal injection for five consecutive days as described previously [37].

Animal use

$Ndrg1^{CreERT2/+}$ mice were mated with *R26ECFP* mice [38] (a gift from F Costantini of Colombia University, New York, New York, USA) and *Rosa26R* indicator mice (*R26R* mice [39]) to obtain double-transgenic mice. $USAG\text{-}1^{+/LacZ}$ mice have been described previously [40]. 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 (NIH) guidelines.

BrdU staining

5-Bromo-2′-deoxyuridine (BrdU) (Sigma-Aldrich) (100 mg/kg BW) was administered to mice intraperitoneally 5 h before sacrifice. BrdU was visualized using the anti-BrdU mouse monoclonal antibody conjugated with Alexa FluorR dye (Molecular Probes, Eugene, OR, USA) as previously described [41].

Histological studies and immunostaining

Histological studies were performed as described previously [40,42,43]. Primary antibodies against the following proteins were used for immunostaining: anti-GFP (Invitrogen, Carlsbad, CA, USA), aquaporin 1 (Abcam, Cambridge, MA, USA), Tamm–Horsfall Protein (Biomedical Technologies Inc, Stoughton, MA, USA), thiazide-sensitive NaCl cotransporter

(NCC) (Chemicon, Temecula, CA, USA), calbindin D28K (Sigma, St Louis, MO, USA), aquaporin 2 (Calbiochem, San Diego, CA, USA), V-ATPase B1/2 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), E-cadherin (Sigma), nestin (Abcam), Ki67 (Vision-Biosystems, Norwell, MA, USA), PDGFRβ (eBioscience, San Diego,0 CA, USA), Kim1 (eBioscience), and Ndrg1 [23]. FITC-conjugated lotus lectin (LTL) (J-Oil Mills, Akashi-cho, Tokyo, Japan) and Alexa-conjugated phalloidin (Invitrogen) were utilized to visualize the brush borders of proximal tubules. LacZ staining was performed as described previously [40]. For double staining with BrdU staining or immunostaining, LacZ staining was performed first. The S3 segment was distinguished both by anatomic location and by morphological differences as described previously [25].

Quantification of mRNA by real-time RT-PCR

Real-time RT-PCR was performed as previously described [44]. Specific primers were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). Serially diluted cDNA was used to generate the standard curves for each primer. Primer sequences for *Ndrg1* were as follows: ACGTATCACGACATCGGCA; CCACATGGCAGACAGCAAA.

Kidney disease model

An ischaemic reperfusion (IR) injury was performed as previously described [45]. Briefly, the IR injury was induced by clamping the left renal artery and vein for 45 min (Bear disposable microvascular clip: AS-1-40). Core body temperature was maintained at 37 °C using a homeothermic table during surgery. The FA nephrotoxicity model was performed as previously described [40].

Quantitative assessment of labelling efficiency and cell proliferation

The labelling efficiency (Figures 2L and 4E) was assessed by counting the number of segment marker-positive cells and GFP/segment marker double-positive cells in four cortical fields and two cortico-medullary fields randomly selected at ×200 magnification in each mouse. Five mice were examined before the IR injury and three mice were examined after the IR injury.

Cell proliferation was analysed by counting the number of BrdU-positive or BrdU/LacZ double-positive cells in ten randomly selected cortical fields at ×200 magnification in each mouse (Figure 3C). Four mice were examined in each group.

Quantitative assessment of proximal tubule complexity

C57BL/6 J mice were subjected to IR injury and the injured kidneys were analysed 45 days after the operation ($n = 4$). Age-matched C57BL/6 J mice were analysed as controls ($n = 4$). Transverse sections of the kidneys were prepared around the hilus and were stained with LTL, AQP1, Kim1 (proximal tubule markers), and nestin (a podocyte marker). We counted the number of proximal tubular sections positive for at least one of these proximal tubule markers, and the number of glomeruli positive for nestin in ten sections in each mouse. We divided the number of proximal tubular sections by the number of glomeruli and defined the divided number as the 'complexity' of proximal tubules.

Quantitative assessment of atubular and connected glomeruli

The prevalence of atubular glomeruli was evaluated as previously described [46–48]. Briefly, paraffin-embedded kidneys were sectioned at 4 μm intervals for 27 slices for each sample. All of the glomeruli in the 13th section were analysed for continuity with proximal tubules utilizing the consecutive sections, and the percentages of atubular and nephron-connected glomeruli were determined in each sample [46]. We adjusted the number of nestin-positive glomeruli in the analysis of complexity by multiplying the percentage of 'connected' glomeruli, and defined the number of proximal tubular sections divided by the adjusted number of glomeruli as 'adjusted complexity'.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Significance was assessed by the Student's *t*-test for two group comparisons. $p < 0.05$ was considered significant.

Results

Generation of proximal tubule-specific inducible Cre mice, *Ndrg1*$^{CreERT2/+}$ mice

To generate proximal tubule-specific inducible Cre mice, we searched for proximal tubule-specific genes and identified *Ndrg1*. The expression of *Ndrg1* mRNA was by far the most abundant in the kidney (Figure 1A), and renal expression increased towards the later stage of development (Figure 1B). Although Wakisaka *et al* reported that Ndrg1 expression in proximal tubules decreased during maturation in rats [49], immunostaining of Ndrg1 in the mouse kidney demonstrated strong expression in the proximal tubules (Figures 1C and 1D). Ndrg1 did not co-localize with either calbindin D28K, a marker of the distal convoluted tubule, or USAG-1, a marker of the thick ascending limb and distal convoluted tubule [40] (Figures 1E, 1F, and 1H). Ndrg1 was positive in some E-cadherin-positive epithelial cells (a marker of the distal convoluted tubule and collecting ducts), indicating the spotted expression in the collecting ducts (Figures 1G and 1H).

Next, we generated an *Ndrg1*$^{CreERT2/+}$ knock-in allele with the *CreER*^{T2} cassette introduced into the *Ndrg1* locus at the position of the initiation codon (Figure 2A). ES clones were tested for correct recombination by Southern blotting (data not shown) and

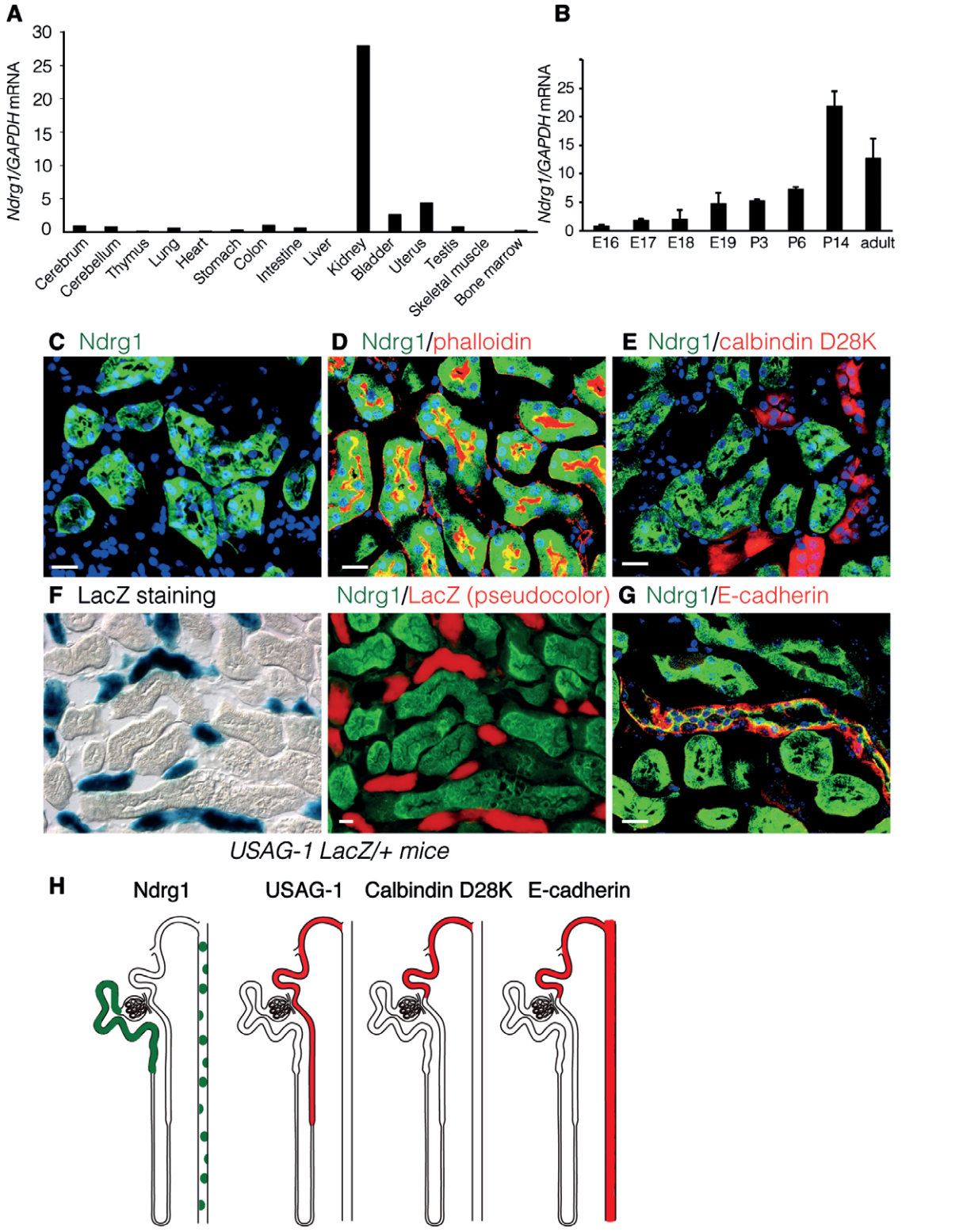

Figure 1. Expression of Ndrg1 in the kidney. (A) Real-time PCR analysis of *Ndrg1* mRNA in wild-type mice. Expression levels were normalized to those of GAPDH. The expression of *Ndrg1* was by far the most abundant in the kidney. (B) The expression of *Ndrg1* during kidney development. Embryonic kidneys, neonatal kidneys, and adult kidneys were subjected to real-time PCR analysis of *Ndrg1*. $n = 3$ in each group. (C–G) Immunostaining of Ndrg1 in the adult kidney. Ndrg1 co-localized with phalloidin-positive proximal tubules (D), but not with calbindin D28K-positive distal convoluted tubules (E). (F) In the kidneys of *USAG-1*$^{+/LacZ}$ mice, LacZ transcripts were observed in the thick ascending limb, distal convoluted tubules, and connecting ducts, which did not co-localize with Ndrg1. (G) Ndrg1 partly co-localized with E-cadherin (a marker for distal convoluted tubules and collecting ducts). (H) Schematic illustration demonstrating the expression of Ndrg1, USAG-1, calbindin D28K, and E-cadherin in a nephron. Scale bars = 20 μm.

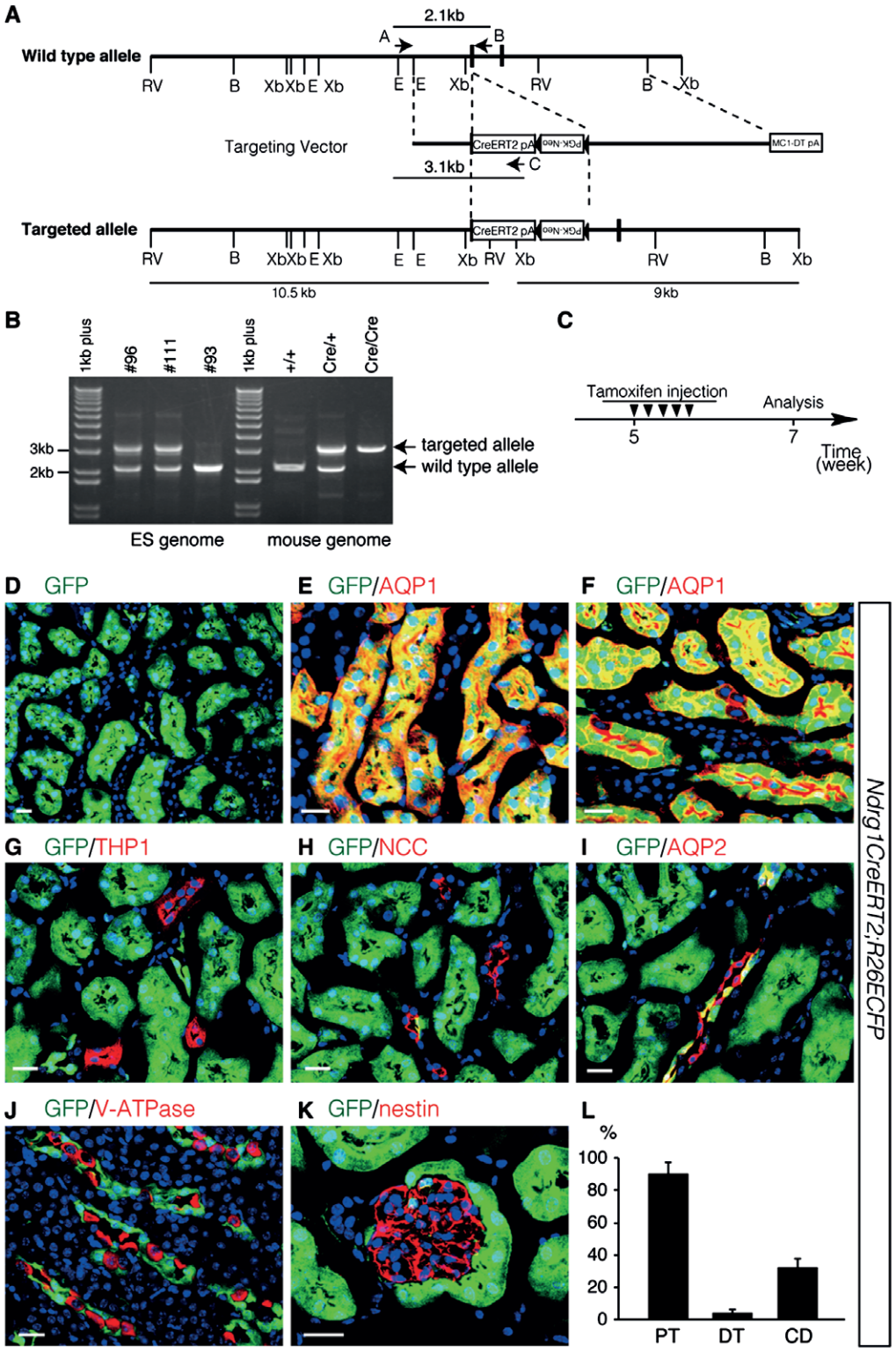

Figure 2. Generation of *Ndrg1*$^{CreERT2/+}$ mice and the efficient Cre-mediated recombination in the mouse strain. (A) Targeting strategy for *Ndrg1*$^{CreERT2/+}$ mice. We generated the *Ndrg1*^{CreERT2} knock-in allele with the CreER^{T2} cassette introduced to the *Ndrg1* locus at the position of the *Ndrg1* initiation codon. (B) Correctly targeted clones (#96 and #111) were selected from G418-resistant 161 ES colonies by Southern blotting (data not shown) and genomic PCR. We also performed genomic PCR of *Ndrg1*$^{CreERT2/+}$, *Ndrg1*$^{CreERT2/CreERT2}$, and *Ndrg1*$^{+/+}$ mice. The ladder shown is 1 kb Plus DNA Ladder (Invitrogen). (C) *Ndrg1*$^{CreERT2/+}$ mice were mated with *R26ECFP* mice to obtain *Ndrg1*$^{CreERT2/+}$*:R26ECFP* mice. These mice were intraperitoneally administered 150 mg/kg tamoxifen for 5 consecutive days at 5 weeks and sacrificed at 7 weeks. (D–K) ECFP expression in the kidneys of *Ndrg1*$^{CreERT2/+}$*:R26ECFP* mice was visualized by immunostaining with anti-GFP antibody. (E) Proximal tubules in the cortex (S1 and S2 segments) were positive for GFP staining. (F) Some of the proximal tubule cells in the cortico-medullary junction (S3 segment) were negative for GFP. (G) Tamm–Horsfall Protein (THP)-positive thick ascending limbs were negative for GFP. (H) Thiazide-sensitive NaCl cotransporter (NCC)-positive distal convoluted tubules were almost negative for GFP. (I, J) The GFP signal in the collecting ducts co-localized with AQP2 (I), but not with V-ATPase (J). (K) Very few GFP signals were observed in glomeruli. (L) Recombination efficiencies in proximal tubules (PT), distal tubules (DT), and collecting ducts (CD). $n = 5$. Scale bars = 20 μm.

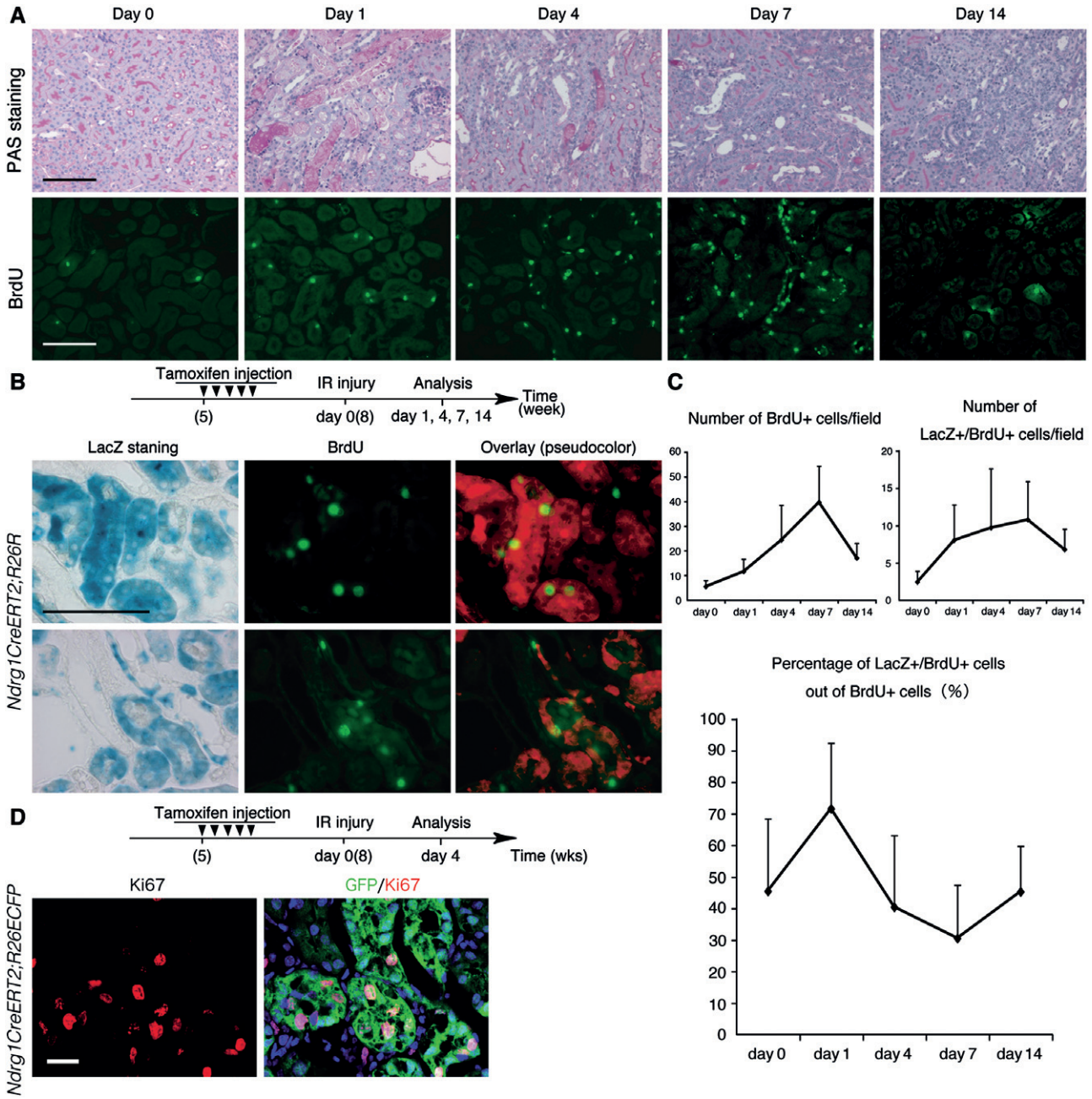

Figure 3. Proliferation of mature proximal tubule cells after IR injury. (A) Histological changes and BrdU staining after IR injury. Scale bar = 100 μm. (B) Genetically labelled *Ndrg1*$^{CreERT2/+}$*:R26R* mice underwent IR injury and were administered BrdU at various time points. Many LacZ/BrdU double-positive cells were detected within injured tubules on day 1 of the IR injury. Scale bar = 100 μm. (C) The time course of the number of BrdU-positive cells per field (×200), the number of LacZ/BrdU double-positive cells per field (×200), and the percentage of LacZ/BrdU double-positive cells out of BrdU-positive cells in the cortex of *Ndrg1*$^{CreERT2/+}$*:R26R* mice. $n = 4$ for each experiment. (D) *Ndrg1*$^{CreERT2/+}$*:R26ECFP* mice administered tamoxifen underwent IR injury and were subjected to double immunostaining of Ki67 and GFP on day 4. Scale bar = 20 μm.

genomic polymerase chain reaction (PCR) (Figure 2B), and were injected into the oocytes of C57BL/6 J mice. The knock-in allele disrupted the first exon, thereby deleting the expression of *Ndrg1*. Recombination in the mouse tail genome was also confirmed by genomic PCR (Figure 2B). Whereas *Ndrg1*$^{CreERT2/CreERT2}$ mice recapitulated the demyelinating phenotype of germline *Ndrg1 null* mice [23], the peripheral nerves of *Ndrg1*$^{CreERT2/+}$ mice are phenotypically normal (Supplementary Figure 1A). We also confirmed that the response to kidney injury was not modified in the absence of a single allele of *Ndrg1* (Supplementary Figures 1B and 1C).

To analyse the specificity and efficiency of the Cre recombination, we bred *Ndrg1*$^{CreERT2/+}$ mice with *Rosa26ECFP* indicator mice (*R26ECFP* mice) [38], in which ECFP was expressed after Cre-mediated recombination of a *loxP*-flanked stop sequence, and other indicator mice, and administered tamoxifen to induce recombination (Figure 2C). No recombination was

observed in the kidneys of *Ndrg1$^{CreERT2/+}$:R26ECFP* mice before the administration of tamoxifen even in female mice (data not shown). Recombination in the kidney was efficiently achieved in the proximal tubules (Figures 2D–2F). Reflecting the high expression of Ndrg1 in the S1 and S2 segments and the moderate expression in the S3 segment in the proximal tubules, the recombination efficiency was 100% in the S1 and S2 segments (Figure 2E), but 58% in the S3 segment (Figure 2F). In total, the recombination efficiency in proximal tubules was approximately 90% (Figure 2L). The recombination efficiency in other segments was 4% in the distal tubules (Figures 2G, 2H, and 2L) and 32% in the collecting ducts (Figures 2I, 2J, and 2L). Occasional recombination was also observed in a few glomeruli (Figure 2K). Slight recombination was observed in the colon and small intestine of *Ndrg1$^{CreERT2/+}$:R26ECFP* mice, although the efficiency was significantly lower than that in the kidney (Supplementary Figure 2). Although we previously reported severe haematological toxicity due to the systemic activation of CreER^{T2} [37], no toxicity was observed in the kidney after the administration of tamoxifen (data not shown).

Mature proximal tubular cells proliferate after kidney injury

Degeneration of injured tubule cells and a subsequent massive proliferative response were observed with ischaemic reperfusion (IR) injury (Figure 3A). To explore the origin of the proliferating cells after acute tubular injury, we genetically labelled the proximal tubules of *Ndrg1$^{CreERT2/+}$:R26R* mice by the administration of tamoxifen, induced an IR injury, and analysed the kidney at various time points (Figure 3B). The recombination efficiency of *Ndrg1$^{CreERT2/+}$:R26R* mice was 86% in proximal tubules (Supplementary Figure 3). BrdU was administered 5 h before sacrifice. To achieve complete elimination of tamoxifen from the body, surgery was postponed until 2 weeks after the last administration of tamoxifen. Double staining of the kidneys of *Ndrg1$^{CreERT2/+}$:R26R* mice demonstrated the existence of many LacZ/BrdU double-positive cells (LacZ$^+$/BrdU$^+$ cells) within injured tubules, indicating that mature proximal tubule cells proliferated after acute tubular injury (Figure 3B). Quantitative analysis of these sections demonstrated that the number of BrdU$^+$ cells as well as the number of LacZ$^+$/BrdU$^+$ cells increased around days 4–7. The percentage of LacZ$^+$/BrdU$^+$ cells was more than 60% of BrdU$^+$ cells on day 1 and around 40% on day 4, and remained high until day 14 (Figure 3C), which might be explained by the sustained proliferation of proximal tubules compared with other segments in the nephron. We further confirmed the presence of many ECFP/Ki67 double-positive cells in the kidneys of *Ndrg1$^{CreERT2/+}$:R26ECFP* mice on day 4 of the IR injury (Figure 3D), indicating the massive proliferation of proximal tubules after acute tubular injury.

Regeneration of proximal tubule cells by their own proliferation

Even after significant tubular damage and the denudation of the basement membrane, proximal tubules were repaired after the IR injury. Regeneration of proximal tubules was proven by the reappearance of PAS-positive, phalloidin-positive brush borders in the apical membrane (Figures 4A and 4B), although prominent fibrosis emerged in the interstitium (Figure 4B). By analysing the genetic label in the proximal tubules of the repaired kidneys of *Ndrg1$^{CreERT2/+}$:R26ECFP* mice, we showed the cells' origin responsible for regeneration. The existence of ECFP-negative cells (ECFP$^-$ cells) within the repaired proximal tubules [(1) in Figure 4C] suggests the contribution of cells other than proximal tubule cells to the regeneration of the proximal tubule, whereas the absence of ECFP$^-$ cells indicates that proximal tubule cells are repaired mainly by their own proliferation [(2) in Figure 4C]. The appearance or increase of ECFP$^+$ cells outside of the proximal tubules after repair supports the possible contribution of proximal tubule cells to the regeneration of other segments [(3) in Figure 4C].

We induced IR injury to genetically labelled *Ndrg1$^{CreERT2/+}$:R26ECFP* mice, sacrificed the mice on day 45 when proximal tubules were repaired (Figures 4A and 4B), and demonstrated that almost all proximal tubules of the repaired kidney were positive for ECFP (Figure 4D), which supports the idea that proximal tubule cells contribute to the regeneration of proximal tubules [(2) in Figure 4C]. We also confirmed that the prevalence of ECFP$^+$ cells in the proximal tubules, distal tubules, and collecting ducts after IR injury was comparable to their prevalence before IR injury (Figures 4D and 4E), suggesting that the idea of proximal tubule cells migrating out and repairing other segments in the kidney [(3) in Figure 4C] was less likely.

We also examined the regenerative capacity of proximal tubules in another kidney disease model, folic acid (FA) nephrotoxicity. Injected FA causes crystallization in distal tubules, which results in the dilatation and degeneration of proximal tubules, leading to AKI (Supplementary Figure 4A) [40]. After 2 weeks, proximal tubules are mostly repaired and renal function returns to normal ranges [40]. We induced FA nephrotoxicity in genetically labelled *Ndrg1$^{CreERT2/+}$:R26ECFP* mice twice and confirmed that the genetic label in the proximal tubules was not diluted even after repeated FA nephrotoxicity (Supplementary Figure 4B).

Limitations in the repair capacity of proximal tubules

Next, we analysed whether the repair capacity of proximal tubules was sufficient to achieve complete repair. To analyse the length of proximal tubules, we employed the concept of 'complexity' of the proximal convoluted tubules [50]. Long proximal convoluted tubules tend to pass through a certain slice repeatedly and produce more

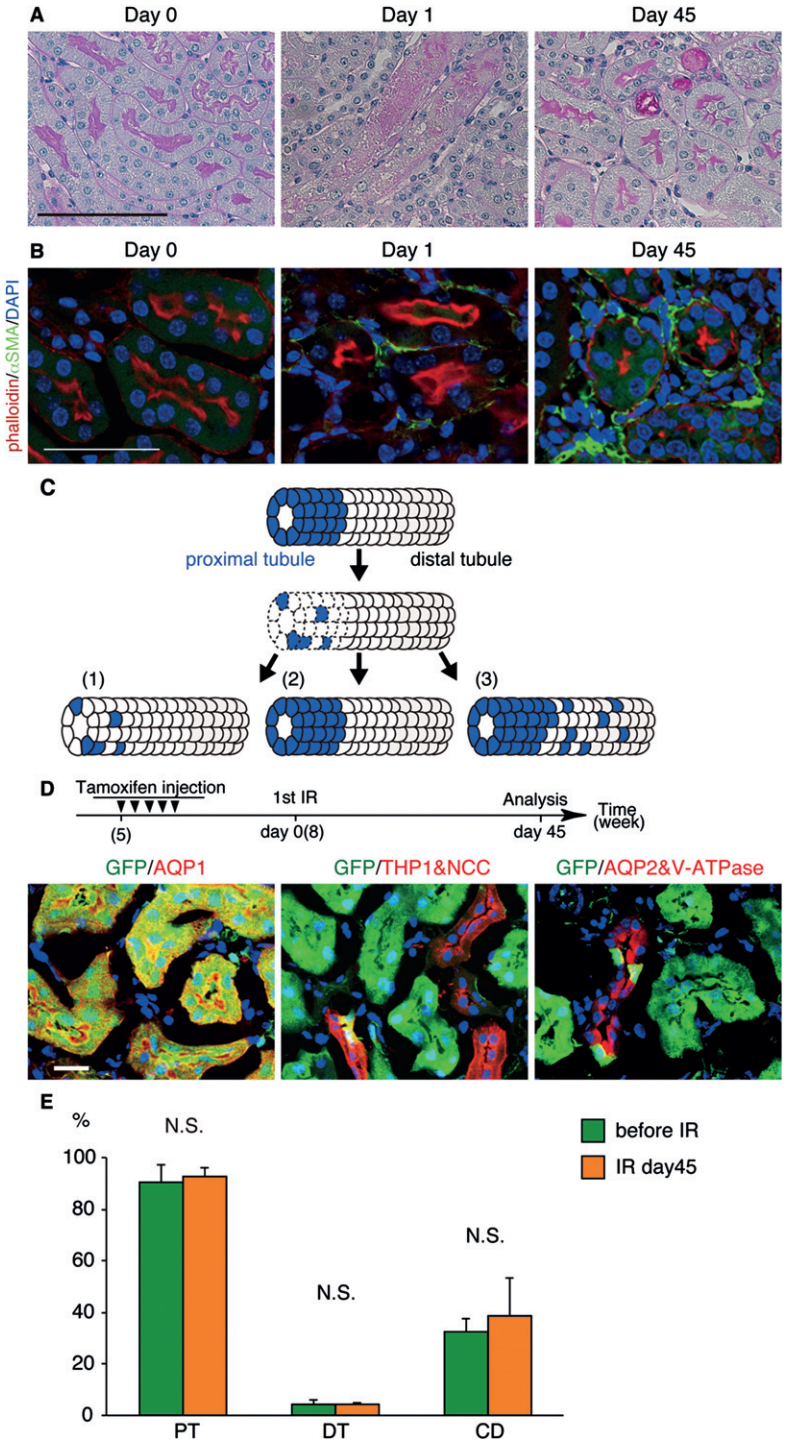

Figure 4. Proximal tubule cells repair themselves by their own proliferation. (A) Histological findings of the kidneys on days 0, 1, and 45 of IR injury. In spite of a severe injury at day 1, tubules were mostly regenerated by day 45, which was proven by the reappearance of PAS-positive brush borders. Scale bar = 100 μm. (B) Phalloidin staining and αSMA immunostaining on days 0, 1, and 45 of IR injury. Regeneration of proximal tubules was proven by the emergence of phalloidin-positive brush borders in the apical membrane, although significant fibrosis was observed in the interstitium. Scale bar = 50 μm. (C) Schematic models for kidney repair. Proximal tubule cells were genetically labelled in blue in the model. If proximal tubules were regenerated by the incorporation of unlabelled cells, dilution of the label would be observed in the repaired proximal tubules (1). If proximal tubules were repaired by surviving labelled proximal tubule cells, no dilution of the label would be observed in the repaired proximal tubules (2). If proximal tubules repaired other segments in the nephron, the genetic label in the other segments should be increased (3). (D) The GFP signals in the kidneys of *Ndrg1*$^{CreERT2/+}$*:R26ECFP* mice were analysed after IR injury. Proximal tubules positive for AQP1 were almost positive for GFP after the injury, whereas distal tubules positive for THP1 or NCC were almost negative for GFP. Collecting ducts positive for AQP2 or V-ATPase were sparsely positive for GFP. Scale bars = 20 μm. (E) Graph showing the recombination efficiencies in proximal tubules (PT), distal tubules (DT), and collecting ducts (CD) before (green, $n = 5$, same samples as in Figure 2L) and after IR injury (orange, $n = 3$). Recombination efficiencies in each segment were not significantly changed after IR injury. N.S. = not significant.

tubular sections than short proximal convoluted tubules (Figure 5F).

We induced IR injury to C57BL/6J mice and analysed the histology of the injured kidneys 45 days after the operation. To quantitate the number of all proximal tubular sections, we first validated the markers of proximal tubules in the repaired kidney. Triple immunostaining of LTL (the marker for the brush border of proximal tubules), AQP1 (the marker for proximal tubules), and Kim1 (the marker of injured proximal tubules) revealed that most of the repaired proximal tubules were positive for LTL and AQP1, whereas around 10% of proximal tubules were positive for Kim1 (Figures 5A and 5B). Thirty-seven per cent of Kim1-positive cells were negative for both LTL and AQP1, indicating the possible underestimation of proximal tubules when analysed only with LTL and AQP1 (Supplementary Figure 5). Therefore, we stained the repaired kidneys with these three markers in the same colour and counted the number of proximal tubular sections that were positive for at least one of these markers. At the same time, we stained the repaired kidney with antibodies against nestin (the marker for podocytes) and counted the number of nestin-positive glomeruli in the section.

We defined the number of proximal tubular sections divided by the number of glomeruli as the 'complexity index' of the proximal tubules and found that the complexity was significantly decreased after IR injury (Figure 5C).

We also considered the possibility that the presence of atubular glomeruli reduces the number of proximal tubular sections. To exclude the influence of atubular glomeruli, we analysed the prevalence of atubular glomeruli in each sample according to previous reports [46–48] and estimated the number of 'connected glomeruli'. We further divided the number of proximal tubular sections in each sample by the number of 'connected glomeruli' and found that the adjusted complexity of proximal tubule was still lower after injury (Figure 5D).

Taken together, the proximal tubule seems to become shorter after repair, indicating that the repair capacity of proximal tubules is not sufficient (Figures 5E and 5F). The shortening of proximal tubules associated with interstitial fibrosis in the repaired kidney might at least partially explain the predisposition to CKD after AKI.

Discussion

In this study, we generated a novel inducible Cre mouse strain in which mature proximal tubules are genetically labelled at desired time points. Using this mouse strain, we directly demonstrated that surviving mature proximal tubule cells proliferate and regenerate injured proximal tubules in acute tubular injury. We also demonstrated that mature proximal tubule cells are the main source to restore damaged proximal tubules after acute tubular injury and that these cells did not contribute to the regeneration of other segments in the nephron. We further clarified the limitations in the regenerative capacity of mature proximal tubule cells, demonstrating the shortening of regenerated proximal tubules associated with interstitial fibrosis.

The proximal tubule is the most important segment in a nephron for the reabsorption of various solutes such as salt, glucose, phosphate, and amino acids. Because of its active reabsorption and massive ATP consumption, the proximal tubule is, at the same time, the most vulnerable segment in a nephron. Equally important is its ability to function as an immune responder to various immunological, ischaemic, or toxic injuries. Therefore, it is not surprising that proximal tubule-related phenomena are closely related to the pathogenesis of a vast array of kidney diseases [51].

The *Ndrg1*$^{CreERT2/+}$ mouse strain is a novel tool to achieve efficient recombination in more than 90% of proximal tubules in a strictly tamoxifen-dependent manner. In addition to the *Ndrg1* promoter, we tried using other known proximal tubule-specific promoters to generate transgenic Cre mouse strains [52]; however, recombination in these strains was not sufficient even at the maximum dose of tamoxifen. High expression of CreER^{T2} is required to achieve efficient inducible recombination in adult proximal tubule cells, which was enabled by the *Ndrg1*$^{CreERT2/+}$ mouse strain. Although the recombination in some collecting ducts might interfere with the interpretation of the data obtained with this mouse strain, the efficient and strictly tamoxifen-dependent recombination in proximal tubules might overcome the problem.

In spite of their vulnerability to various insults, proximal tubules are capable of regeneration. The source of regeneration was a matter of controversy until Humphreys *et al* concluded, using Six2-Cre mice, that metanephric mesenchyme-derived nephron epithelial cells, which include Bowman's capsule, podocytes, proximal tubules, the loop of Henle, and distal tubules, repair injured kidneys [21]. This study provided conclusive evidence that the nephron was repaired by the nephron itself and negated the contribution of non-tubular adult stem cells to regeneration. However, this does not eliminate the possibility of stem/progenitor cells being derived from the Six2$^+$ progenitor population during development surviving after birth and contributing to kidney regeneration [53]. In addition, the possibility of intratubular stem/progenitor cells existing in the adult kidney and contributing to kidney regeneration remains.

Several groups report the existence of possible intratubular progenitor populations, such as CD24$^+$ CD133$^+$ parietal epithelial cells and proximal tubules [12–14,54–56], and Lgr5$^+$ cells [57]. Another group demonstrated that NFATc1$^+$ cells in mature proximal tubules repair damaged proximal tubules [18].

On the other hand, Smeets *et al* demonstrated that CD24$^+$CD133$^+$ cells are formed *de novo* after injury in a rodent experimental model and are positive for tubular injury markers, suggesting that these cells are transiently

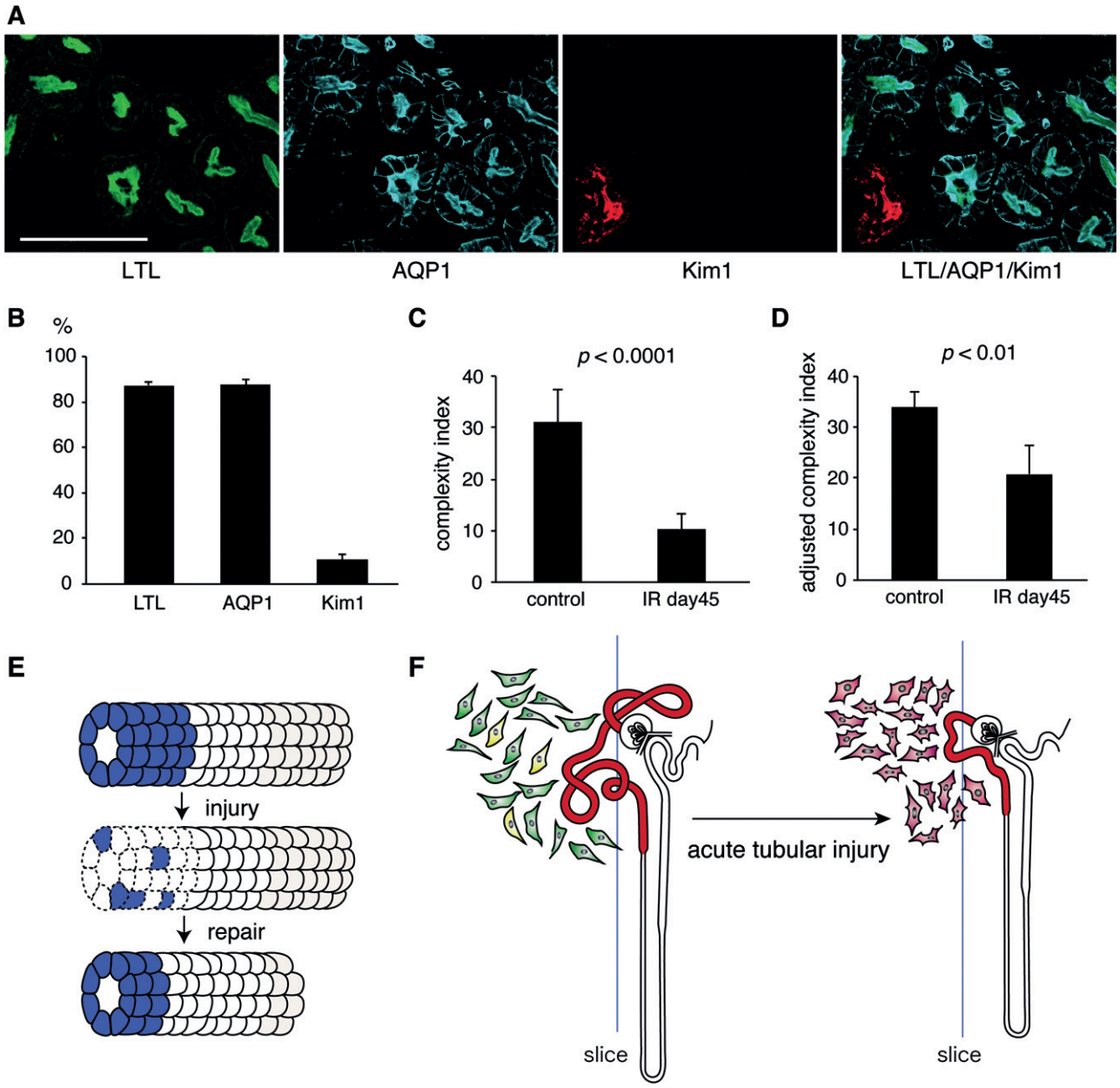

Figure 5. Limitations in the repair capacity of proximal tubules. (A) Triple immunostaining of LTL, AQP1, and Kim1 of the repaired kidney revealed that most of the repaired proximal tubules were positive for LTL and AQP1, whereas a few proximal tubules were positive for Kim1 and negative for LTL and/or AQP1. Scale bars = 100 μm. (B) Percentage of LTL, AQP1, and Kim1-positive tubules in the proximal tubules positive for at least one of these markers. Although 90% of the repaired proximal tubules were positive for LTL and AQP1, 10% of the proximal tubules were positive for Kim1. (C) The 'complexity index' of the proximal tubules, which was defined as the number of proximal tubular sections divided by the number of glomeruli, was significantly decreased after IR injury. (D) The 'adjusted complexity index', in which the influence of atubular glomeruli was minimized, was still significantly lower after IR injury. (E) Schematic illustration demonstrating that the length of the proximal tubules becomes shorter after the injury, indicating a limitation in the repair capacity of proximal tubules. (F) Schematic illustration demonstrating the shortening of proximal tubules and fibrosis after the recovery from acute tubular injury.

dedifferentiated tubular cells [58]. More recently, the transgenic mouse approach demonstrated that scattered proximal tubule cells are not fixed progenitors, but rather injured proximal tubule cells [26]. Utilizing a new inducible Cre mouse strain that labels mature proximal tubules, Kusaba *et al* demonstrated the massive expansion of labelled cells, the *de novo* expression of CD24, CD133 in injured proximal tubules, and no dilution of the genetic label after repair [25]. They concluded that terminally differentiated epithelial cells re-express stem cell markers such as CD24 and CD133 during injury.

In this study, by utilizing the *Ndrg1*$^{CreERT2/+}$ mouse strain which faithfully labels mature proximal tubules at desired time points, we provided direct evidence of the proliferation of mature proximal tubule cells (Figure 3). The prevalence of the genetic label of proximal tubules, distal tubules, and collecting ducts in the repaired kidney is comparable to that of the uninjured kidney (Figure 4E), indicating that mature proximal

tubules are mainly repaired by their own proliferation. Although the contribution of labelled collecting ducts to the repair process of the proximal tubule cannot be excluded, it is less likely considering the very low prevalence of labelled cells in distal tubules located between proximal tubules and collecting ducts. In addition, the contribution of proximal tubules to the repair process of other segments is also shown to be less likely in this study.

We also demonstrated for the first time the limitation of the repair capacity of proximal tubules in this study. Because it is technically challenging to measure the length of proximal tubules in individual nephrons, we employed a concept of proximal tubule complexity. During development and postnatal maturation, substantial elongation and increased convolution of the proximal convoluted tubules are observed [59,60]. The number of proximal tubular sections per glomerulus (complexity index) is lower in the immature kidney [50], indicating that the complexity index might be a good indicator to monitor the elongation and convolution of proximal convoluted tubules. Because the repair process after AKI shares considerable similarities with the maturation process of a nephron, we assume that the complexity index could be indicative of the extent of the regeneration of proximal tubules. If the repair process after AKI is incomplete and the repaired proximal tubule is shortened, the complexity index should be lower in the repaired kidney. We further considered the possibility that the presence of atubular glomeruli after AKI [48] reduces the complexity index and found that the adjusted complexity is still lower after acute tubular injury.

Our study has the following limitations: our study left the possibility that intratubular progenitor cells expressing Ndrg1 exist in proximal tubules and contribute to the regeneration of proximal tubules. In addition, we could not measure the direct length of the shortened nephrons because it is technically challenging.

Our current findings demonstrated that the episode of acute tubular injury leads to the shortening of proximal tubules and fibrosis, and clarified the limited regenerative capacity of mature proximal tubule cells. These results showed that although the repaired proximal tubule is similar, it is not identical to a healthy proximal tubule. These findings may explain, at least in part, the underlying mechanism of CKD progression after AKI. The reason for the limited regenerative capacity of proximal tubules might be explained by the limited proliferation or massive apoptosis. The limited regenerative capacity of proximal tubules could also be attributable to the severe injury model employed in this study, supported by the clinical observation linking the severity of AKI to the progression to CKD [61]. A better understanding of the mechanism underlying this limited regenerative capacity may lead to specific therapies to block the AKI-to-CKD continuum.

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Author contribution statement

TE, JN, and YS conceived and carried out experiments. MA, RY, MT, KT, AO, TI, AYH, TO, and TN carried out experiments. EM and TK were involved in writing the paper. MY designed the research plan, conceived the experiments, analysed the data, and wrote the paper. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article:

Figure S1. Phenotypic analysis of *Ndrg1* $^{CreERT2/+}$ mice.

Figure S2. Cre-mediated recombination in other tissues of *Ndrg1*$^{CreERT2/+}$*:R26ECFP* mice.

Figure S3. Recombination efficiency in the kidneys of *Ndrg1*$^{CreERT2/+}$*:R26R* mice.

Figure S4. No dilution of the genetic label in the kidneys of *Ndrg1*$^{CreERT2/+}$*:R26ECFP* mice after recovery from FA nephrotoxicity.

Figure S5. The expression of proximal tubule markers in Kim1-positive proximal tubules.