Lineage tracing analysis defines erythropoietin-producing cells as a distinct subpopulation of resident fibroblasts with unique behaviors

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Erythropoietin (Epo) is produced by a subpopulation of resident fibroblasts in the healthy kidney. We have previously demonstrated that, during kidney fibrosis, kidney fibroblasts including Epo-producing cells transdifferentiate into myofibroblasts and lose their Epo-producing ability. However, it remains unclear whether Epo-producing cells survive and transform into myofibroblasts during fibrosis because previous studies did not specifically label Epo-producing cells in pathophysiological conditions. Here, we generated *Epo*$^{CreERT2/+}$ mice, a novel mouse strain that enables labeling of Epo-producing cells at desired time points and examined the behaviors of Epo-producing cells under pathophysiological conditions. Lineage-labeled cells that were producing Epo when labeled were found to be a small subpopulation of fibroblasts located in the interstitium of the kidney, and their number increased during phlebotomy-induced anemia. Around half of lineage-labeled cells expressed Epo mRNA, and this percentage was maintained even 16 weeks after recombination, supporting the idea that a distinct subpopulation of cells with Epo-producing ability makes Epo repeatedly. During fibrosis caused by ureteral obstruction, *Epo*$^{CreERT2/+}$-labeled cells were found to transdifferentiate into myofibroblasts with concomitant loss of Epo-producing ability, and their numbers and the proportion among resident fibroblasts increased during fibrosis, indicating their high proliferative capacity. Finally, we confirmed that *Epo*$^{CreERT2/+}$-labeled cells that lost their Epo-producing ability during fibrosis regained their ability after kidney repair due to relief of the ureteral obstruction. Thus, our analyses have revealed previously unappreciated characteristic behaviors of Epo-producing cells, which had not been clearly distinguished from those of resident fibroblasts.

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Translational Statement

Although we and others showed previously that kidney fibroblasts, including erythropoietin (Epo)-producing cells, transdifferentiate into myofibroblasts in kidney diseases, the behavior of Epo-producing cells remains unclear, because previous studies do not specifically label Epo-producing cells. Here, we generated *Epo*$^{CreERT2/+}$ mice to label Epo-producing cells at desired time points and thereby revealed the unique behaviors of Epo-producing cells, such as their sustained Epo-producing ability in healthy kidneys, their loss of Epo-producing ability and rapid proliferation during fibrosis, and their reacquisition of Epo-producing ability after kidney repair. Further analysis of this subpopulation will provide insights that may yield new therapeutic approaches to renal anemia.

The hormone erythropoietin (Epo) is essential for erythropoiesis.[1] In adults, Epo is produced mainly by resident fibroblasts in the kidney and is regulated at transcriptional levels through a hypoxia-inducible factor–dependent mechanism under physiological conditions.[2–5] Epo-producing cells are distributed mainly in the deep cortex and outer medulla,[4,6] the regions that are physiologically hypoxic and sensitive to subtle changes in oxygen delivery.[7] Under physiological conditions, Epo-producing cells are a small subpopulation of resident fibroblasts detectable around the corticomedullary junctions, whereas under hypoxic conditions, they become detectable in a broader area of the cortex.[8,9]

Although the transcriptional regulation of Epo has been analyzed intensively, a remaining unanswered question is whether Epo-producing cells constitute a distinct and specialized subpopulation of resident fibroblasts, or in contrast, all resident fibroblasts possess the capacity to produce Epo.

Yamazaki *et al.* have demonstrated that most resident fibroblasts in the kidneys of inherited super anemic mice (ISAM), which lack Epo-producing ability in the kidneys and are severely anemic, are lineage-labeled with *Epo-Cre*.[10] Although this finding suggests that all kidney fibroblasts have the potential to produce Epo, the lineage-labeled cells in the study are the cells with a history of *Epo* expression from the developmental period.

Indeed, lineage-tracing studies analyzing the cells currently producing Epo in the adult kidneys are lacking, and the behaviors of Epo-producing cells under pathologic conditions also remain unknown. In our previous study, we demonstrated that resident fibroblasts including Epo-producing cells are lineage-labeled with *myelin protein zero Cre* (*P0-Cre*), and that, during kidney injury, they transdifferentiate into myofibroblasts and lose their potential to produce Epo, resulting in kidney fibrosis and renal anemia.[11,12] We also confirmed that Epo-producing ability can be regained in myofibroblasts by the induction of severe anemia. However, what is not fully clear from our previous study is whether the cells that had been capable of producing Epo in the healthy kidney die, or rather, survive and transdifferentiate into myofibroblasts during kidney fibrosis. This lack of clarity is due to the fact that all kidney fibroblasts, including Epo-producing cells, are labeled in *P0-Cre* mice in the same way, so we could not trace Epo-producing cells specifically.

Previous studies have identified Epo-producing cells by means of *in situ* hybridization or by using transgenic mice in which green fluorescent protein is knocked-in at the Epo locus.[4,13,14] Therefore, Epo-producing cells could not be observed in the kidneys with impaired Epo-producing ability, and the behavior of Epo-producing cells could not be monitored while Epo production was paused.

In the present study, to address these problems, we established $Epo^{CreERT2/+}$ mice, a novel mouse line that allows us to label Epo-producing cells at desired time points and to trace Epo-producing cells even while Epo production is paused. Utilizing this novel mouse line, we traced the fate of Epo-producing cells under physiological and pathologic conditions and identified Epo-producing cells as being a distinct subpopulation of resident fibroblasts with unique phenotypes.

METHODS

Study approval

All animal studies were approved by the Animal Research Committee, Kyoto University Graduate School of Medicine, and the Institutional Animal Care and Use Committee of the RIKEN Center for Biosystems Dynamic Research, Kobe branch, and performed in accordance with the guidelines of Kyoto University and the RIKEN Kobe branch, as well as US National Institutes of Health guidelines.

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

To construct a targeting vector, genomic fragments containing the mouse *Epo* gene were isolated from a bacterial artificial chromosome (BAC) clone (RP23-129L22, BACPAC Resources). We inserted a Cre^{ERT2} cassette (Artemis Pharmaceuticals), polyA tail, and a *FRT*-flanked *PGK-Neo* cassette into the *Epo* ATG start site of exon 1 (Figure 1a). TT2 embryonic stem (ES) cells were electroporated with targeting vector.[15] G418-resistant ES colonies were selected, and correctly targeted clones were identified by Southern blotting (Figure 1b). Two clones of ES cells (#21 and #55) were injected into 8-cell stage embryos to obtain mouse chimeras, which were crossed with wild-type C57BL/6J mice for germline transmission. Correct targeting was also confirmed by genomic polymerase chain reaction (PCR) of the tail genome (Figure 1c). Primers utilized were as follows: primer A: CTACAGAACTTCCAAGGATG; primer B: ACTTCTCGGCCAAACTTCAC; primer C: CTCGACCAGTTTAGTTACCC. $Epo^{CreERT2/+}$ mice (Accession No. CDB1003K: http://www2.clst.riken.jp/arg/mutant%20mice%20list.html) were backcrossed to C57BL/6J mice at least 7 times. The $Epo^{CreERT2/+}$ mouse strain can be used by other researchers, subject to agreement with the corresponding author on terms and conditions of use.

A detailed description is provided in the Supplementary Full Methods of protocols used for the following: (i) animals; (ii) anemia induction and the administration of tamoxifen; (iii) kidney injury models; (iv) immunostaining; (v) *in situ* hybridization; (vi) quantitative assessment; (vii) real-time reverse transcription quantitative (RT-q) PCR analysis; (viii) enzyme-linked immunosorbent assay (ELISA); and (ix) statistical analysis.

RESULTS

Generation of Epo-producing cell-specific inducible Cre mice, $Epo^{CreERT2/+}$ mice

To generate Epo-producing cell-specific inducible Cre mice, we generated an $Epo^{CreERT2/+}$ knock-in allele with the Cre^{ERT2} cassette introduced into the *Epo* locus at the position of the initiation codon (Figure 1a). ES clones were tested for correct recombination by Southern blotting (Figure 1b), and correctly targeted ES cells were injected into blastocysts to obtain mouse chimeras. Chimeras were mated with C57BL/6J mice to obtain an N1 generation. Correct recombination was also confirmed by PCR of the tail genome (Figure 1c). The knock-in allele disrupted the first exon, thereby deleting the expression of *Epo*. Although no $Epo^{CreERT2/CreERT2}$ mouse was born from the mating between $Epo^{CreERT2/+}$ mice, in agreement with the previous report showing that *Epo* knockout mice are embryonically lethal,[16] $Epo^{CreERT2/+}$ and $Epo^{+/+}$ littermates were obtained in the predicted ratios (Table 1). Body weight (Bw), hemoglobin (Hb), hematocrit (Hct; Figure 1d and e), and *Epo* mRNA expression in the kidney and cerebrum (Figure 1f) were comparable between $Epo^{CreERT2/+}$ mice and wild-type littermates, although the expression levels in these tissues were significantly lower compared to those in anemic kidneys (Figure 1f). High-sensitivity *in situ* hybridization using RNAscope detected very few *Epo* mRNA–expressing cells in the kidney and did not detect any *Epo* mRNA–expressing cells in the cerebrum of either genotype in non-anemic conditions (Supplementary Figure S1). Next, we assessed whether there was a difference in Epo responsiveness to anemia between wild-type and $Epo^{CreERT2/+}$ mice, and found that the Epo reactivity to anemia was attenuated in $Epo^{CreERT2/+}$ mice, probably due to

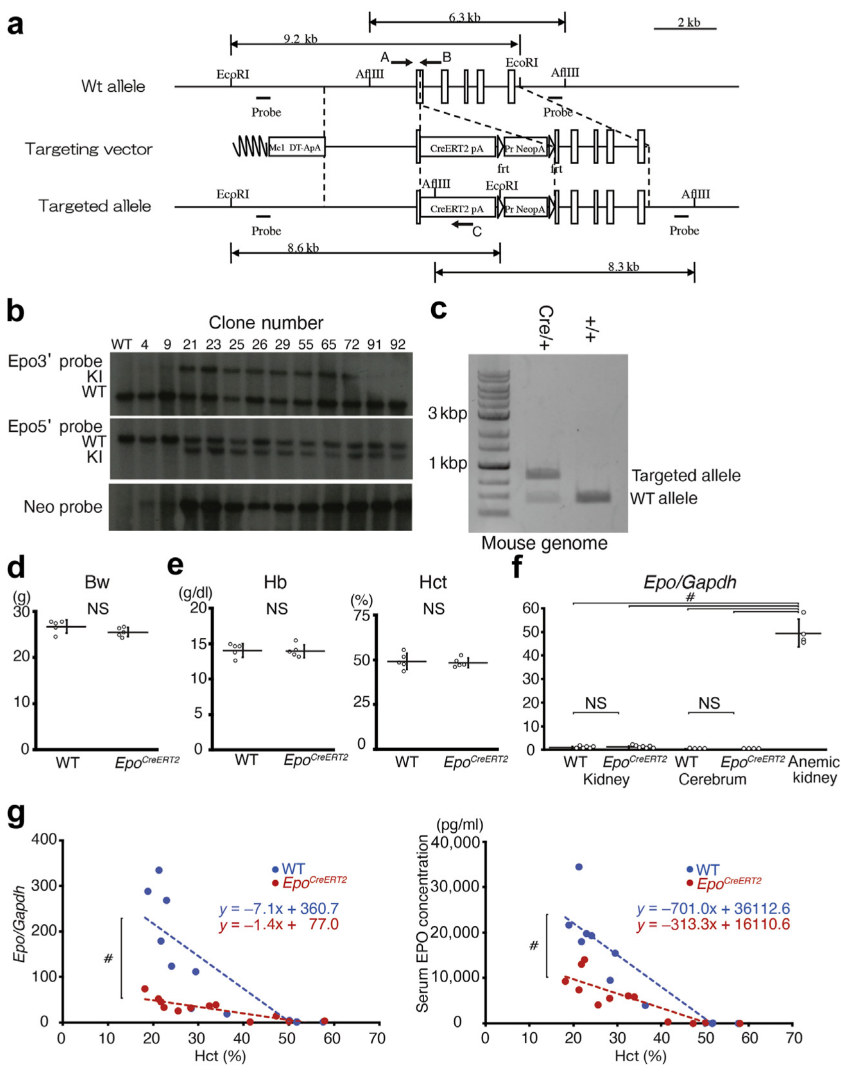

Figure 1 | Generation of $Epo^{CreERT2/+}$ mice. (**a**) Targeting strategy for $Epo^{CreERT2/+}$ mice. We generated the $Epo^{CreERT2/+}$ knock-in allele by introducing a Cre^{ERT2} cassette to the *Epo* locus at the position of the *Epo* initiation codon. (**b**) Correctly targeted embryonic stem (ES) cell clones were selected by Southern blotting. Epo3′ probe: KI (knock-in) 8.3 kb, wild-type (WT) 6.3 kb. Epo5′ probe: KI 8.6 kb, WT 9.2 kb. (**c**) Genomic polymerase chain reaction (PCR) of $Epo^{CreERT2/+}$ mice and $Epo^{+/+}$ mice. PCR products of WT and targeted alleles were 458 and 754 base pairs (bp) in size, respectively. The ladder shown is 1 kbp DNA ladder One. (**d**) Body weight (Bw) and (**e**) hemoglobin (Hb) and hematocrit (Hct) of WT and $Epo^{CreERT2/+}$ mice ($Epo^{CreERT2}$) at age 9 weeks. (**f**) *Epo* mRNA in the indicated organs from WT and $Epo^{CreERT2/+}$ mice at age 9 weeks. The expression levels of *Epo* mRNA were normalized to those of *Gapdh,* and the average levels of WT kidneys were set as 1. Data are given as mean $\pm$ SD, $n = 4$–7 in WT and $Epo^{CreERT2/+}$ mice. Anemic kidneys were from the 4 most severely anemic mice of Figure 2c. (**g**) Erythropoietin (Epo) responsiveness to various degrees of anemia in WT and $Epo^{CreERT2/+}$ mice. The left graph shows the correlation between *Epo* mRNA expression and Hct, and the right graph shows the correlation between serum EPO concentration (pg/ml) and Hct. Epo responsiveness was defined as the slope of the regression line. Hypoxic Epo response of $Epo^{CreERT2/+}$ mice was attenuated in both *Epo* mRNA expression and serum EPO concentration. Multiple group comparisons were performed using analysis of covariance. ($P = 0.002$ in *Epo* mRNA expression; $P = 0.009$ in serum EPO concentration). $^{\#}P < 0.05$. The statistical analysis used was the Student *t* test in (**d**) and (**e**), 1-way analysis of variance, followed by Tukey-Kramer *post hoc* analysis in (**f**), and analysis of covariance in (**g**). Mc1 DT-ApA, Diphtheria toxin A gene; EcoRI, sequence that is cut by the restriction enzyme EcoRI; Pr NeopA, Neomycin-resistance gene; NS, not significant.

heterozygous deletion of the *Epo* gene (Figure 1g). However, *Epo* mRNA expression and serum EPO concentration increased with the severity of anemia.

Characterization of lineage-labeled cells in $Epo^{CreERT2/+}$ mice

To analyze the specificity and efficiency of Cre recombination, we bred $Epo^{CreERT2/+}$ mice with *Rosa26-tdTomato* (*R26tdTomato*) indicator mice, in which tdTomato is expressed after Cre-mediated recombination of the loxP-flanked stop sequence, and administered tamoxifen to $Epo^{CreERT2/+}$*:R26tdTomato* mice to induce this recombination (Figure 2a). To enhance *Epo* expression, we induced anemia by phlebotomy during tamoxifen administration. Although very few tdTomato-expressing cells (tdTomato^{+} cells) existed in the kidney without anemia induction (Figure 2b), the number of tdTomato^{+} cells increased in parallel with the severity of

Table 1 | Number and percentage of pups in each genotype

N/%	*Epo*$^{CreERT2/CreERT2}$	*Epo*$^{CreERT2/+}$	*Epo*$^{+/+}$	Total
Number of pups	0	44	19	63
%	0	69.8	30.2	100

anemia and *Epo* mRNA expression in the kidney (Figure 2b and c). tdTomato^{+} cells in severely anemic mice were located mainly in the corticomedullary region, where they formed clusters (Figure 2d). *Epo* mRNA-expressing cells were also observed by high-sensitivity *in situ* hybridization in the corticomedullary region, where they formed clusters similar to those of tdTomato^{+} cells (Supplementary Figure S2).

To exclude the possibility of spontaneous recombination, 3 *Epo*$^{CreERT2/+}$*:R26tdTomato* mice were vehicle-treated with anemia induction, and 3 slice sections of the kidney per mouse were examined. In the kidneys, tdTomato^{+} cells were present at 0.09 ± 0.19/mm^2, whereas tdTomato^{+} cells in tamoxifen-administered *Epo*$^{CreERT2/+}$*:R26tdTomato* mice with the same volume of bleeding were present at 22.9 ± 8.9/mm^2 (data of 4 samples shown in Figure 2c), indicating that the spontaneous recombination of *Epo*$^{CreERT2/+}$ was very limited (Supplementary Figure S3).

Immunostaining of tdTomato^{+} cells showed that these cells were located in the interstitium and that they expressed platelet-derived growth factor receptor beta (PDGFRβ) and

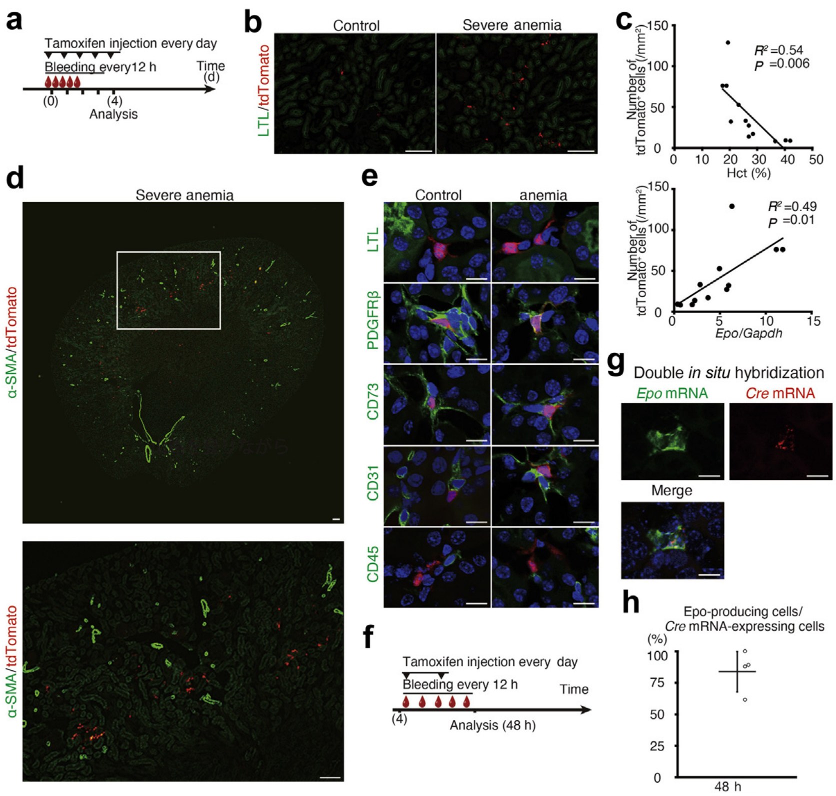

Figure 2 | Analysis of recombination in *Epo*$^{CreERT2/+}$*:R26tdTomato* mice. (**a**) Experimental protocol of (**b–e**). *Epo*$^{CreERT2/+}$ mice were mated with *R26tdTomato* mice to obtain *Epo*$^{CreERT2/+}$*:R26tdTomato* mice. Four-week-old *Epo*$^{CreERT2/+}$*:R26tdTomato* mice were i.p. administered 120 mg/kg body-weight tamoxifen for 5 consecutive days with anemia induction, and euthanized on the last day of tamoxifen administration. (**b**) The presence of tdTomato^{+} cells with or without anemia induction. Bar = 100 μm. (**c**) The upper graph shows the inverse correlation between the number of tdTomato^{+} cells and hematocrit (Hct). (R^2 = 0.54, P = 0.006). The number of tdTomato^{+} cells is expressed as the number of cells per square millimeter (mm^2). The lower graph shows the correlation between the number of tdTomato^{+} cells and the expression of *Epo* mRNA (R^2 = 0.49; P = 0.01). The correlation was determined by Pearson's correlation analysis. (**d**) The localization of tdTomato^{+} cells in the kidneys of *Epo*$^{CreERT2/+}$*:R26tdTomato* mice with severe anemia. The upper panel shows the distribution of tdTomato^{+} cells in the corticomedullary region. The lower panel shows a higher magnification of the boxed region in the upper panel. Bar = 100 μm. (**e**) Immunostaining of tdTomato^{+} cells in the kidneys of *Epo*$^{CreERT2/+}$*:R26tdTomato* mice with or without anemia. The left panels show images from mice without anemia, and the right panels show images from mice with severe anemia. tdTomato^{+} cells were located in the interstitium and expressed the fibroblast markers platelet-derived growth factor receptor beta (PDGFRβ) and cluster of differentiation (CD)73, but not CD31 (endothelial marker) or CD45 (hematopoietic cell marker). Bar = 10 μm. (**f**) Experimental protocol of (**g**) and (**h**). *Epo*$^{CreERT2/+}$*:R26tdTomato* mice were i.p. administered 120 mg/kg body-weight tamoxifen for 2 consecutive days with anemia induction (with Hct between 20% and 30%), and euthanized 48 hours after the first administration of tamoxifen. (**g**) High-sensitivity double *in situ* hybridization showed the colocalization of *Cre* mRNA (red) and *Epo* mRNA (green). Bar = 10 μm. (**h**) Graph illustrating the proportion of *Cre* mRNA–expressing cells that expressed *Epo* mRNA (erythropoietin [Epo]-producing cells/Cre mRNA–expressing cells). The proportion of Epo-producing cells in Cre mRNA–expressing cells was 84.3% ± 16.4%. n = 4. Data are given as mean ± SD. LTL, lotus tetragonolobus lectin; SMA, smooth muscle actin. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Table 2 | Number and proportion of Epo-producing cells and *Cre* mRNA–expressing cells 48 hours after the first administration of tamoxifen

Cells	Number and proportion
Epo-producing cells	66.0 ± 24.9
Cre mRNA–expressing cells	6.4 ± 4.7
Coexpressing cells	5.0 ± 3.6
Epo-producing cells in *Cre* mRNA–expressing cells, %	84.3 ± 16.4
Cre mRNA–expressing cells in Epo-producing cells, %	6.5 ± 3.6

Epo, erythropoietin.
Units are number/mm^2, except proportions, which are specified as %. Data are given as mean ± SD.

CD73, indicating that tdTomato$^+$ cells were resident fibroblasts (Figure 2e). tdTomato$^+$ cells did not express CD31, an endothelial cell marker, or CD45, a common leucocyte antigen (Figure 2e). Occasional recombination was also observed in a small number of cells in the glomeruli and collecting ducts (Supplementary Figure S4).

To confirm that Epo-producing cells were accurately labeled in *Epo*$^{CreERT2/+}$ mice, we performed high-sensitivity double *in situ* hybridization for *Epo* mRNA and *Cre* mRNA, and found that 84.3% ± 16.4% of *Cre* mRNA-expressing cells expressed *Epo* mRNA (Figure 2f–h; Table 2). The proportion of *Cre* mRNA–expressing cells in Epo-producing cells was 6.5% ± 3.6%, and the number of *Cre* mRNA–expressing cells and *tdTomato* mRNA–expressing cells was low compared with the number of Epo mRNA–expressing cells (Tables 2 and 3; Supplementary Figure S5), indicating the relatively low recombination efficiency of *Epo*$^{CreERT2/+}$ mice. Taken together, these findings clearly show the following characteristic features of lineage-labeled cells in *Epo*$^{CreERT2/+}$ mice: (i) *Epo*$^{CreERT2/+}$-labeled cells were fibroblasts in the corticomedullary interstitium, as previously reported[4,17]; (ii) anemia induction increased the number of *Epo*$^{CreERT2/+}$-labeled cells; and (iii) most *Cre* mRNA–expressing cells in *Epo*$^{CreERT2/+}$ mice expressed *Epo* mRNA.

A certain subpopulation of fibroblasts maintains the ability to produce Epo over a long period

Next, we examined whether the same subpopulation of fibroblasts repeatedly produces Epo over a long period upon ischemia of their microenvironment, or rather whether different subpopulations of fibroblasts produce Epo stochastically at different time points (Figure 3a). We administered tamoxifen to 4-week-old *Epo*$^{CreERT2/+}$*:R26tdTomato* mice, and euthanized them at various time points after anemia induction (48 hours, 5 weeks, or 16 weeks after the first tamoxifen administration; Figure 3b, upper illustration). The Hct levels at each time point were 27.1 ± 2.0%, 21.5 ± 1.6%, and 22.6 ± 3.1%, respectively. A slightly milder anemia was induced for analysis at 48 hours, to prevent death due to simultaneous administration of tamoxifen, and because the mice were small at 4 weeks of age. We performed high-sensitivity double *in situ* hybridization for *Epo* mRNA and *tdTomato* mRNA, and found that 56.7% ± 15.7% of *tdTomato* mRNA–expressing cells expressed *Epo* mRNA (Figure 3c and d; Table 3), whereas 10.7% ± 2.9% of *Epo* mRNA–expressing cells were positive for *tdTomato* mRNA 48 hours after the first tamoxifen administration. Interestingly, about 50% of *tdTomato* mRNA–expressing cells maintained the ability to produce Epo for as long as 16 weeks: the proportions of Epo-producing cells among the *tdTomato* mRNA–expressing cells were 56.7% ± 15.7%, 51.0% ± 11.8%, and 47.3% ± 14.3% at 48 hours, 5 weeks, and 16 weeks, respectively (Figure 3c and d). There was no significant difference among these 3 time points ($P = 0.65$ by analysis of variance). These results support "hypothesis 1" that there exists a certain subpopulation among resident fibroblasts that repeatedly produces Epo in response to anemic conditions (Figure 3a). We also examined the proportion of Epo-producing cells among tdTomato$^+$ cells without anemia induction at 5 weeks and at16 weeks after the tamoxifen administration (Figure 3b, lower illustration), and the averages, respectively, were 3.3% ± 4.7% and 5.4% ± 3.6% (Figure 3d).

Epo-producing cells transdifferentiate into myofibroblasts and have high proliferating ability during fibrosis

We further investigated the behaviors of Epo-producing cells during kidney injury utilizing the unilateral ureteral obstruction (UUO) model. To exclude the effects of tamoxifen administration and anemia, we performed UUO 5 weeks after the administration of tamoxifen and phlebotomy (Figure 4a). tdTomato$^+$ cells were a rare population before injury, and their number was 47.9 ± 23.6/mm^2, and the proportion of tdTomato$^+$ cells to PDGFRβ-positive fibroblasts in the inner cortex was 1.9% ± 0.8% (Figure 4b, d, e; Table 4). Three days

Table 3 | Number and proportion of Epo-producing cells and *tdTomato* mRNA–expressing cells 48 hours, 5 weeks, and 16 weeks after the first administration of tamoxifen

Cells	Analysis with anemia			Analysis without anemia	
	48 h	5 wk	16 wk	5 wk	16 wk
Epo-producing cells	58.5 ± 29.1	59.2 ± 12.5	71.7 ± 18.2	4.7 ± 3.8	9.0 ± 4.9
tdTomato mRNA–expressing cells	10.8 ± 5.8	14.0 ± 3.5	17.0 ± 1.6	13.7 ± 5.6	23.8 ± 15.9
Coexpressing cells, number/mm^2	6.2 ± 4.1	7.1 ± 2.1	8.1 ± 3.0	0.2 ± 0.3	1.4 ± 1.3
Epo-producing cells in *tdTomato* mRNA–expressing cells, %	56.7 ± 15.7	51.0 ± 11.8	47.3 ± 14.3	3.3 ± 4.7	5.4 ± 3.6
tdTomato mRNA–expressing cells in Epo-producing cells, %	10.7 ± 2.9	12.1 ± 3.7	11.7 ± 3.9	31.2 ± 47.3	12.8 ± 10.3

Epo, erythropoietin.
Units are number/mm^2, except proportions, which are specified as %. Data are given as mean ± SD.

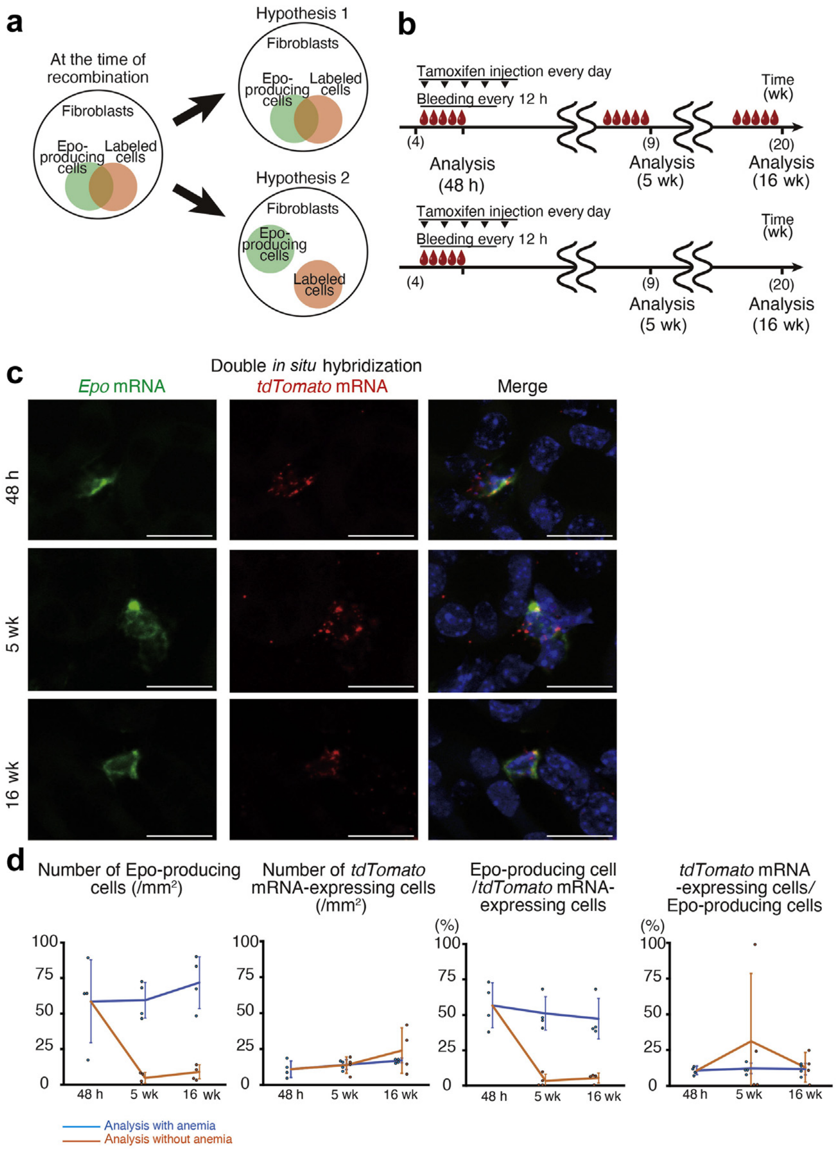

Figure 3 | $Epo^{CreERT2/+}$ lineage-labeled cells maintain erythropoietin (Epo)-producing ability for a long period. (**a**) Hypothetical model of Epo-producing cells. Green circles show Epo-producing cells, and red circles show $Epo^{CreERT2/+}$-labeled cells. The left panel describes the cell populations at the time of recombination. The right upper panel illustrates one hypothesis in which Epo is consistently produced by a certain subpopulation of kidney fibroblasts (hypothesis 1). In this situation, $Epo^{CreERT2/+}$-labeled cells maintain Epo-producing ability. The right lower panel illustrates another hypothesis in which all fibroblasts possess the potential to produce Epo, yet only a random subset of them actually produces Epo at any given time (hypothesis 2). In this situation, $Epo^{CreERT2/+}$-labeled cells cannot maintain Epo-producing ability. (**b**) Experimental protocol of (**c**) and (**d**). Four-week-old $Epo^{CreERT2/+}$:*R26tdTomato* mice were administered tamoxifen with anemia induction. They were euthanized 48 hours, 5 weeks, or 16 weeks after the administration of tamoxifen. In some mice, anemia was induced again before euthanasia at 5 or 16 weeks after the recombination (upper illustration). Other mice were euthanized at 5 or 16 weeks without anemia induction (lower illustration). (**c**) High-sensitivity double *in situ* hybridization of the kidney of $Epo^{CreERT2/+}$:*R26tdTomato* mice euthanized 48 hours, 5 weeks, or 16 weeks after the recombination with anemia induction. Bars = 10 μm. (**d**) Graphs illustrating the number of Epo-producing cells, the number of *tdTomato* mRNA–expressing cells, the proportion of *Epo* mRNA in *tdTomato* mRNA–expressing cells, and the proportion of *tdTomato* mRNA–expressing cells in Epo-producing cells. The blue line shows the results with anemia induction at analysis, and the orange line shows the results without anemia induction. The proportions of Epo-producing cells in tdTomato mRNA–expressing cells with anemia induction were 56.7% ± 15.7%, 51.0% ± 11.8%, and 47.3% ± 14.3% at 48 hours, 5 weeks, and 16 weeks after the recombination, respectively ($n = 4$). There was no significant difference among these 3 time points ($P = 0.65$ by analysis of variance). The proportions of Epo-producing cells in *tdTomato* mRNA–expressing cells without anemia induction 5 weeks and 16 weeks after the recombination were 3.3% ± 4.7% and 5.4% ± 3.6%, respectively ($n = 4$). Data are given as mean ± SD. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

after UUO, the number of tdTomato$^+$ cells increased dramatically (Figure 4b). The number of tdTomato$^+$ cells per high-power field was 324.4 ± 103.0/mm^2 3 days after UUO (Figure 4d), and the proportion of tdTomato$^+$ cells to PDGFRβ-positive fibroblasts had increased 4.8-fold (1.9% ± 0.8% before injury, and 9.2% ± 2.4% after UUO; Figure 4e).

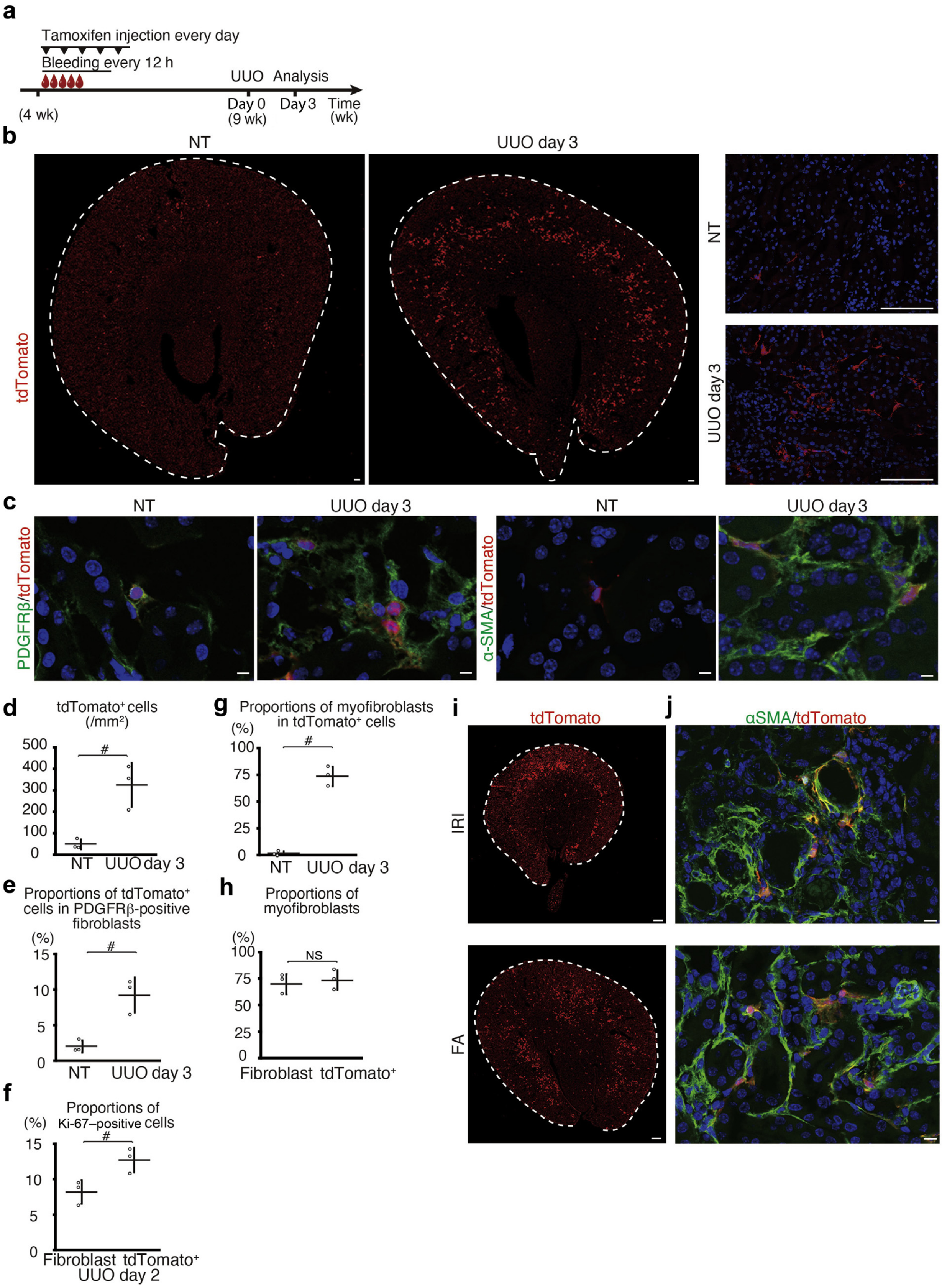

Figure 4 | *Epo*$^{CreERT2/+}$ lineage-labeled cells transdifferentiate into myofibroblasts and proliferate. (**a**) Experimental protocol of (**b–h**). Four-week-old *Epo*$^{CreERT2/+}$*:R26tdTomato* mice were administered tamoxifen with anemia induction. Five weeks after the administration of tamoxifen, *Epo*$^{CreERT2/+}$*:R26tdTomato* mice were subjected to unilateral ureteral obstruction (UUO) and were analyzed 3 days later, (continued)

Table 4 | Number and proportion of tdTomato$^+$ cells, PDGFRβ-positive cells, αSMA–positive cells, and Ki67$^+$ cells during UUO experiment

Cells	NT	UUO, day 2	UUO, day 3
tdTomato-positive cells	47.9 ± 23.6	114.8 ± 17.2	324.4 ± 103.0
α-SMA-positive cells	124.6 ± 35.2	n.d.	2862.1 ± 409.9
PDGFRβ-positive cells	2410.4 ± 205.2	3161.3 ± 254.8	3462.9 ± 215.1
PDGFRβ-positive tdTomato$^+$ cells	46.3 ± 21.6	106.6 ± 21.2	293.8 ± 98.7
PDGFRβ-positive tdTomato$^-$ cells	2364.1 ± 191.0	3054.6 ± 276.1	3169.1 ± 122.8
tdTomato$^+$ cells in PDGFRβ-positive fibroblasts, %	1.9 ± 0.8	3.6 ± 0.8	9.2 ± 2.4
α-SMA-positive myofibroblasts in tdTomato$^+$ cells, %	1.6 ± 2.0	n.d.	73.4 ± 9.2
α-SMA-positive myofibroblasts in PDGFRβ-positive tdTomato$^-$ fibroblasts, %	1.9 ± 0.8	n.d.	69.6 ± 9.4
Ki67$^+$ cells in PDGFRβ-positive tdTomato$^+$ fibroblast	n.d.	12.9 ± 4.9	n.d.
Ki67$^+$ cells in PDGFRβ-positive tdTomato- fibroblast	n.d.	247.2 ± 32.9	n.d.
Ki67+ cells in PDGFRβ-positive tdTomato$^+$ fibroblasts, %	n.d.	12.7 ± 1.7	n.d.
Ki67+ cells in PDGFRβ-positive tdTomato$^-$ fibroblasts, %	n.d.	8.1 ± 1.6	n.d.

αSMA, alpha smooth muscle actin; n.d., not determined; NT, not treated; PDGFRβ, platelet-derived growth factor receptor beta; UUO, unilateral ureteral obstruction. Units are number/mm^2, except proportions, which are specified as %. Data are given as mean ± SD.

Additionally, 2 days after UUO, the proportion of Ki67$^+$ cells among tdTomato$^+$ cells (12.7% ± 1.7%) was significantly higher than that among PDGFRβ-positive fibroblasts without tdTomato expression (8.1% ± 1.6%; Figure 4f; Supplementary Figure S6). These results indicate that tdTomato$^+$ cells had a high capacity to proliferate during fibrosis. Although tdTomato$^+$ cells in healthy kidneys expressed PDGFRβ, but not alpha smooth muscle actin (α-SMA; a marker of myofibroblasts), 73.4% ± 9.2% of tdTomato$^+$ cells began to express α -SMA after UUO, indicating their transdifferentiation into myofibroblasts (Figure 4c and g). The proportions of myofibroblasts in tdTomato$^+$ cells and in PDGFRβ-positive fibroblasts without tdTomato expression were comparable (Figure 4h; the data of tdTomato$^+$ cells shown in Figure 4h are the same data shown in Figure 4g), indicating that *Epo^{CreERT2}*-labeled cells transdifferentiated into myofibroblasts at the same rate as other fibroblasts.

A significant increase in the number of tdTomato$^+$ cells was also observed in other models of kidney injury, ischemic reperfusion injury, and folic acid nephropathy (Figure 4i). Additionally, most of the tdTomato$^+$ cells in these models expressed α-SMA, indicating their transdifferentiation into myofibroblasts (Figure 4j).

Epo-producing cells lose their ability to produce Epo after transdifferentiation into myofibroblasts

Next, we examined whether tdTomato$^+$ cells are able to produce Epo after transdifferentiation into myofibroblasts 14 days after UUO. Even under anemic conditions, when about 43.4% ± 10.7% of *tdTomato* mRNA–expressing cells in the contralateral kidney expressed *Epo* mRNA, only 2.2% ± 3.9% of *tdTomato* mRNA–expressing cells in the diseased kidney expressed *Epo* mRNA, and the proportion of Epo-producing cells among *tdTomato* mRNA–expressing cells was significantly reduced (Figure 5a and b; Table 5). These results show that most *tdTomato* mRNA–expressing cells lost their Epo-producing ability after transdifferentiation into myofibroblasts.

Epo-producing cells that lose their ability to produce Epo during fibrosis regain their ability after kidney repair

Finally, we examined whether *tdTomato* mRNA–expressing cells have the capacity to restore Epo-producing ability

Figure 4 | (continued) except in the experiment depicted in (**f**). (**b**) The left and middle large panels are images of whole-kidney cross-sections. The number of tdTomato$^+$ cells increased dramatically after UUO. The white dotted lines show outlines of the kidney. The right small panels are the same image with higher magnification. The upper panel shows tdTomato$^+$ cells in non-treated (NT) kidney, and the lower panel shows those in UUO kidney. Bars = 100 μm. (**c**) The left panels show that tdTomato$^+$ cells expressed platelet-derived growth factor receptor beta (PDGFRβ) in NT and UUO kidneys. The right panels show that tdTomato$^+$ cells in UUO kidneys, but not those in NT kidneys, expressed alpha smooth muscle actin (α-SMA). Bars = 5 μm. (**d**) Graph illustrating the numbers of tdTomato$^+$ cells in NT and UUO kidneys. The numbers were 47.9 ± 23.6/mm^2 and 324.4 ± 103.0/mm^2, respectively. (**e**) Graph illustrating the proportions of tdTomato$^+$ cells in PDGFRβ-positive fibroblasts in NT and UUO kidneys. The proportions were 1.9% ± 0.8% and 9.2% ± 2.4% in NT and UUO kidneys, respectively. (**f**) Graph illustrating the proportions of Ki67$^+$ cells in PDGFRβ-positive tdTomato$^-$ fibroblasts and tdTomato$^+$ cells in UUO kidneys 2 days after the operation. The proportions were 8.1% ± 1.6% and 12.7% ± 1.7% in PDGFRβ-positive tdTomato$^-$ fibroblasts and tdTomato$^+$ cells, respectively. (**g**) Graph illustrating the proportions of α-SMA-positive myofibroblasts in tdTomato$^+$ cells. The proportions were 1.6% ± 2.0% and 73.4% ± 9.2% in NT and UUO kidneys, respectively. (**h**) Graph illustrating the proportions of α-SMA-positive myofibroblasts in PDGFRβ-positive tdTomato$^-$ fibroblasts and PDGFRβ-positive tdTomato$^+$ cells. The proportions were 69.6% ± 9.4% and 73.4% ± 9.2% in tdTomato$^-$ fibroblasts and tdTomato$^+$ cells, respectively. The data on tdTomato$^+$ cells in (**h**) are the same data shown in (**g**). (**i**) *Epo$^{CreERT2/+}$*-labeled cells were increased and transdifferentiated into myofibroblasts after unilateral ischemic reperfusion injury (IRI) and folic acid (FA) nephropathy. The upper panels show the images of IRI, and the lower panels show the images of FA nephropathy. The numbers of tdTomato$^+$ cells were increased 14 days after 45-minute IRI, and 3 days after FA administration in the whole-kidney cross-section. Bars = 300 μm. (**j**) tdTomato$^+$ cells were positive for α-SMA in IRI and FA nephropathy. Bars = 10 μm. Statistical analysis was performed using Student's *t* test. Data are given as mean ± SD. $n = 3$. $^{\#}P < 0.05$. NS, not significant. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

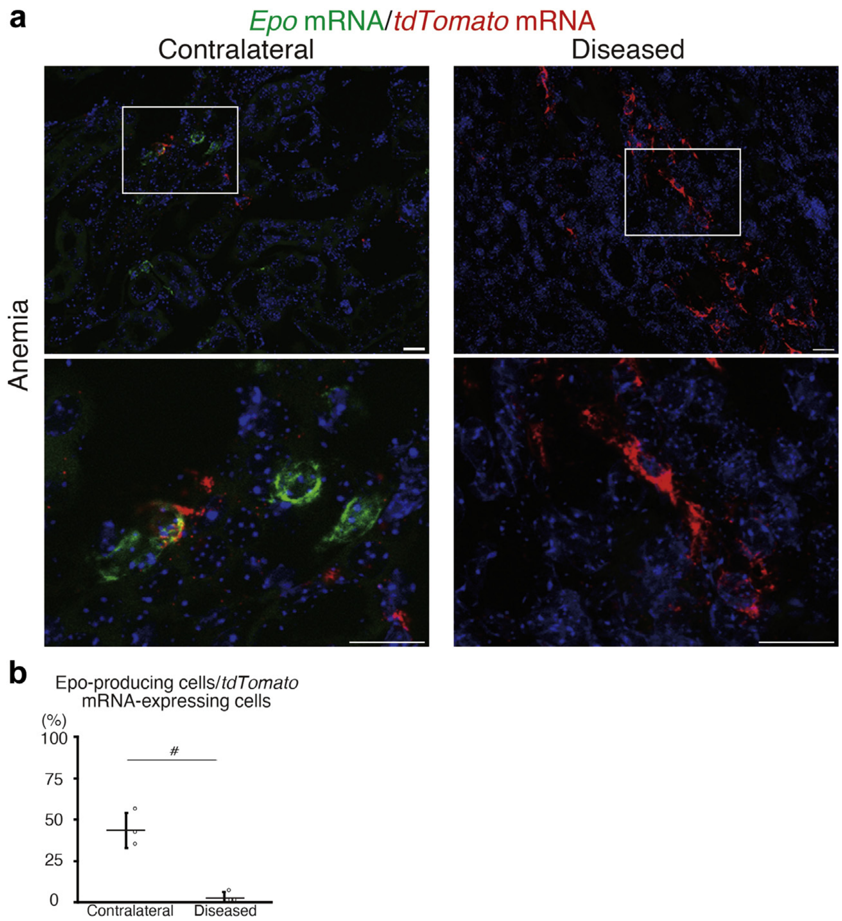

Figure 5 | $Epo^{CreERT2/+}$-labeled cells lose erythropoietin (Epo) production ability after unilateral ureteral obstruction (UUO). High-sensitivity double *in situ* hybridization of the kidney 14 days after UUO with induction of anemia. (**a**) The left panels show the contralateral kidney, and the right panels show the diseased kidney 14 days after UUO. Boxed regions in the upper panels are shown at higher magnification in the lower panels. About half of all $tdTomato^+$ cells in the contralateral kidney expressed *Epo* mRNA, whereas very few $tdTomato^+$ cells in the diseased kidney expressed *Epo* mRNA. Bars = 20 μm. (**b**) Graph illustrating the proportion of *tdTomato*$^+$ cells coexpressing *Epo* mRNA (Epo-producing cells/$tdTomato^+$ cells). The proportion of Epo-producing cells in *tdTomato* mRNA–expressing cells was 43.4% ± 10.7% in the contralateral kidney and 2.2% ± 3.9% in the diseased kidney. $n = 3$, #: contralateral versus diseased, $P < 0.05$. Statistical analysis was performed using Student's *t* test. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

when kidney injury is repaired. We utilized a reversible UUO model, in which a vascular clip was used for ureter obstruction instead of ureter ligation, and hydronephrosis would be reversed by clip removal 3 days after UUO. Because the anemia induction protocol of 5 bleedings would result in minimal hydronephrosis due to dehydration, we changed the anemia-induction protocol in this model—instead, mice underwent a single bleeding followed by peritoneal injection of the same amount of saline (Figure 6a). We performed analysis at 3 time points, with anemia induced by a single bleeding—before injury (non-treat; NT), 3 days after ureteral obstruction by the clip (Injury), and 14 days after clip removal (Repaired). The numbers of Epo-producing cells and the proportions of Epo-producing cells among *tdTomato* mRNA–expressing cells decreased from 57.4 ± 12.8/mm^2 to 7.0 ± 3.6/mm^2, and 19.0% ± 7.8% to 2.6% ± 0.6%, 3 days after ureter obstruction, but rebounded 14 days after clip removal to 49.8 ± 2.4/mm^2 and 20.1% ± 6.0%, respectively (Figure 6b and c; Table 6). The Hct levels were 31.1 ± 1.6% in the NT group, 34.1 ± 2.0% in the Injury group, and 28.6 ± 2.6% in the Repaired group (Figure 6d), and there was no significant difference between the NT and Repaired groups.

Table 5 | Number and proportion of Epo-producing cells and *tdTomato* mRNA–expressing cells 14 days after UUO with anemia induction

Cells	Contralateral	Diseased
Epo-producing cells	90.6 ± 57.3	1.9 ± 3.2
tdTomato mRNA-expressing cells	25.9 ± 6.8	46.1 ± 21.7
Coexpressing cells	11.6 ± 5.7	0.5 ± 0.9
Epo-producing cells in *tdTomato* mRNA–expressing cells, %	43.4 ± 10.7	2.2 ± 3.9
tdTomato mRNA–expressing cells in Epo-producing cells, %	13.7 ± 3.3	10.0 ± 17.3

Epo, erythropoietin; UUO, unilateral ureteral obstruction.
Units are number/mm^2, except proportions, which are specified as %. Data are given as mean ± SD.

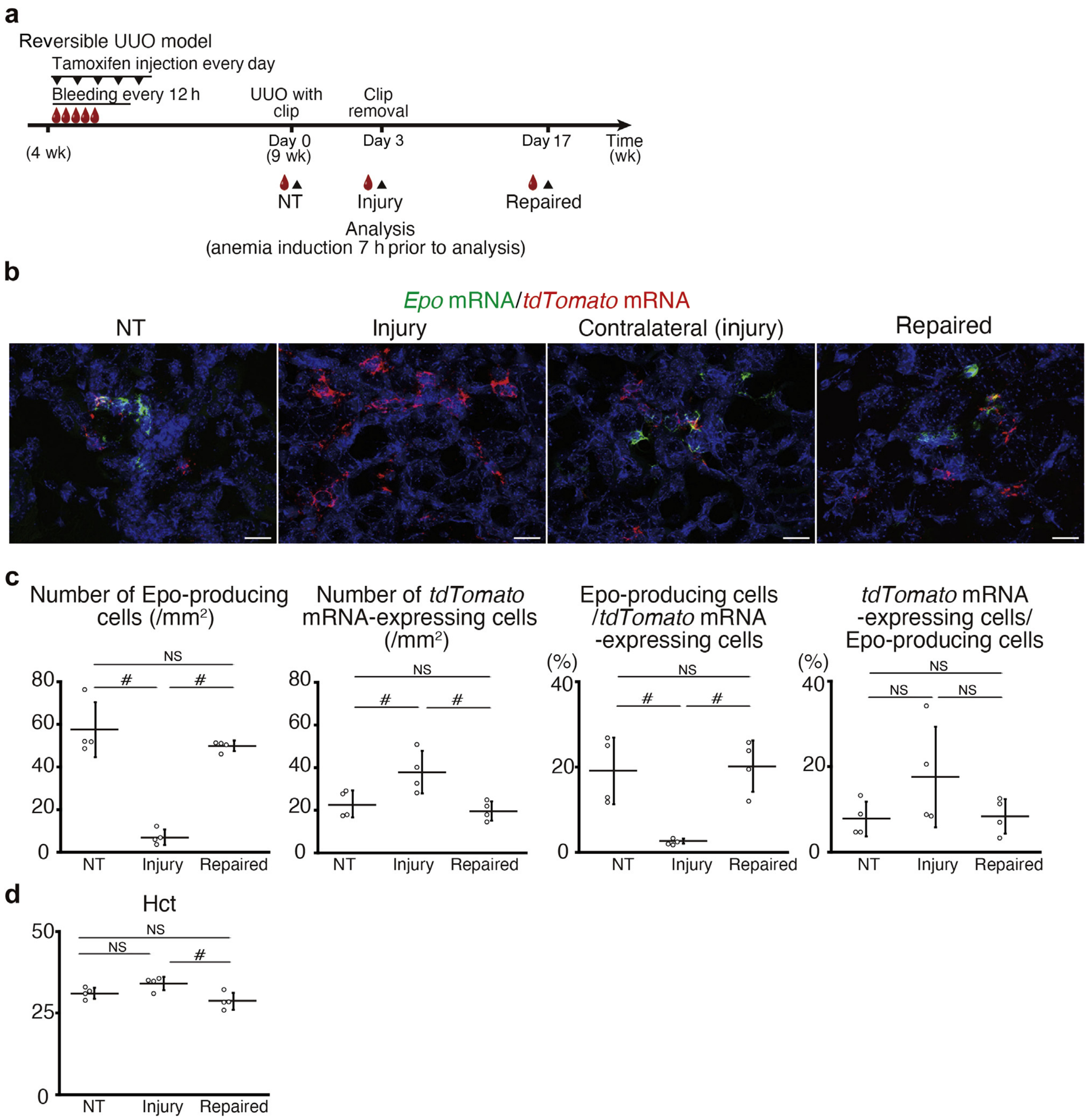

Figure 6 | *Epo*$^{CreERT2/+}$-labeled cells that lose their erythropoietin (Epo)-producing ability during fibrosis regain this ability after kidney repair. (**a**) Experimental protocol of (**b–d**). Four-week-old *Epo*$^{CreERT2/+}$*:R26tdTomato* mice were administered tamoxifen with anemia induction. Five weeks after the administration of tamoxifen, *Epo*$^{CreERT2/+}$*:R26tdTomato* mice were subjected to reversible unilateral ureteral obstruction (UUO) with vascular clip. We performed analysis at 3 time points: before injury (not treated [NT]), on the third day of UUO by the clip (Injury), and 14 days after clip removal (Repaired). Mice underwent a single bleeding followed by peritoneal injection of the same amount of saline. (**b**) High-sensitivity double *in situ* hybridization of the kidney of *Epo*$^{CreERT2/+}$*:R26tdTomato* mice euthanized at the 3 time points: NT, Injury, and Repaired. The center right panel shows the contralateral kidney of Injury mice. Bars = 20 µm. (**c**) Graph showing the numbers of Epo-producing cells, the numbers of *tdTomato* mRNA–expressing cells, the proportion of Epo-producing cells in *tdTomato* mRNA–expressing cells, as well as the proportion of *tdTomato* mRNA–expressing cells in Epo-producing cells. The numbers of Epo-producing cells were 57.4 ± 12.8/mm^2, 7.0 ± 3.6/mm^2, and 49.8 ± 2.4/mm^2 at the NT, Injury, and Repaired time points, respectively. The numbers of tdTomato$^+$ cells at each time point were 22.8 ± 6.3/mm^2, 37.8 ± 9.9/mm^2, and 19.5 ± 4.5/mm^2, respectively. The proportions of Epo-producing cells in *tdTomato* mRNA–expressing cells were 19.0% ± 7.8%, 2.6% ± 0.6% and 20.1% ± 6.0%, respectively. The proportions of *tdTomato* mRNA–expressing cells in Epo-producing cells were 7.7% ± 4.0%, 17.5% ± 11.8% and 8.3% ± 4.0%, respectively. (**d**) Graph illustrating hematocrit (Hct) at the NT, Injury, and Repaired time points. Hct was 31.1 ± 1.6% in NT, 34.1 ± 2.0% in Injury, and 28.6 ± 2.6% in Repaired. Statistical analysis was performed using 1-way analysis of variance followed by Tukey-Kramer *post hoc* analysis in (**c**) and (**d**). Data are given as mean ± SD. $n = 4$. NS, not significant. $^{\#}P < 0.05$. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Table 6 | Number and proportion of Epo-producing cells and *tdTomato* mRNA–expressing cells in reversible UUO

Cells	NT	Injury	Repaired
Epo-producing cells	57.4 ± 12.8	7.0 ± 3.6	49.8 ± 2.4
tdTomato mRNA–expressing cells	22.8 ± 6.3	37.8 ± 9.9	19.5 ± 4.5
Coexpressing cells	4.2 ± 1.9	1.0 ± 0.2	4.1 ± 2.0
Epo-producing cells in *tdTomato* mRNA–expressing cells, %	19.0 ± 7.8	2.6 ± 0.6	20.1 ± 6.0
tdTomato mRNA–expressing cells in Epo-producing cells, %	7.7 ± 4.0	17.5 ± 11.8	8.3 ± 4.0

Epo, erythropoietin; NT, not treated; UUO, unilateral ureteral obstruction.
Units are number/mm^2, except proportions, which are specified as %. Data are given as mean ± SD.

The number of *tdTomato* mRNA–expressing cells was increased in the Injury group, possibly due to proliferation, but also returned to the basal levels in the Repaired group (Figure 6c). These results show that *tdTomato* mRNA–expressing cells lose Epo-producing ability during kidney injury but regain Epo-producing ability after kidney repair.

DISCUSSION

In the present study, we succeeded in labeling and tracing Epo-producing cells at specific time points and clarified the behavior of Epo-producing cells. In our previous study, we demonstrated that Epo is produced by some fibroblasts in the kidney, and that Epo-producing ability is lost in the process of fibroblast-to-myofibroblast transdifferentiation during fibrosis.[11] Although these previous results clarified the behavior of fibroblasts, the behavior of Epo-producing cells remained unclear. In the present study, for the first time, we have proven directly that Epo-producing cells under physiological conditions survive and transform into myofibroblasts during fibrosis and lose their Epo-producing ability. In addition, we showed that the same Epo-producing cells regain their Epo-producing ability after kidney repair.

Until now, the question of whether Epo-producing cells constitute a specialized population among fibroblasts, or whether all fibroblasts possess Epo-producing ability and a small number of fibroblasts stochastically produce Epo, has remained unanswered. In the current study, we found that *Epo*$^{CreERT2/+}$-labeled fibroblasts constituted only 2% of PDGFRβ-positive fibroblasts (Figure 4e), and that this subpopulation of PDGFRβ-positive fibroblasts repeatedly produced Epo in response to hypoxic insult over a period of 16 weeks (Figure 3). Furthermore, *Epo*$^{CreERT2/+}$-labeled cells lose their Epo-producing ability during fibrosis but regain the ability after kidney repair. These results support the possibility that Epo-producing cells are a unique subpopulation among the fibroblasts in the kidney.

By using this unique mouse line, we were able to elucidate the behavior of Epo-producing cells, which has not been analyzed clearly in previous reports. Souma *et al.* previously reported that cells with a history of Epo production expressed fibroblast markers such as PDGFRβand CD73 in healthy kidneys, and that those cells transdifferentiated into myofibroblasts and lost their Epo production ability during fibrosis.[18] However, as their work used *Epo-Cre* mice mated with another mouse line inducing severe fetal anemia,[10] all cells with a history of Epo production *in utero* were labeled, resulting in the lineage labeling of almost all fibroblasts, which may not reflect the behavior of Epo-producing cells in adult mice. Recently, another group published a paper using *EpoCre*^{ERT2} mice similar to ours, but as the goal of that study was to generate an Epo-producing cell line, the analysis was done mostly *in vitro*.[19] Our *Epo*$^{CreERT2/+}$ mice enabled us for the first time to trace the behavior of Epo-producing cells at any given time point and to distinguish them from other fibroblasts.

Our novel mouse line has some weaknesses. First, Epo reactivity to anemia was attenuated in *Epo*$^{CreERT2/+}$ mice, compared with that in wild-type littermates. To induce recombination faithfully in Epo-producing cells, the *Cre*^{ERT2} cassette was knocked-in into the *Epo* locus at the position of the initiation codon, which makes it inevitable for *Epo* genes to be heterozygous. Another possibility is that the knock-in might have affected the regulation of Epo production, as well as the study outcome. Nevertheless, considering that *Epo* mRNA expression in the kidney and EPO concentrations in the blood increased in response to anemia in this mouse line (Figure 1g), and that the numbers of labeled cells increased in response to anemia (Figure 2b and c), the results of our experiments using this mouse line are reliable.

In our novel mouse strain, about 50% of *Epo*$^{CreERT2/+}$-labeled cells expressed *Epo* mRNA 48 hours after tamoxifen administration. One possible reason that only 50% of *tdTomato* mRNA–expressing cells coexpressed *Epo* mRNA is ectopic *Cre* expression. However, we demonstrated the following: (i) *Epo*$^{CreERT2/+}$-labeled cells were fibroblasts in the corticomedullary interstitium, as previously reported (Figure 2e)[4,17]; (ii) anemia induction increased the number of *Epo*$^{CreERT2/+}$-labeled cells (Figure 2c); and (iii) most *Cre* mRNA–expressing cells in *Epo*$^{CreERT2/+}$ mice expressed *Epo* mRNA (Figure 2h). We also showed that almost no recombination occurred in the absence of tamoxifen (Supplementary Figure S3). These data indicate that ectopic recombination is very unlikely. Another possible explanation is the time lag between the recombination and the analysis. Epo-producing cells are considered to be regulated with an on–off mode, as evidenced by the fact that the number of Epo-producing cells in the kidney increases with anemia.[4,13] Imeri *et al.* developed an immortalized cell line of Epo-producing cells and showed that Epo production activated by hypoxia *in vitro* decreased within 24 hours, even in continuous hypoxia.[19] Therefore, some cells that had been producing Epo at the time of recombination might have turned off Epo production by the time of analysis, which was 48 hours after the first tamoxifen administration. Additionally, the expression of *Cre*^{ERT2} in *Epo*$^{CreERT2/+}$ mice, which is driven by authentic *Epo* promotor, might not be sufficient for complete recombination in all Epo-expressing cells, and might be active only in the cells expressing higher amounts of Epo. These results might alternatively indicate the possibility that a fixed pool expresses Epo, but with a more plastic subset that is either on or off depending on the hypoxic conditions in their microenvironment. Indeed, some data do support the heterogeneity of Epo-producing cells. Kobayashi *et al.* have shown that induction of anemia in fibroblast-specific *Phd2*-deficient mice further increased the number of Epo-producing cells, indicating that a hierarchy of Epo-producing cells may be present.[20] Another group showed that different subpopulations of kidney fibroblasts produced Epo in response to different stimuli, such as genetic stabilization of hypoxia-inducible factor and hypoxia.[21]

Another technical issue is the low level of labeling efficiency of this mouse strain. Only about 10% of Epo-producing cells were labeled in the $Epo^{CreERT2/+}$ mice, indicating that the recombination in this mouse line was not very effective. This labeling inefficiency may be an inevitable property of $Epo^{CreERT2/+}$ mice, because in a previous report describing $Epo^{CreERT2/+}$ mice, the number of labeled cells was around 0.3/mm^2 after hypoxic insult,[19] whereas the number of labeled cells in our study was around 10/mm^2. Given that the hypoxic insults are different, we cannot make a simple comparison, but we can tell that the recombination efficiency in $Epo^{CreERT2/+}$ mice is generally low. The recombination efficiency of Cre^{ERT2} is well known to depend on the amount of Cre^{ERT2} protein,[22] and expression induced by the Epo promoter alone may not be sufficient for efficient recombination. If we had knocked-in some additional potent promoter in the *Epo* locus, we might have been able to increase the efficiency of recombination, but the regulation of *Cre* expression might have been affected. In spite of the limitations of this study, the $Epo^{CreERT2/+}$ mice revealed the unique behaviors of Epo-producing cells, and these mice will serve as a useful tool for the analysis of Epo-producing cells.

We previously reported that neuroprotective agents such as dexamethasone and neurotrophins restore Epo production in cultured renal myofibroblasts,[11] and another group demonstrated that human growth hormone and insulin-like growth factor-I inhibit erythropoietin secretion from rat kidneys.[23] A recent study suggests that transforming growth factor-β signaling decreases Epo production in fibrotic kidneys.[24] These results suggest that factors other than hypoxia could additionally regulate Epo production or the condition of Epo-producing cells. Prolyl hydroxylase domain (PHD) inhibitors are expected to be a new therapeutic agent for renal anemia, and recent phase 3 trials of these inhibitors have demonstrated their effectiveness in the treatment of renal anemia.[25–29] However, a concern is that the systemic activation of hypoxia-inducible factor could induce side effects such as tumorigenesis, thrombosis, or pulmonary hypertension.[30–32] The analysis of $Epo^{CreERT2/+}$ mice will provide important insights into the behaviors and regulatory mechanisms of Epo-producing cells, and may lead to the development of novel treatments for renal anemia.

DISCLOSURE

YS is employed by the TMK Project, which is a collaboration project between Kyoto University and Mitsubishi Tanabe Pharma. MY receives research grants from Mitsubishi Tanabe Pharma and Boehringer Ingelheim. All the other authors declared no competing interests.

DATA STATEMENT

For original data, please contact motoy@kuhp.kyoto-u.ac.jp.

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AUTHOR CONTRIBUTIONS

KK and MY designed the experiments and wrote the article. MS and HK generated the $Epo^{CreERT2/+}$ mice. KK, YS, and SE carried out the experiments. KK, EU, NT, and SF analyzed the data. MY supervised the project. All authors contributed to the discussion and approved the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary File (PDF)

Supplementary Full Methods.

Figure S1. *Epo* mRNA–expressing cells in the kidneys and cerebrum in non-anemic conditions.

Figure S2. *Epo* mRNA–expressing cells are located in the corticomedullary region with or without anemia.

Figure S3. Very rare spontaneous recombination in the kidneys of $Epo^{CreERT2/+}$:*R26tdTomato* mice.

Figure S4. Very few tdTomato-positive cells in the glomeruli and collecting ducts in the kidneys of $Epo^{CreERT2/+}$:*R26tdTomato* mice.

Figure S5. The distribution of *Epo* mRNA–expressing cells and *tdTomato* mRNA–expressing cells in the kidneys of $Epo^{CreERT2/+}$:*R26tdTomato* mice.

Figure S6. Triple immunostaining of Ki67, platelet-derived growth factor receptor beta (PDGFRβ), and tdTomato in the kidneys of $Epo^{CreERT2/+}$:*R26tdTomato* mice.

REFERENCES

1. Wojchowski DM, Sathyanarayana P, Dev A. Erythropoietin receptor response circuits. *Curr Opin Hematol*. 2010;17:169–176.
2. Haase VH. Hypoxic regulation of erythropoiesis and iron metabolism. *Am J Physiol Renal Physiol*. 2010;299:F1–F13.
3. Koury MJ, Haase VH. Anaemia in kidney disease: harnessing hypoxia responses for therapy. *Nat Rev Nephrol*. 2015;11:394–410.
4. Obara N, Suzuki N, Kim K, et al. Repression via the GATA box is essential for tissue-specific erythropoietin gene expression. *Blood*. 2008;111:5223–5232.
5. Sato Y, Yanagita M. Renal anemia: from incurable to curable. *Am J Physiol Renal Physiol*. 2013;305:F1239–F1248.
6. Suzuki N, Obara N, Yamamoto M. Use of gene-manipulated mice in the study of erythropoietin gene expression. *Methods Enzymol*. 2007;435:157–177.

7. Brezis M, Heyman SN, Dinour D, et al. Role of nitric oxide in renal medullary oxygenation. Studies in isolated and intact rat kidneys. *J Clin Invest*. 1991;88:390–395.
8. Kurtz A. Endocrine functions of the renal interstitium. *Pflugers Arch*. 2017;469:869–876.
9. Suzuki N, Yamamoto M. Roles of renal erythropoietin-producing (REP) cells in the maintenance of systemic oxygen homeostasis. *Pflugers Arch*. 2016;468:3–12.
10. Yamazaki S, Souma T, Hirano I, et al. A mouse model of adult-onset anaemia due to erythropoietin deficiency. *Nat Commun*. 2013;4:1950.
11. Asada N, Takase M, Nakamura J, et al. Dysfunction of fibroblasts of extrarenal origin underlies renal fibrosis and renal anemia in mice. *J Clin Invest*. 2011;121:3981–3990.
12. Mack M, Yanagita M. Origin of myofibroblasts and cellular events triggering fibrosis. *Kidney Int*. 2015;87:297–307.
13. Koury ST, Bondurant MC, Koury MJ. Localization of erythropoietin synthesizing cells in murine kidneys by in situ hybridization. *Blood*. 1988;71:524–527.
14. Lacombe C, Da Silva JL, Bruneval P, et al. Peritubular cells are the site of erythropoietin synthesis in the murine hypoxic kidney. *J Clin Invest*. 1988;81:620–623.
15. Yagi T, Tokunaga T, Furuta Y, et al. A novel ES cell line, TT2, with high germline-differentiating potency. *Anal Biochem*. 1993;214:70–76.
16. Wu H, Liu X, Jaenisch R, et al. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell*. 1995;83:59–67.
17. Bachmann S, Le Hir M, Eckardt KU. Co-localization of erythropoietin mRNA and ecto-5′-nucleotidase immunoreactivity in peritubular cells of rat renal cortex indicates that fibroblasts produce erythropoietin. *J Histochem Cytochem*. 1993;41:335–341.
18. Souma T, Yamazaki S, Moriguchi T, et al. Plasticity of renal erythropoietin-producing cells governs fibrosis. *J Am Soc Nephrol*. 2013;24:1599–1616.
19. Imeri F, Nolan KA, Bapst AM, et al. Generation of renal Epo-producing cell lines by conditional gene tagging reveals rapid HIF-2 driven Epo kinetics, cell autonomous feedback regulation, and a telocyte phenotype. *Kidney Int*. 2019;95:375–387.
20. Kobayashi H, Liu Q, Binns TC, et al. Distinct subpopulations of FOXD1 stroma-derived cells regulate renal erythropoietin. *J Clin Invest*. 2016;126:1926–1938.
21. Broeker KAE, Fuchs MAA, Schrankl J, et al. Different subpopulations of kidney interstitial cells produce erythropoietin and factors supporting tissue oxygenation in response to hypoxia in vivo. *Kidney Int*. 2020;98:918–931.
22. Swonger JM, Liu JS, Ivey MJ, et al. Genetic tools for identifying and manipulating fibroblasts in the mouse. *Differentiation*. 2016;92:66–83.
23. Sohmiya M, Kato Y. Human growth hormone and insulin-like growth factor-I inhibit erythropoietin secretion from the kidneys of adult rats. *J Endocrinol*. 2005;184:199–207.
24. Fuchs MAA, Broeker KAE, Schrankl J, et al. Inhibition of transforming growth factor β1 signaling in resident interstitial cells attenuates profibrotic gene expression and preserves erythropoietin production during experimental kidney fibrosis in mice. *Kidney Int*. 2021;100:122–137.
25. Bernhardt WM, Wiesener MS, Scigalla P, et al. Inhibition of prolyl hydroxylases increases erythropoietin production in ESRD. *J Am Soc Nephrol*. 2010;21:2151–2156.
26. Besarab A, Chernyavskaya E, Motylev I, et al. Roxadustat (FG-4592): correction of anemia in incident dialysis patients. *J Am Soc Nephrol*. 2016;27:1225–1233.
27. Chen N, Hao C, Peng X, et al. Roxadustat for anemia in patients with kidney disease not receiving dialysis. *N Engl J Med*. 2019;381:1001–1010.
28. Chen N, Hao C, Liu BC, et al. Roxadustat treatment for anemia in patients undergoing long-term dialysis. *N Engl J Med*. 2019;381:1011–1022.
29. Nangaku M, Kondo K, Kokado Y, et al. Phase 3 randomized study comparing vadadustat with darbepoetin alfa for anemia in Japanese patients with nondialysis-dependent CKD. *J Am Soc Nephrol*. 2021;32:1779–1790.
30. Haase VH. Therapeutic targeting of the HIF oxygen-sensing pathway: lessons learned from clinical studies. *Exp Cell Res*. 2017;356:160–165.
31. Sugahara M, Tanaka T, Nangaku M. Prolyl hydroxylase domain inhibitors as a novel therapeutic approach against anemia in chronic kidney disease. *Kidney Int*. 2017;92:306–312.
32. Kurata Y, Tanaka T, Nangaku M. Hypoxia-inducible factor prolyl hydroxylase inhibitor in the treatment of anemia in chronic kidney disease. *Curr Opin Nephrol Hypertens*. 2020;29:414–422.

Supplementary Materials for

Lineage tracing analysis defines erythropoietin-producing cells as a distinct subpopulation of resident fibroblasts with unique behaviors

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Supplementary Materials

This PDF file includes:

Supplementary materials and methods

Supplementary figure legends

Supplementary reference

Figure S1 to S6

Supporting information

Materials and Methods

Animals

Rosa26tdTomato mice [stock number: 007905] were purchased from Jackson Laboratory. Experiments represented in Figure 1 and Table 1 were performed using *Epo*$^{CreERT2/+}$ mice and their wild-type littermates of the N7 generations. Other experiments were performed using *Epo*$^{CreERT2/+}$ mice of the N8 generations. Experiments in Figure 6 were performed with female mice, and all other experiments were performed with male mice.

Anemia induction and the administration of tamoxifen

To increase *Epo* mRNA expression, we induced anemia by phlebotomy, taking 100μL of peripheral blood five times at 12-hour intervals. We also administered 120mg/kg body weight tamoxifen (Sigma-Aldrich, St. Louis, MO, USA) to the mice by intraperitoneal injection, starting at the time of the first blood draw and continuing once daily for five consecutive days. For Figure 2c, we induced anemia with various severity by taking 100 or 200 μL of peripheral blood five times. For Figure 3, four-week-old *Epo*$^{CreERT2/+}$*:R26tdTomato* mice (48 hours) were induced anemia by taking 100 μL of peripheral blood five times, nine-week-old *Epo*$^{CreERT2/+}$*:R26tdTomato* mice (5 weeks) and twenty-week-old *Epo*$^{CreERT2/+}$*:R26tdTomato* mice (16 weeks) were induced anemia by taking 400 μL of peripheral blood five times. For Figure 6, mice weighing less than 18g were subjected to single bleeding of 400 μL and mice weighing more than 18g were subjected to single bleeding of 500 μL followed by peritoneal injection of the same amount of saline 7 hours before analyze.[1]

Kidney injury models

Unilateral ureteral obstruction (UUO), unilateral ischemic reperfusion injury (IRI) and folic acid (FA) nephropathy were induced as described elsewhere.[2-4] To induce UUO, briefly, the left ureter was exposed by flank incision, ligated at the lower pole of the kidney, and cut between two ligated points. At either three or 14 days after surgery, the mice were euthanized and the obstructed kidneys were harvested. For the reversible UUO model, a vascular clip (B-1V, S and T, Neuhausen Switzerland) was used instead of ureter ligation. After three days of the ureter obstruction, the vascular clip was removed. Mice were euthanized after three days of obstruction or 14 days after clip removal with anemia induction. IRI was induced by clamping the left renal pedicles for 45 min. To induce FA nephropathy, FA (250 mg/kg, Sigma-Aldrich) in 0.15M NaHCO3 was administered by a single intraperitoneal injection.

Immunostaining

Histological studies were performed as described previously.[2] Primary antibodies against the following proteins were used: anti-aquaporin 2 (Cat.No.171612, Calbiochem, San Diego, CA, USA), -CD31 (Cat.No.553370, BD Biosciences, San Jose, CA, USA), -CD45 (Cat.No.14-0451-82, eBioscience, San Diego, CA, USA), -CD73 (Cat.No.550738, BD Biosciences), -Ki67 (Cat.No.ACK02, Leica Biosystems, Buffalo Grove, IL, USA), -nephrin (Cat.No.AF3159, R&D Systems, Minneapolis, MN, USA), -PDGFRβ (Cat.No.14-1401-82, eBioscience), and -α-SMA (Cat.No.ab184675, Abcam, Cambridge, MA, USA). Anti-α-SMA antibody were diluted at 1:500 in 3% BSA and 0.3% Triton in PBS. All other primary antibodies were diluted at 1:200. FITC-conjugated lotus lectin (LTL) (Cat.No.FL-1321, Vector Laboratories, Burlingame, CA, USA) was utilized to visualize the brush borders of proximal tubules at 1:200 dilution. Staining was analyzed under a confocal microscope (FV1000D; Olympus, Tokyo, Japan).

Quantitative assessment

For quantification of tdTomato-positive (tdTomato$^+$) cells, fibroblasts, and myofibroblasts in immunostaining, 18 pictures (400×) were taken continuously in the inner cortex of the kidney. We defined fibroblasts as PDGFRβ-positive cells and myofibroblasts as α-SMA-positive cells in the interstitium. Positive cells were automatically analyzed by MetaMorph software (Universal Imaging Corporation, Downingtown, PA, USA). For quantification of *tdTomato* mRNA-expressing cells and Epo-producing cells in high sensitivity *in situ* hybridization (ISH), 18 pictures (400×) were taken continuously in the inner cortex of the kidney. Positive cells were manually analyzed, because ISH sometimes caused nonspecific signals in the tubules, which were not suitable for counting with MetaMorph software.

High sensitivity in situ hybridization

Target mRNA was detected by using the RNAscope Multiplex Fluorescent v2 kit (Advanced Cell Diagnostics, Newark, CA, USA), according to the manufacturer's instructions. Mice were perfused through the heart with 4% paraformaldehyde and kidneys were harvested. The kidneys were fixed in 4% paraformaldehyde for 24 hours and embedded in OCT compound. Blocks were sectioned at 10 μm thickness. Fluorescence detection was performed using TSA-plus FITC and Cy3 (Perkin Elmer, Wellesley, MA, USA). The probes used were *Epo* (Cat.No.315501), *tdTomato* (Cat.No.317041-C2) and *Cre* (Cat.No.312281-C2 or -C3). *Cre* probe was used to detect Cre^{ERT2} mRNA-expressing cells. The mouse gene *Ppib* (Cat.No.313911) was used as positive control probe and the bacterial gene *Dapb* (Cat.No.310043) was used as negative control probe.

Real-time RT-PCR analysis

RNA extraction and real-time PCR were performed as described previously.[2, 4] The primer sequences were as follows: Epo (Forward GCAGCTAGGCGCGGAGAT; Reverse AGGCCCAGAGGAATCAGTAGCAA), GAPDH (Forward CCAGAACATCATCCCTGCATC; Reverse CCTGCTTCACCACCTTTCTTGA).

ELISA

Mouse Erythropoietin Quantikine ELISA kit (MEP00B) was used to measure serum EPO concentrations according to the manufacturer's instructions.

Statistical Analysis

Data were presented as mean ± standard deviation. The differences between two groups were compared using Student's *t*-test. All tests were two-tailed. To compare Epo responsiveness to anemia between WT and $Epo^{CreERT2/+}$ mice, we used analysis of covariance (Figure 1g). To investigate correlations between the number of $tdTomato^{+}$ cells and anemia or the expression of *Epo* mRNA, we used Pearson's correlation test (Figure 2c). For the analysis of the proportions of certain cell populations, we calculated the mean of proportions in each mouse as continuous variables (ranging from 0 to 100%), and compared them between groups by one way ANOVA in Figure 3d, by Student's *t*-test in Figure 4. Data in Figure 1f, 6c and 6d were analyzed by one way ANOVA followed by Tukey-Kramer post hoc analysis. Data are presented as mean ± standard deviation. Statistical analysis was performed using JMP software (version 15, SAS Institute Inc). For all statistical tests, p values < 0.05 were considered statistically significant.

Supplemental Figure Legends

Figure S1. *Epo* mRNA-expressing cells in the kidneys and cerebrum in non-anemic condition

(a) The distribution of *Epo* mRNA-expressing cells in wild-type and $Epo^{CreERT2/+}$ kidney. Because the signals of high sensitivity in situ hybridization cannot be seen in this magnification, the signals of *Epo* mRNA-expressing cells were visualized by yellow dots. Very few *Epo* mRNA-expressing cells were located in the corticomedullary region. Scale bars: 200μm. (b) High sensitivity in situ hybridization of *Epo* mRNA did not show any signal in wild-type and $Epo^{CreERT2/+}$ cerebrum. Lower panel showed the signals with the positive control probe, mouse gene *Ppib*, in the cerebrum of wild-type mice. Scale bars: 10μm.

Figure S2. *Epo* mRNA-expressing cells are located in the corticomedullary region with or without anemia

(a, b) High sensitivity in situ hybridization of *Epo* mRNA of the kidneys of wild-type mice with (b) or without anemia (a). The right panel in (b) shows a higher magnification of the boxed region in the left panel in (b). *Epo* mRNA-expressing cells were located mainly in the corticomedullary region. (c) There was no signal in the anemic kidney with the negative control probe, the bacterial gene *Dapb*. Arrows: *Epo* mRNA-expressing cells. Scale bars: 50 μm.

Figure S3. Very rare spontaneous recombination in the kidneys of $Epo^{CreERT2/+}$:*R26tdTomato* mice

(a) Experimental protocol. Four-week-old $Epo^{CreERT2/+}$:*R26tdTomato* mice were intraperitoneally administered vehicle for five consecutive days with anemia induction and euthanized on the last day of vehicle administration. (b) Immunostaining of tdTomato in the kidneys of $Epo^{CreERT2/+}$:*R26tdTomato* mice with vehicle administration. The right panel shows a higher magnification of the boxed region in the left panel. Only one tdTomato-positive cell was present in this field. Scale bars: 20 µm. (c) Graph illustrating the numbers of tdTomato-positive cells in *Epo*$^{CreERT2/+}$*:R26tdTomato* mice with vehicle and tamoxifen administration. Data of *Epo*$^{CreERT2/+}$*:R26tdTomato* mice with tamoxifen administration were four samples with the same volume of bleeding in Figure 2c. The number of tdTomato-positive cells were 0.09 ± 0.19/mm^2 with vehicle and 22.9 ± 8.9/mm^2 with tamoxifen. Statistical analysis by Student's *t*-test. #: $p < 0.05$. Data are the means ± s.d. n = 3 in vehicle and 4 in tamoxifen.

Figure S4. Very few tdTomato-positive cells in the glomeruli and collecting ducts in the kidneys of *Epo*$^{CreERT2/+}$*:R26tdTomato* mice

(a, b) Few tdTomato-positive cells existed in the glomeruli (a) and the collecting ducts (b). Arrowheads: tdTomato-positive cells. Scale bars: 10µm.

Figure S5. The distribution of *Epo* mRNA-expressing cells and *tdTomato* mRNA-expressing cells in the kidneys of *Epo*$^{CreERT2/+}$*:R26tdTomato* mice

High sensitivity in situ hybridization of *Epo* mRNA and *tdTomato* mRNA in the kidneys of four-week-old *Epo*$^{CreERT2/+}$:*R26tdTomato* mice 48 hours after the first administration of tamoxifen with anemia induction. The panel showed the distribution of *Epo* mRNA-expressing cells (green dot), *tdTomato* mRNA-expressing cells (red dot) and coexpressing cells (yellow dot). Scale bars: 300µm.

Figure S6. Triple immunostaining of Ki67, PDGFRβ, and tdTomato in the kidneys of *Epo*$^{CreERT2/+}$*:R26tdTomato* mice

Upper left panel showed double immunostaining of Ki67 and PDGFRβ, and lower left panel showed double immunostaining of tdTomato and Ki67 in the same field of the kidney of *Epo*$^{CreERT2/+}$*:R26tdTomato* mice. Upper right panel show the overlay of triple staining. In right lower panel, green dots indicated PDGFRβ positive fibroblasts and red dots indicated *Epo*$^{CreERT2/+}$

lineage-labeled cells. Yellow line circles indicated Ki67 positive cells. Scale bars: 10μm.

References

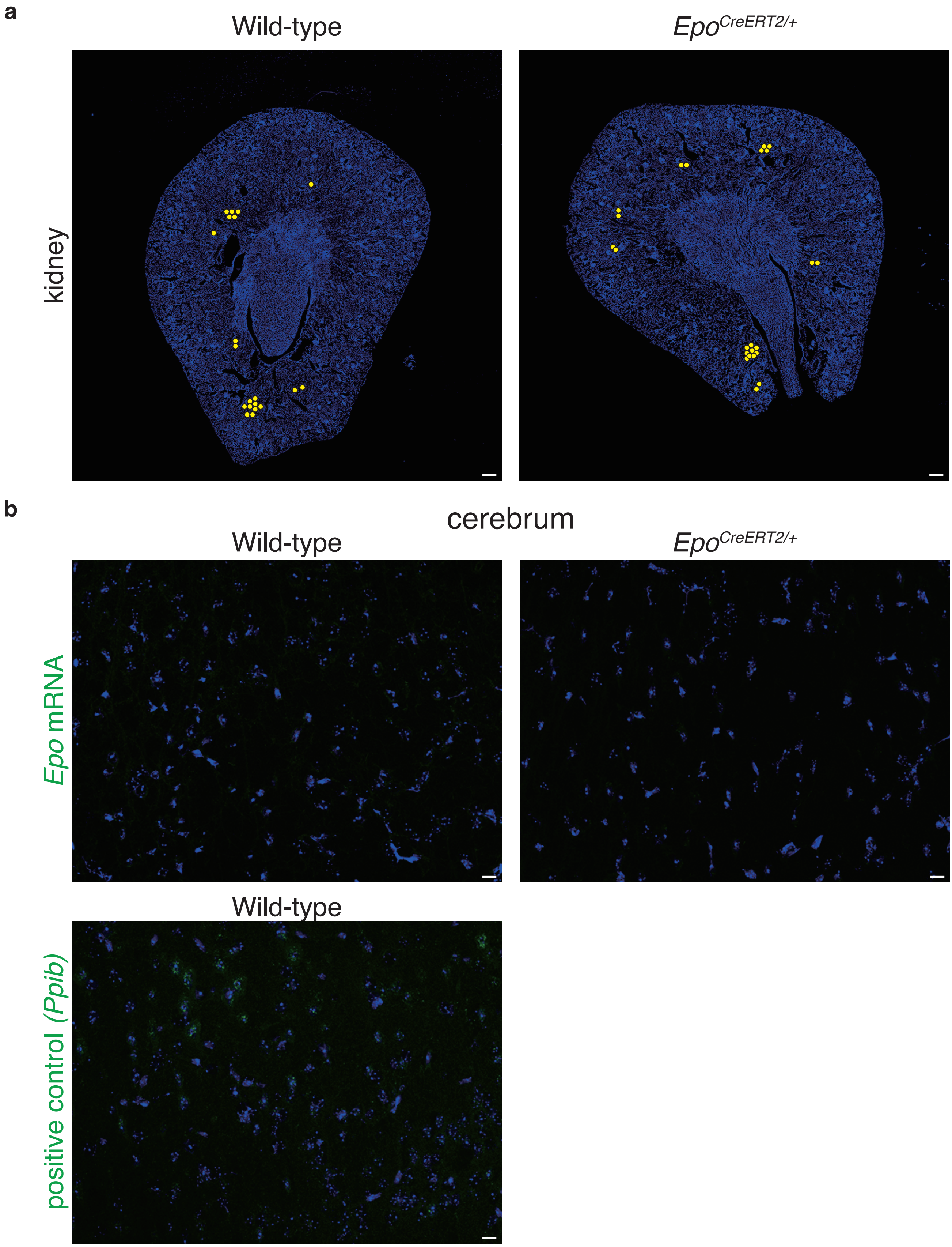
S1. Bondurant MC, Koury MJ. Anemia induces accumulation of erythropoietin mRNA in the kidney and liver. *Mol Cell Biol* 1986; **6:** 2731-2733.

S2. Yanagita M, Okuda T, Endo S, *et al.* Uterine sensitization-associated gene-1 (USAG-1), a novel BMP antagonist expressed in the kidney, accelerates tubular injury. *J Clin Invest* 2006; **116:** 70-79.

S3. Endo T, Nakamura J, Sato Y, *et al.* Exploring the origin and limitations of kidney regeneration. *J Pathol* 2015; **236:** 251-263.

S4. Tanaka M, Endo S, Okuda T, *et al.* Expression of BMP-7 and USAG-1 (a BMP antagonist) in kidney development and injury. *Kidney Int* 2008; **73:** 181-191.

Supplemental Figure S1.



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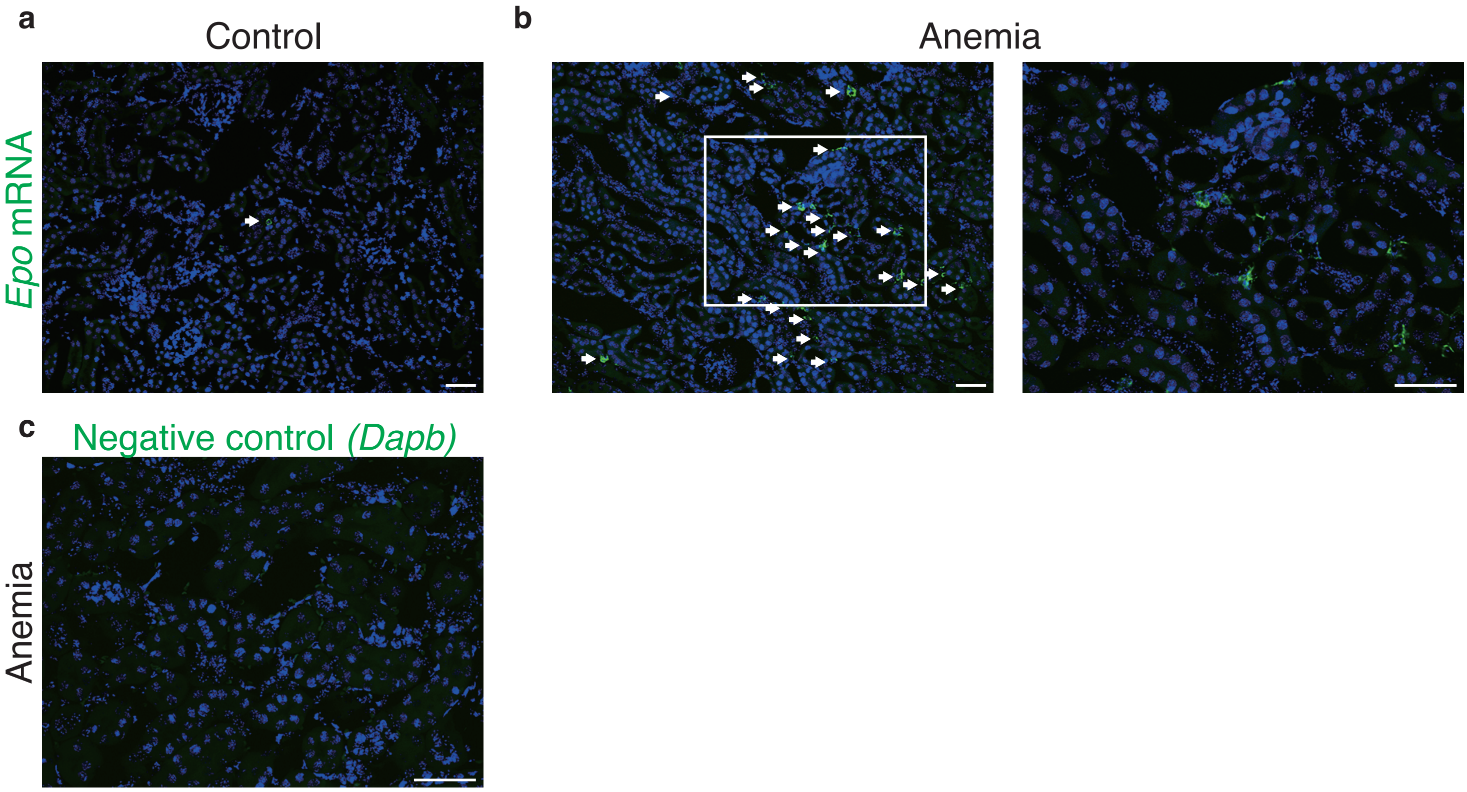
Supplemental Figure S2.



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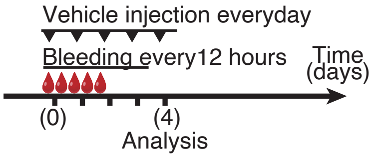
Supplemental Figure S3.

a



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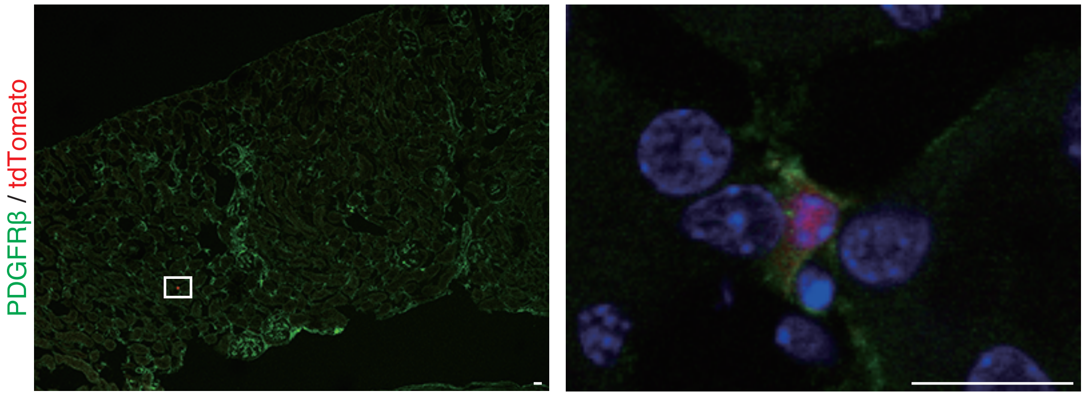

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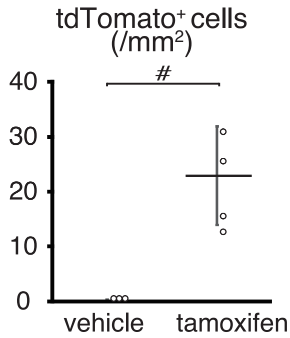

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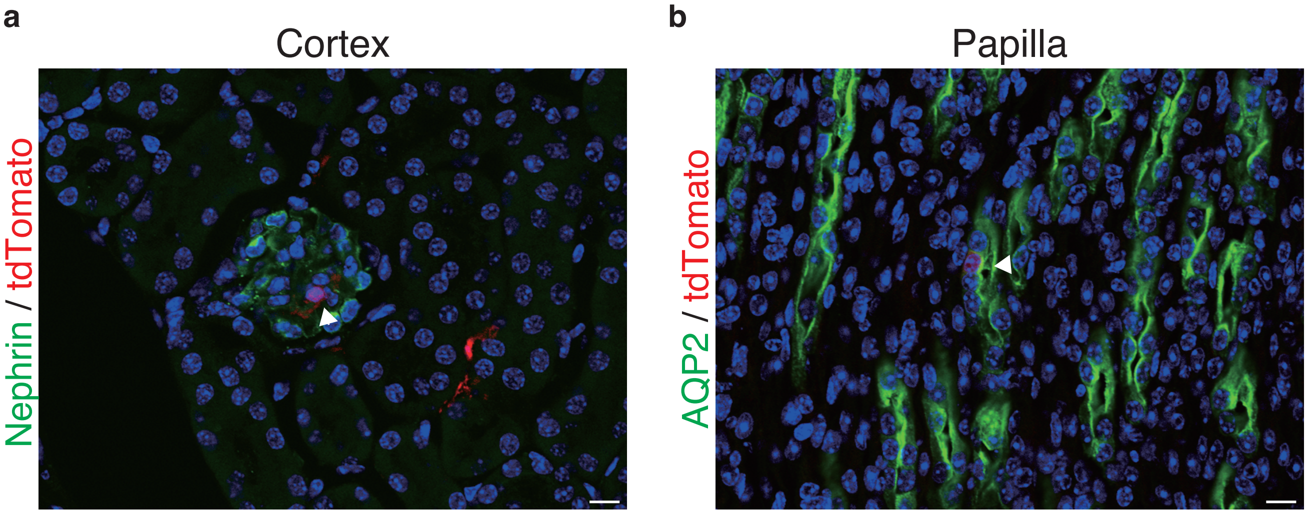
Supplemental Figure S4.



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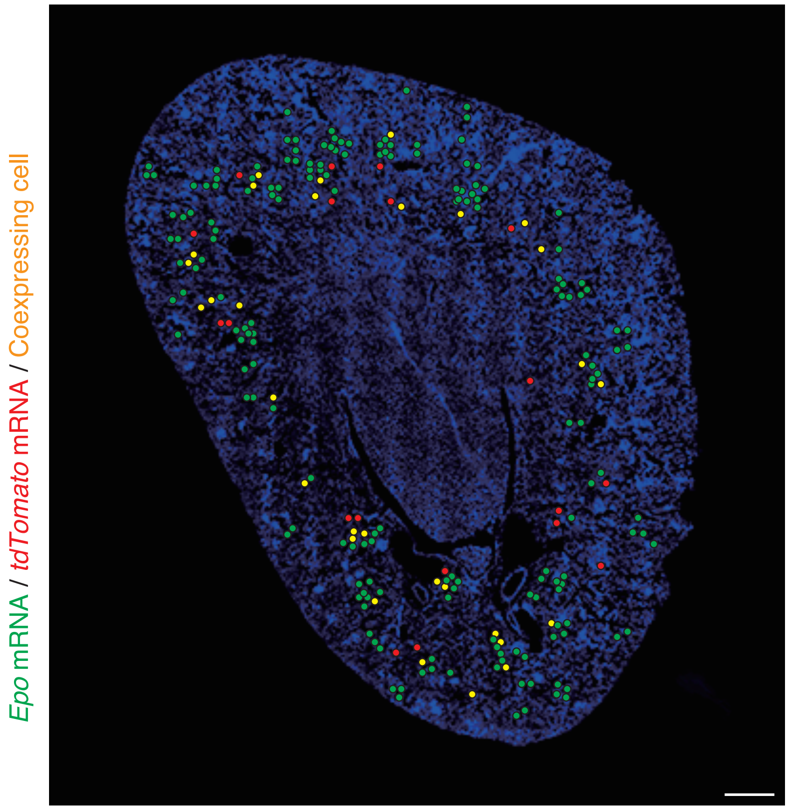
Supplemental Figure S5.



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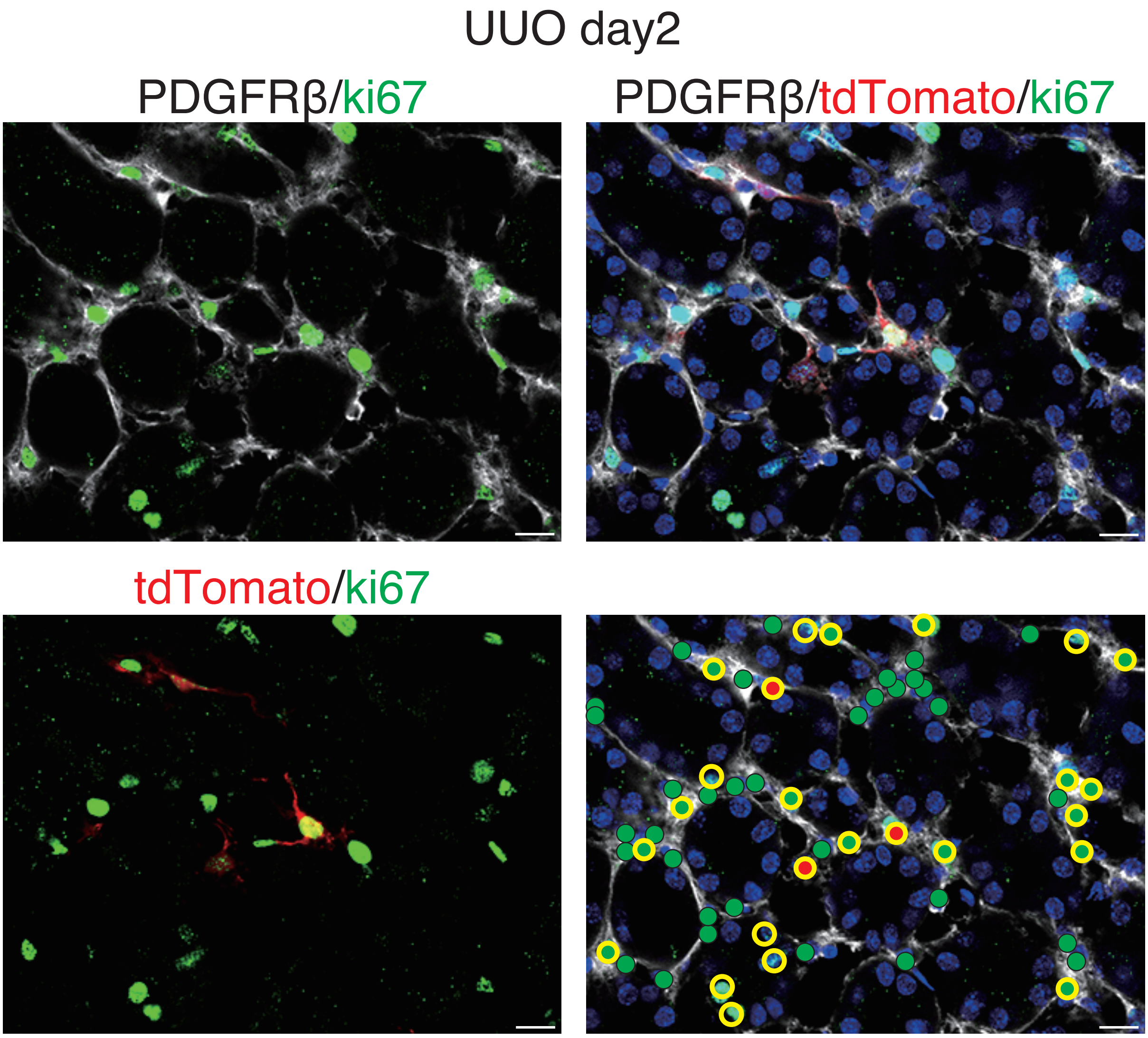
Supplemental Figure S6.



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