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 α1 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.
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 α1 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), PO-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,

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Colla1-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 myofibroblast transition (29, 30), can induce Rasal1, a well-recognized cytokine inducing pericyte-myofibroblast transition (29, 30), 1 and TGF-β, to perpetuation of fibroblast activation and kidney fibrosis (32).

**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, Colla1-GFP+, PDGFRβ+, CD73+, and p75+ cells (Supplemental Figure 2). We crossed EpoIRES-RFP reporter mice to Colla1-GFP+ mice to study the Epo expression in Colla1-GFP+ pericytes. EPO-RFP was detectable in less than 10% of Colla1-GFP+ pericytes in control EpoIRES-RFP−/− Colla1-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 Colla1-GFP+ PDGFRβ+ pericytes isolated from kidneys of Colla1-GFP+ mice after phlebotomy (Figure 1C). We then cultured the isolated kidney pericytes in chambers containing 21% or 0.5% O2 and confirmed

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Figure 1. *Colla1-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+ Colla1-GFP+ mice. Arrowheads indicate EPO-RFP+ Colla1-GFP+ pericytes. T, renal tubules. Original magnification, ×400. Scale bar: 20 μm. (C) Expression of Epo, Phd2, Phd3, and Vegfa in Colla1-GFP-PDGFRβ+ kidney pericytes isolated from Colla1-GFP+ mice. n = 5 per group. (D) Fluorescent (left) and bright-field (right) images of primary cultures of live Colla1-GFP+ kidney pericytes. Original magnification, ×400. Scale bar: 25 μm. (E) Epo expression and supernatant EPO concentration of Colla1-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 Colla1-GFP+ kidney pericytes cultured in the presence of CoCl2, n = 4 per group. (H) Epo expression of Colla1-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.
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). 10X2, a specific PHD2 inhibitor, increased EPO expression in pericytes as well (Figure 1H). IOX2, a specific PHD2 inhibitor, increased EPO expression in mice (Figure 2B). The knockout of Hif2a (Supplemental Figure 4). However, anemia was noted in knockout mice (Figure 2B). The knockout of Hif2a in Foxd1 progenitor-derived kidney pericytes led to a 50% decrease and 130% increase of renal Epo expression, respectively (Figure 2C). Plasma levels of blood urea nitrogen (BUN) and creatinine were not different between Foxd1 Cre/+ Hif2a fl/fl 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 Foxd1 Cre/+ 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 Foxd1 Cre/+ Hif2afl/fl control mice and Foxd1 Cre/+ Hif2afl/fl knockout mice (Supplemental Figure 4). However, anemia was noted in knockout mice (Figure 2B). The knockout of Hif2a in Foxd1 progenitor-derived kidney pericytes led to a 50% decrease and 130% increase of renal Hif2a and Hif1a transcripts, respectively (Figure 2C). Compared with those in control mice, renal Epo transcripts and plasma EPO concentrations were reduced by 90% and 50%, respectively, in the knockout mice (Figure 2, C and D). Renal Epo transcripts and plasma EPO concentrations were markedly induced by phlebotomy in control mice but not in knockout mice (Figure 2, E–G). Vascular smooth muscle cells of hepatic arteries, not hepatocytes, were also Foxd1 progenitor derived (Supplemental Figure 5A). Hepatic Epo expression was increased in knockout mice, suggesting that livers took over EPO production when renal production was decreased (Figure 2, F and G, and Supplemental Figure 5B).

**Transition to myofibroblasts decreases 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 3A 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). 10X2 increased Epo expression in cultured myofibroblasts to a much lesser degree than in pericytes (Figure 3D).

We 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-Azacytidine 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 cell apoptosis or change in cell cycle was noted (Figure 5, D and E). Demethylation by Aza increased not only the basal expression of Epo, Vegfa, and Phd3 of Col1a1-GFP–PDGFRβ+ pericytes and myofibroblasts isolated from CL kidneys and kidneys 7 days after UUO surgery from Col1a1-GFP Tg mice, respectively (Figure 5, F–H). Moreover the expression of Vegfa and repression in pericytes was induced by TGF-β1, which was prevented by Aza (Figure 5, I–K). By contrast, TGF-β1 increased Vegfa expression but did not affect Phd3 (Figure 5K).
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<sup>+</sup> 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 Hif1α and Hif2α 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, Collα1-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 demeth-

Figure 5. Aza restores EPO expression in myofibroblasts and TGF–β1-exposed pericytes. (A) Schema showing CoCl2 stimulation of myofibroblasts after 3-day exposure to Aza or Veh and then 2-day drug withdrawal. (B) Western blot analysis showing the inhibitory effect of 500 nM Aza on DNMT1 expression of UUO kidney myofibroblasts. A representative blot of 4 independent experiments is shown. (C) Epo 5′-UTR methylation in myofibroblasts determined by MSP using the same method as in Figure 4, D and E. Myofibroblasts were analyzed at day 5, as described in A. Representative electrophoresis of 4 independent experiments is shown. (D) Annexin V apoptosis assay of myofibroblasts at day 3 and day 5, as described in A. n = 4 per group per time point. (E) Cell cycle analysis of myofibroblasts by measuring DNA content using propidium iodide staining at day 5, as described in A. The data were means of 4 independent experiments. (F) Expression of Epo, Phd3, and Vegfa in myofibroblasts with or without CoCl2 for 16 hours. Transient exposure to Aza was performed, as described in A. n = 4 per group. (G) Expression of Acta2 in myofibroblasts at day 5, as described in A. n = 4 per group. (H) Expression of Dnmt1, Dnmt3a, Dnmt3b, and Tgfb1 in kidney pericytes cultured in medium containing 5 ng/ml TGF–β1 or Veh for 24 hours. n = 4 per group. (I) Schema illustrating the culture of pericytes with or without TGF–β1 in the presence of 500 nM Aza or Veh. (J) Methylation of Epo 5′-UTR in pericytes determined by MSP at day 3, as described in I. Representative electrophoresis of 4 independent experiments is shown. (K) Expression of Epo, Phd3 and Vegfa in pericytes at day 3, as described in I. n = 4 per group. One-way ANOVA was used for analyses of data in D, F, H, and K, and Student’s t test was used for analyses of data in E and G. *P < 0.05, †P < 0.01, ‡P < 0.001.
ylating Epo 5′-regulatory elements in myofibroblasts, thereby increasing the plasma EPO concentration and ameliorating renal anemia. The potential effect of Aza on the redifferentiation of myofibroblasts into pericytes could also contribute to the restoration of Epo expression. In addition, our data showed that low-dose Aza treatment could prevent kidney fibrosis 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. (B) Confocal images of DNMT1, DNMT3a, DNMT3b, and nidogen staining on kidney sections of Col1a1-GFP3 mice. Arrowheads highlight Col1a1-GFP-DNMT1+ 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.

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-GFP3 mice. Arrowheads highlight Col1a1-GFP-DNMT1+ 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.
clear whether the high doses of Aza used in folic acid nephropathy attenuate fibrosis through cytotoxic effect (32), the low and nontoxic doses used in this study will be clinically applicable and safer for the treatment of renal fibrosis and anemia. In cancer treatment, it is emerging that low-dose demethylating agents can effectively deplete DNMTs, while high doses induce cell cycle arrest and cytotoxicity (36–38). Of the well-recognized profibrotic cytokines, we have previously shown that TGF-β1 induces pericyte-myofibroblast transition during kidney fibrosis (28–30). Here, our data further support that TGF-β1 was one of factors that led to upregulation of DNMTs and methylation and repression of Epo in kidney pericytes. The methylation and repression of Epo induced by TGF-β1 was a unique mechanism during pericyte-myofibroblast transition, because TGF-β1 affected the other HIF-regulated genes Vegfa and Phd3 differently, as shown in our data.

The main reason for anemia in patients with CKD is that plasma EPO levels are unable to rise proportionately upon the decrease of hemoglobin concentration (39). Although the accumulated toxic metabolites as well as proinflammatory cytokines might inhibit the anemic induction of EPO production (11, 14, 40), intense dialysis does not restore EPO concentrations appreciably (41). Our data do not support that uremic toxins directly inhibit Epo expression in kidney myofibroblasts because plasma levels of BUN and creatinine are maintained within the normal range by normally functioning CL kidneys after UUO surgery. However, our data could not exclude the possibility that CL kid-

Figure 7. Aza restores EPO expression and ameliorates anemia in an adenine-induced kidney fibrosis model. (A) Schema illustrating Aza or Veh treatment in mice fed with regular or adenine chow, as indicated in Supplemental Figure 9A. Phlebotomy was or was not performed 1 day before analyses at week 10. n = 12 per group. (B and C) Hematocrit, renal Epo expression, and plasma EPO levels of mice fed with regular or adenine chow and treated with Veh or Aza according to the schema in A. (D) Plasma levels of BUN and creatinine in mice fed with regular or adenine chow and treated with Veh or Aza according to the schema in A. (E) Renal expression of Actα2 and Colα1 in mice fed with regular or adenine chow and treated with Veh or Aza according to the schema in A. One-way ANOVA was used for data analyses. †P < 0.01, ‡P < 0.001.

neys, as the major source of EPO, could inhibit 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).

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 repression in both kidney myofibroblasts and TGF-β1–treated kidney pericytes, leading to a rise of plasma EPO and hematocrit in CKD mice. Future studies are needed to explore the applicability of demethylating agents for renal anemia treatment in patients with CKD.

Methods

Animals. Collα1-GFP mice, with Collα1-expressing cells that expressed GFP, were generated and validated as previously described (22). B6;129S4-Foxd1tm1(GFP/cre)(referred to herein as Foxd1tm1), B6.Cg-Gt(Rosa)26Sortm14(CAG-tdTomato)Hze/J, and STOCK Epas1tm1erf (referred to herein as Hif2αtm1) mice were obtained from The Jackson Laboratory (23, 34, 45). Epo(ΔEXS-RFP) mice on the C57BL/6 background were used to evaluate the effects of Aza or Veh treatment in an adenine-induced kidney fibrosis model. (A) Schema illustrating Aza or Veh treatment in mice fed with regular or adenine chow, as indicated in Supplemental Figure 9A. Phlebotomy was or was not performed 1 day before analyses at week 10. n = 12 per group. (B and C) Hematocrit, renal Epo expression, and plasma EPO levels of mice fed with regular or adenine chow and treated with Veh or Aza according to the schema in A. (D) Plasma levels of BUN and creatinine in mice fed with regular or adenine chow and treated with Veh or Aza according to the schema in A. (E) Renal expression of Actα2 and Colα1 in mice fed with regular or adenine chow and treated with Veh or Aza according to the schema in A. One-way ANOVA was used for data analyses. †P < 0.01, ‡P < 0.001.
ground were generated by knocking in IRES-RFP between nucleotides 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 without 0.25% adenine alternately according to the protocol shown in Supplemental Figure 9A. Analyses of hemocrit, 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 subcutaneous 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, clone 1A4, Sigma-Aldrich), DNMT1 (5032, Cell Signaling Technology), DNMT3b (20-205, Cosmo Bio Co. LTD.), DNMT3a (sc-20703), nitogen (sc-33706, Santa Cruz Biotechnology), p75 NGF receptor (ab8875, Abcam), CD73 (550738, BD Biosciences), and PDGFRα (a gift from William Stallcup, Burnham Institute, La Jolla, California, USA). Fluorescence-conjugated secondary antibody labeling (111-310-003, Jackson Immunoresearch Laboratories), DAPI staining, VECTASHIELD (Vector Laboratories) mounting, 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 colabeled with DAPI. Col1a1-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 fluorescence, 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. Intertstitial fibrosis was quantified in Picrosirius red-stained paraffin sections.

Isolation and culture of kidney pericytes and myofibroblasts. Pericytes 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-PDGFRα-C313 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, cells were washed with 1× PBS (pH 7.4) and renewed culture medium and then placed in an incubator with 21% O2 or in a hypoxia chamber (INVIVO200, Ruskinn Technology Ltd.) with 0.5% O2 for 48 hours. Cellular RNA was harvested by adding the RLT buffer provided in the RNeasy Mini Kit (Qiagen) immediately after cells were taken out of the incubator and the supernatant was removed for storage. In CoCl2 (Sigma-Aldrich) or IOX2 (Tocris Bioscience) experiments, cells were washed 1× PBS and renewed culture medium and with or without CoCl2 or IOX2. Cellular RNA was harvested at indicated time points. In TGF-β1 stimulation experiments, cells were washed 1× PBS and renewed culture medium and with or without 5 ng/ml TGF-β1 (R&D Systems) in the presence of 500 ng/ml Aza (Sigma-Aldrich) or Veh. Cellular RNA and genomic DNA were harvested at indicated time points. In myofibroblast culture, cells were treated with 500 ng/ml Aza for 3 days and then harvested for Western blot analysis of DNMT1 or treated with CoCl2 or Veh after 2-day Aza-free period. Cellular RNA was then harvested after CoCl2 or Veh treatment.

PCR. Total RNA was extracted using the RNeasy Mini Kit (Qiagen). The purity of each sample was determined based on the ratio of A260 to A280. cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen). Conventional and quantitative PCR were performed using methods described previously (28). Expression levels were normalized by ubiquitin C (Ubc) or Gapdh. The specific primer pairs used for PCR are listed in Supplemental Tables 1 and 2.

COBRA. Genomic DNA was prepared from Col1a1-GFP-PDGFRα/C313 E-cadherin pericytes and myofibroblasts isolated from normal kidneys and kidneys 14 days after UUO surgery of Col1a1-GFP mice, respectively. Sodium bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation Kit according to the manufacturer’s protocol (ZYMO Research). PCR using bisulfite-converted genomic DNA as the template and the primers shown in Supplemental Table 2 would amplify the genomic DNA fragments containing promoter and 5′-UTR of Epo. Unmethylated and methylated controls were from mouse sperm genomic DNA and Methylated Mouse Genomic DNA Standard, respectively (ZYMO Research). Equal amount of the PCR products were incubated in buffer with or without restriction enzyme BstUI at 60°C for 3 hours (New England Biolabs), and then electrophoresis was performed in a 1% agarose gel.

BGS. Genomic DNA was prepared and sodium bisulfite conversion of genomic DNA was performed as described for those in COBRA. PCR products of the genomic DNA fragment containing promoter and 5′-UTR or distal 5′-enhancer amplified from bisulfite-converted genomic DNA using the primers shown in Supplemental Table 2 were gel purified with the QAquick Gel Extraction Kit (QIAGEN). The eluted DNA fragments were ligated into pGEM-T Easy Vector (Promega Corporation) for sequencing. Four colonies for each mouse were randomly chosen for sequencing.

MSP. Genomic DNA was prepared and sodium bisulfite conversion of genomic DNA was performed as described for those in COBRA. Bisulfite-converted genomic DNA was amplified with methylation-specific or unmethylation-specific primer pairs shown in Supplemental Table 3. Unmethylated and methylated controls were from mouse sperm genomic DNA and Methylated Mouse Genomic DNA Standard, respectively (ZYMO Research). The PCR products were analyzed by electrophoresis. The electrophoresis result was shown as a virtual gel. The percentage of methylation of...
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 at 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.

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