DNA methyltransferase inhibition restores erythropoietin production in fibrotic murine kidneys

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Renal erythropoietin-producing cells (REPCs) remain in the kidneys of patients with chronic kidney disease, but these cells do not produce sufficient erythropoietin in response to hypoxic stimuli. Treatment with HIF stabilizers rescues erythropoietin production in these cells, but the mechanisms underlying the decreased response of REPCs in fibrotic kidneys to anemic stimulation remain elusive. Here, we show that fibroblast-like FOXD1+ progenitor-derived kidney pericytes, which are characterized by the expression of α type I collagen and PDGFRβ, produce erythropoietin through HIF2α regulation but that production is repressed when these cells differentiate into myofibroblasts. DNA methyltransferases and erythropoietin hypermethylation are upregulated in myofibroblasts. Exposure of myofibroblasts to nanomolar concentrations of the demethylating agent 5-azacytidine increased basal expression and hypoxic induction of erythropoietin. Mechanistically, the profibrotic factor TGF-β1 induced hypermethylation and repression of erythropoietin in pericytes; these effects were prevented by 5-azacytidine treatment. These findings shed light on the molecular mechanisms underlying erythropoietin repression in kidney myofibroblasts and demonstrate that clinically relevant, nontoxic doses of 5-azacytidine can restore erythropoietin production and ameliorate anemia in the setting of kidney fibrosis in mice.

Introduction

Low levels of plasma erythropoietin (EPO) that are disproportional to the degree of anemia are often observed in patients with chronic kidney disease (CKD) (1, 2). However, the oxygen-EPO-hemoglobin feedback loop is still operating, even if at a lower set point (3). Indeed, plasma EPO concentrations in patients with CKD decline after blood transfusion and measurably increase after hemorrhage, even while levels remain low to the point of anemia (4, 5). Although hepatocytes can produce EPO in patients with CKD after hemorrhage, it is possible that renal EPO-producing cells (REPCs) continue functioning in fibrotic kidneys, but their response to anemic stimulation decreases (6). A recent clinical trial studied an inhibitor of prolyl-hydroxylase domain (PHD) enzyme, FG-2216, which stabilizes HIFs independent of oxygen availability in hemodialysis (HD) patients and healthy volunteers (7). FG-2216 increases plasma EPO levels 30.8-fold in HD patients with fibrotic kidneys, 14.5-fold in anephric HD patients, and 12.7-fold in healthy volunteers, demonstrating that enhancement of HIFs can stimulate endogenous EPO production and retain REPC function in fibrotic kidneys (7).

REPCs, which have long projections between tubules and blood vessels, are detected in the interstitium (8–13). Lineage-tracing studies have revealed that the majority of REPCs in the healthy kidney are derived from myelin protein 0–expressing (PO-expressing) cells, which are positive for CD73 (also known as ecto-5′-nucleotidase), PDGFRβ, and p75 nerve growth factor receptor and negative for PECAM-1 (also known as CD31) (11). In kidney fibrosis induced by unilateral ureteral obstruction (UUO), P0-derived cells differentiate into α smooth muscle actin (αSMA)+ myofibroblasts, whose Epo expression decreases (11, 14). Even though various treatments can increase EPO in patients or animals with CKD (7, 11, 14), the mechanisms underlying the decreased response of myofibroblasts to the anemic stimulation remain elusive.

The regulation of Epo transcription is tissue specific (15, 16). While the hypoxia response element–positive (HRE+) 3′-enhancer of the Epo gene has been confirmed to be liver specific in mice beyond embryonic day 14.5, the HRE+ kidney-specific element has remained unexplored until recently (15, 16). Storti and colleagues reported that a functional HIF2α-dependent HRE in the distal 5′-enhancer is REPC specific (16). In vitro analyses have shown that methylation of the CpG islands in the promoter and 5′-untranslated region (5′-UTR) can inhibit Epo expression through recruiting methyl-CpG binding proteins to the promoter and hindering the binding of nuclear proteins in Hep3B human hepatoma cell line (17, 18). Moreover, methylation-free regulatory elements are a prerequisite for Epo expression in many human cancer cell lines (19, 20).

FOXD1+ progenitors arise in the area of the neural crest and appear earlier in the same locations as PO+ progenitors. FOXD1+ progenitors give rise to essentially the same cells in the mature kidney as PO+ progenitors (11, 21). FOXD1+ progenitor-derived,
**Figure 1.** Col1a1-GFP+ pericytes are REPCs. (A) Hematocrit (Hct) and plasma EPO concentrations and renal expression of Epo, Phd2, Phd3, and Vegfa normalized by Ubc in mice with and without phlebotomy (Con). Phlebotomy was performed 1 day before analysis, n = 5 per group. (B) Confocal images of kidney sections of EpoIRES-RFP/Col1a1-GFP+ reporter mice. Arrowheads indicate EPO-RFP+ and COL1A1-GFP+ pericytes. T, renal tubules. Original magnification, ×400. Scale bar: 20 μm. (C) Expression of Epo, Phd2, Phd3, and Vegfa in Col1a1-GFP+/PDGFRβ+ kidney pericytes isolated from Col1a1-GFP+ mice. n = 5 per group. (D) Fluorescent (left) and bright-field (right) images of primary cultures of live Col1a1-GFP+ kidney pericytes. Original magnification, ×400. Scale bar: 25 μm. (E) Epo expression and supernatant EPO concentration of Col1a1-GFP+ kidney pericytes cultured in chambers with normoxia (21% O2) or hypoxia (0.5% O2) for 24 hours, n = 4 per group. (F and G) Epo expression of Col1a1-GFP+ kidney pericytes cultured in the presence of CoCl2, n = 4 per group. (H) Epo expression of Col1a1-GFP+ kidney pericytes cultured in the presence of IOX2 for 24 hours. n = 4 per group. Student’s t test and 1-way ANOVA were used for analyses of data in A, C, and E and F–H, respectively. *P < 0.05, †P < 0.01, ‡P < 0.001.

Col1a1-GFP+PDGFRβ+ pericytes are perivascular collagen-producing cells that surround the endothelia of capillaries. These pericytes deserve attention due to their potential to produce EPO in healthy kidneys, and, as these cells stand at the junction between the circulation and the kidney, they are primed to sense the change of oxygenation and hemoglobin concentration (21–27). During fibrogenic injury, the pericytes proliferate and differentiate into myofibroblasts that produce pathogenic extracellular collagenous matrix, which leads to kidney fibrosis and function failure (22–31). In accordance with previous studies that refer to REPCs as fibroblast-like cells that might transit to myofibroblasts and contribute to kidney fibrosis (10, 11, 14), FOXD1+ progenitor-derived, Col1a1-GFP+PDGFRβ+ pericytes might provide a good model for studying the molecular mechanisms underlying the regulation of EPO expression in healthy and fibrotic kidneys. Moreover, TGF-β1, a well-recognized cytokine inducing pericyte-myofibroblast transition (29, 30), can induce Rasal1 methylation through DNA methyltransferase 1 (DNMT1), thereby leading to perpetuation of fibroblast activation and kidney fibrosis (32). We propose that TGF-β1-induced methylation of Epo 5′-regulatory elements may provide a molecular basis for a decreased Epo response of REPCs to anemic stimulation in CKD.

**Results**

*Kidney pericytes produce EPO.* Renal Epo mRNA and plasma EPO concentrations were increased in mice after phlebotomy (Figure 1A). Renal expression of the other HIF-regulated genes, including prolyl-hydroxylase 2 (Phd2), Phd3, and Vegfa, was not changed (Figure 1A). We generated EpoIRES-RFP+ reporter mice by knocking IRES-RFP into Epo 3′-UTR (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI82819DS1). Renal expression of RFP and Epo increased in parallel after phlebotomy (Supplemental Figure 1B). EPO-RFP+ cells were detected and increased in the peritubular interstitium (Supplemental Figure 1C). Kidney pericytes were FOXD1+ progenitor-derived, Col1a1-GFP+, PDGFRβ+, CD73+, and p75+ cells (Supplemental Figure 2). We crossed EpoIRES-RFP+ reporter mice to Col1a1-GFP+ mice to study the Epo expression in Col1a1-GFP+ pericytes. EPO-RFP was detectable in less than 10% of Col1a1-GFP+ pericytes in control EpoIRES-RFP+Col1a1-GFP+ mice; however, the percentage increased to more than 80% after phlebotomy (Figure 1B and Supplemental Figure 3, A and B). The increase of Epo expression was confirmed in Col1a1-GFP+PDGFRβ+ pericytes isolated from kidneys of Col1a1-GFP+ mice after phlebotomy (Figure 1C). We then cultured the isolated kidney pericytes in chambers containing 21% or 0.5% O2 and confirmed...
the induction of EPO by hypoxia in pericytes (Figure 1, D and E). Cobalt chloride (CoCl₂), an inducer of hypoxia-like responses, increased Epo expression in cultured kidney pericytes (Figure 1, F and G). IOX2, a specific PHD2 inhibitor, increased EPO expression in pericytes as well (Figure 1H).

**Kidney pericytes produce EPO through HIF2α regulation.** To verify whether FOXD1+ progenitor-derived pericytes produced EPO through HIF2α regulation that has been demonstrated in REPCs (33–35), we crossed Foxd1Cre/+ mice with mice with a homozygous conditional Hif2a allele (Hif2afl/fl mice) to knockout Hif2a in pericytes specifically. The recombination of the conditional Hif2a allele in Foxd1Cre/+ Hif2afl/fl mice was confirmed by PCR using kidney genomic DNA as the template (Figure 2A). Plasma levels of blood urea nitrogen (BUN) and creatinine were not different between control mice and Foxd1 Cre/+ Hif2afl/fl mice confirmed by PCR using kidney genomic DNA and genotyping primers in kidney pericytes. (Supplemental Figure 5A). Hepatic Epo expression was increased in knockout mice, suggesting that livers took over EPO production when renal production was decreased (Figure 2A and Supplemental Figure 5A).

**Phlebotomy-induced EPO expression.** We then performed UUO surgery in a mouse model used to study the contralateral (CL) control and UUO fibrotic kidneys simultaneously. Phlebotomy induced robust Epo expression in CL kidneys but failed to do so in UUO kidneys (Figure 3, A and B). Phlebotomy did not affect Phd3, which had increased expression in UUO kidneys (Figure 3B). Renal Phd2 expression was not changed by UUO injury or phlebotomy (Supplemental Figure 6). Pericytes differentiated to αSMA+ myofibroblasts that retained PDGFRβ after UUO injury (Supplemental Figure 7). Analysis of pericytes and myofibroblasts isolated from CL and UUO kidneys, respectively, showed that phlebotomy-induced Epo expression was only noted in pericytes (Figure 3C). The expression of Vegfa and Phd3, but not Phd2, was higher in myofibroblasts, and expression was not changed by phlebotomy (Figure 3C). IOX2 increased Epo expression in cultured myofibroblasts to a much lesser degree than in pericytes (Figure 3D).

**Transition to myofibroblasts decreases EPO expression.** We then induced the second model of kidney fibrosis by feeding mice with chow containing 0.25% adenine for 21 days (Supplemental Figure 8, A–D, and Figure 3E). Compared with that in mice fed with regular chow, renal Epo expression failed to increase significantly after phlebotomy in mice fed with adenine chow, although their plasma level increased to a lesser level (Figure 3, F–H). Renal expression of Vegfa and Phd3 was not changed by adenine feeding and phlebotomy (Supplemental Figure 8E). Analysis of kidney pericytes and myofibroblasts isolated from mice fed with regular and adenine chow, respectively, reconfirmed that myofibroblasts failed to have a significant increase in Epo expression after phlebotomy (Figure 3I).
Because most mice fed with adenine chow daily did not survive after 3 weeks, we used a protocol of alternate feeding to establish a chronic model with anemia (Supplemental Figure 9A). With the elevated levels of plasma BUN and creatinine, hematocrit decreased progressively, without an increase of plasma EPO concentration in mice fed with regular and adenine chows in alternate weeks (Supplemental Figure 9, B–D). Epo expression decreased in kidneys but increased in livers (Supplemental Figure 9E).

Hypermethylation of Epo 5′-regulatory elements in kidney myofibroblasts. To study the mechanisms underlying the repression of Epo in kidney myofibroblasts, genomic DNA obtained from normal kidney pericytes and UUO kidney myofibroblasts isolated from Col1a1-GFP Tg mice were subjected to methylation assay. Combined bisulfite restriction analysis (COBRA) showed that Epo promoter and 5′-UTR amplified from sodium bisulfite–converted genomic DNA of myofibroblasts was digested by restriction enzyme BstUI, suggesting the presence of hypermethylation (Figure 4, A and B). We confirmed the hypermethylation of Epo promoter and 5′-UTR in myofibroblasts again by bisulfite genomic sequencing (BGS) and methylation-specific PCR (MSP) (Figure 4, C–F). Hypermethylation of the distal HRE 5′-enhancer in myofibroblasts was confirmed by BGS as well (Figure 4G).

5-Azagytidine restores Epo expression in myofibroblasts and TGF-β1-exposed pericytes. To gain insights into the role of hypermethylation in Epo expression of myofibroblasts, we isolated and cultured kidney myofibroblasts 14 days after UUO surgery and treated these cells with 500 nM 5-azacytidine (Aza) (Figure 5A). We found that transient 3-day exposure of myofibroblasts to Aza followed by 2-day drug-free culture led to evident inhibition on β1–exposed pericytes

*P < 0.05, †P < 0.01, ‡P < 0.001.
Aza restores EPO expression and ameliorates anemia in mouse models of kidney fibrosis. We then confirmed the expression of Dnmt isoforms by quantitative PCR in mouse models of kidney fibrosis induced by UUO and adenine (Figure 6A and Supplemental Figure 8F). Confocal microscopy detected DNMT1 in kidney myofibroblasts of Col1a1-GFP mice (Figure 6B). DNMT3a, not DNMT3b, was expressed in both pericytes and myofibroblasts (Figure 6B). Because the UUO mouse model, which showed normal hematocrit and plasma levels of BUN and creatinine, could be used to study the CL control and UUO fibrotic kidneys simultaneously, we first...
treated mice with Aza or PBS vehicle (Veh) after UUO surgery to study whether DNA demethylation restored Epo expression in fibrotic kidneys (Figure 6C). The demethylating effect of Aza on Epo 5′-regulatory elements of kidney myofibroblasts was confirmed (Supplemental Figure 10). Phlebotomy induced Epo expression in CL kidneys but failed to do so in UUO kidneys of mice treated with Veh (Figure 6, D and E). In UUO kidneys, Aza treatment increased not only the basal expression of Epo but also phlebotomy-induced expression (Figure 6, D and E). However, the expression of Hif1a and Hif2a was not changed by Aza treatment (Supplemental Figure 11). Epo expression in CL kidneys and plasma EPO concentration were not changed by Aza treatment, suggesting normally functioning CL kidneys as the major source of plasma EPO in the UUO kidney fibrosis model (Figure 6, D–F).

We next studied the effect of Aza treatment on anemia and EPO expression in the adenine-induced kidney fibrosis model (Figure 7A). Indeed, Aza treatment attenuated the decrease of hematocrit and increased renal Epo expression and plasma EPO concentration in adenine-induced CKD mice, without adverse effects on white cell and platelet counts in peripheral blood (Figure 7, B and C, and Supplemental Figure 12). Further analyses revealed that Aza treatment led to a greater EPO response than phlebotomy (Figure 7, B and C). In addition, Aza treatment attenuated kidney fibrosis and the elevation of plasma BUN and creatinine levels (Figure 7, D and E).

Discussion

These studies report that FOXD1+ progenitor-derived, Coll1a-GFP–PDGFRβ+ kidney pericytes provide a good model for studying the molecular mechanisms underlying EPO regulation in healthy and fibrotic kidneys. Our data indicate that pericytes produce EPO through HIF2α regulation, but their EPO production capability is repressed by methylation of Epo 5′-regulatory elements when they differentiate into myofibroblasts during kidney fibrosis. We show compelling evidence that Aza at low nontoxic doses can restore EPO production and ameliorate anemia in mouse CKD models by targeting DNA methylation.

Our data support previous studies that demonstrated REPCs in fibrotic kidneys and their EPO production capability activated by HIF stabilizers (7, 11, 14). Although many in vitro studies have shown the association between methylation of Epo 5′-regulatory elements and inhibition of Epo expression in human cancer cell lines (17–20), our data provide the first evidence to our knowledge that methylation of Epo 5′-regulatory elements inhibited the baseline expression and anemic induction of Epo in fibrotic kidneys and myofibroblasts. Demethylation of in vitro cultured myofibroblasts and TGF-β1–exposed pericytes by Aza at a clinically relevant and nontoxic concentration increased baseline expression and hypoxic induction of Epo. Moreover, low-dose Aza treatment in mouse CKD models restored baseline expression and enhanced anemic induction of Epo in fibrotic kidneys, possibly through demethylation.
The antifibrotic property of Aza could be ascribed to not only the potential effect of Aza on redifferentiating myofibroblasts back into pericytes, but also to the effect of Rasal1 demethylation in myofibroblasts that was reported previously (32). Although Rasal1 demethylation was shown to deactivate myofibroblasts, we are not sure whether the dose of 10 mg/kg Aza every other day in mice with folic acid nephropathy attenuated fibrosis through cytotoxic effect (32). Our own pilot experiments have shown adverse effects, including myelosuppression and body weight loss in mice after UUO surgery and in adenine-induced CKD mice treated with daily injection of Aza for 5 days per week at doses higher than or equal to 2 mg/kg and 0.5 mg/kg, respectively. Although it is not

Figure 6. Aza restores EPO expression in fibrotic kidneys induced by UUO. (A) Expression of Dnmt isoforms in CL and UUO kidneys after surgery. n = 10 per group. (B) Confocal images of DNMT1, DNMT3a, DNMT3b, and nidogen staining on kidney sections of Col1a1-GFP mouse. Arrowheads highlight Col1a1-GFP+ or Col1a1-GFP+DNMT3a+ cells. Original magnification, ×400. Scale bar: 20 μm. (C) Schema illustrating Aza or Veh treatment in mice after UUO surgery. Phlebotomy was or was not performed 1 day before analyses at day 14 after UUO surgery. n = 10 per group. (D–F) Hematocrit, renal Epo expression, and plasma EPO levels in mice after UUO surgery and treatment with Veh or Aza according to the schema in C. One-way ANOVA was used for data analyses. *P < 0.05, †P < 0.01, ‡P < 0.001.
It is emerging that low-dose demethylating agents can effectively attenuate fibrosis through cytotoxic effect (32), the low and non-toxic doses used in this study will be clinically applicable and safer for the treatment of renal fibrosis and anemia. In cancer treatment, methylation and repression of Epo expression in kidney myofibroblasts because plasma EPO levels are unable to rise proportionately upon the rise of hemoglobin concentration (39). Although the accumulation of toxic metabolites as well as proinflammatory cytokines might inhibit the anemic induction of EPO production (11, 14, 17, 18). Apparently the PHD inhibitor might attenuate the methylation-induced inhibition of Epo expression in kidney myofibroblasts through robust increase of HIFs for transcription initiation. However, one of the mechanisms underlying the absence of response to the PHD inhibitor in some of the patients might be hypermethylation that is too extensive to be overcome by enhanced HIFs (7). The other possible mechanism would be renal fibrosis so extensive that no viable myofibroblasts existed to produce EPO. Moreover, we should be concerned about the possible downside of long-term HIF overactivation induced by PHD inhibitor (44).

In summary, kidney pericytes produce EPO through HIF2α regulation, but this function is repressed by methylation of Epo 5′-regulatory elements when pericytes transit to myofibroblasts during kidney fibrosis. At low nontoxic doses, the demethylating agent Aza has produced a reversal of EPO expression of UUO kidneys through produced EPO. In accordance with previous evidence (33-35), our data support the crucial role of HIF2α in Epo regulation of kidney pericytes. Although the PHD inhibitor can stabilize HIFs and increase plasma EPO levels in some of HD patients with atrophic kidneys (7), absolute insufficiency of HIFs should not be the reason for renal anemia, because hypoxia in the renal interstitium has been considered a hallmark of injury and mediator of CKD progression (42, 43). Our data support that methylation of Epo 5′-regulatory elements plays a crucial role in the disproportionate Epo expression in CKD by hindering the association of HIFs and the other transacting proteins with the regulatory elements (17, 18). Apparently the PHD inhibitor might attenuate the methylation-induced inhibition of Epo expression in kidney myofibroblasts through robust increase of HIFs for transcription initiation. However, one of the mechanisms underlying the absence of response to the PHD inhibitor in some of the patients might be hypermethylation that is too extensive to be overcome by enhanced HIFs (7). The other possible mechanism would be renal fibrosis so extensive that no viable myofibroblasts existed to produce EPO. Moreover, we should be concerned about the possible downside of long-term HIF overactivation induced by PHD inhibitor (44).

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Methods

Animals. Coll1a1-GFP mice with Coll1a1-expressing cells that expressed GFP, were generated and validated as previously described (22). B6:129S4-Foxd1tm1Gfp/+ (referred to herein as Foxd1GFP), B6.Cg-Gt(Rosa)26Sortm6(CAG-tdTomato)Hze/J, and STOCK Epas1tm1svr) (referred to herein as Hif2α) mice were obtained from The Jackson Laboratory (23, 34, 45). EpoR(βEXA-RFP) mice on the C57BL/6 back-
ground were generated by knocking in IRES-RFP between nucleo-
tides 13432 and 13433 at Epo 3′-UTR of chromosome 5 (Ensembl
ENSMUSG00000029711) (Supplemental Figure 1A).

**Mouse models of kidney fibrosis.** UUO was performed in adult (8- to
12-week-old) mice as previously described (22). Briefly, the left ureter
was ligated twice using 4-0 nylon surgical sutures at the level of the
lower pole of kidney. Acute adenine nephropathy was induced in adult
mice fed a regular one-half inch pellet diet of LabDiet 5001 (TestDiet)
containing 0.25% adenine for 21 days (Sigma-Aldrich) (46). Mice fed a
regular pellet diet (LabDiet 5001) served as control. Chronic adenine
nephropathy was induced in adult mice fed a regular diet with or with-
out 0.25% adenine alternately according to the protocol shown in
Supplemental Figure 9A. Analyses of hematoxilin, plasma BUN, and
creatinine were performed in the Laboratory Animal Center, National
Taiwan University College of Medicine, Taipei, Taiwan.

**Administration of Aza to mouse models of kidney fibrosis.** Mice
received subcutaneous daily injections of PBS Veh or Aza (0.5 mg/kg,
Sigma-Aldrich) for 5 days per week after UUO surgery, as outlined in
Figure 6C. Mice fed with regular or adenine chow received subcuta-
nous daily injections of Veh or Aza (0.125 mg/kg, reduced dose for
decreased kidney function) for 5 days per week starting at week 3, as
outlined in Figure 7A.

**Tissue preparation and histology.** Mouse tissues were prepared and
stained as previously described (29). Primary antibodies against the
following proteins were used for immunolabeling: αSMA-Cy3 (C6198,
clon 1A4, Sigma-Aldrich), DNMT1 (5032, Cell Signaling Technol-
ogy), DNMT3b (20-205, Cosmo Bio Co. LTD.), DNMT3a (sc-20703),
nidogen (sc-33706, Santa Cruz Biotechnology), p75 NGF receptor
(ab8875, Abcam), CD73 (500738, BD Biosciences), and PDGFRβ (a
gift from William Stallcup, Burnham Institute, La Jolla, California,
USA). Fluorescence-conjugated-secondary antibody labeling (1:1165-144, 112-605-167, Jackson Immunoresearch Labora-
tories), DAPI staining, VECTASHIELD (Vector Laboratories) mount-
ing, and image capture and processing were carried out as previously
described (29). Quantification of specific cells in tissue sections was
carried out as follows. In brief, sections were co-labeled with DAPI,
Coll1a1-GFP and EPO-RFP cells were identified by DAPI (blue),
green, and red colocalization, respectively. αSMA− cells were identified
by the presence of greater than 75% of the cell area immediately
surrounding nuclei (detected by DAPI) staining positive with Cy3 fluo-
rescence, which is indicative of antigen expression. Specific cells were
counted in 10 randomly selected cortical interstitial fields at ×400
magnification (high-powered field) per mouse. Interstitial fibrosis was
quantified in Picrosirius red-stained paraffin sections.

**Isolation and culture of kidney pericytes and myofibroblasts.** Peri-
cytes and myofibroblasts were isolated from normal and day 14 UUO
kidneys, respectively, as described previously (29). In brief, kidney
was diced and incubated at 37°C for 45 minutes with Liberase (0.5
mg/ml, Roche Applied Science) and DNase (100 U/ml, Roche Applied
Science) in HBSS. After centrifugation, cells were resuspended in 5
ml PBS/1% BSA and filtered (40 μm). Pericytes and myofibroblasts
were isolated by sorting GFP−PDGFRβ− CD31 E-cadherin+ cells using
a FACSARia cell sorter (BD Biosciences) and cultured in DMEM with
10% FBS. The percentages of pericytes and myofibroblasts of the
total kidney cells gated in FACS plots isolated from normal and day
14 UUO kidneys were 0.7% ± 0.3% and 12.9% ± 0.8%, respectively.
Passage 0 cells were used for experiments. In hypoxia experiments,
**Epo 5′-UTR** for the indicated cells was determined by densitometric analysis of MSP products (methylated products divided by the sum of methylated and unmethylated products).

**Western blot analysis.** Total cellular protein extracted using RIPA buffer was subjected to Western blot analysis using methods described previously (28). The following primary antibodies were used to detect protein: DNMT1 (5032, Cell Signaling Technology) and β-actin (4967, Cell Signaling Technology).

**Detection of EPO in plasma and culture media.** Mouse heparin plasma and pericyte culture supernatant stored in a −80°C freezer after collection were transferred into a −20°C Freezer 12 to 16 hours prior to analysis and thawed on ice before analysis. The analysis was performed according to the protocol of provided in the Mouse Erythropoietin Quantikine ELISA Kit (R&D Systems).

**Statistics.** Data are expressed as mean ± SEM. Statistical analyses were carried out using GraphPad Prism (GraphPad Software). Statistical significance was evaluated by Student's t tests or 1-way ANOVA. P values of less than 0.05 were considered significant.

**Study approval.** All animal studies were carried out under a protocol approved by the Institutional Animal Care and Use Committee of the National Taiwan University College of Medicine.

**Author contributions**

YTCC, CCP, YHC, FCC, CLI, MHT, and YMC participated in experiment design and data analysis. YMC and SLC designed and directed the project, carried out experiments, analyzed data, and wrote the manuscript.

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