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Renal peritubular interstitial fibroblast-like cells are critical for adult erythropoiesis, as they are the main source of erythropoietin (EPO). Hypoxia-inducible factor 2 (HIF-2) controls EPO synthesis in the kidney and liver and is regulated by prolyl-4-hydroxylase domain (PHD) dioxygenases PHD1, PHD2, and PHD3, which function as cellular oxygen sensors. Renal interstitial cells with EPO-producing capacity are poorly characterized, and the role of the PHD/HIF-2 axis in renal EPO-producing cell (REPC) plasticity is unclear. Here we targeted the PHD/HIF-2/EPO axis in FOXD1 stroma-derived renal interstitial cells and examined the role of individual PHDs in REPC pool size regulation and renal EPO output. Renal interstitial cells with EPO-producing capacity were entirely derived from FOXD1-expressing stroma, and *Phd2* inactivation alone induced renal *Epo* in a limited number of renal interstitial cells. EPO induction was submaximal, as hypoxia or pharmacologic PHD inhibition further increased the REPC fraction among *Phd2−/−* renal interstitial cells. Moreover, *Phd1* and *Phd3* were differentially expressed in renal interstitium, and heterozygous deficiency for *Phd1* and *Phd3* increased REPC numbers in *Phd2−/−* mice. We propose that FOXD1 lineage renal interstitial cells consist of distinct subpopulations that differ in their responsiveness to *Phd2* inactivation and thus regulation of HIF-2 activity and EPO production under hypoxia or conditions of pharmacologic or genetic PHD inactivation.

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Distinct subpopulations of FOXD1 stroma-derived cells regulate renal erythropoietin

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Introduction

The hypoxic induction of erythropoietin (EPO), a hypoxia-inducible factor–regulated (HIF-regulated) glycoprotein hormone that is essential for normal erythropoiesis, represents one of the most sensitive systemic hypoxia responses in humans (1). In the bone marrow, EPO acts on CFU, pro-, and early basophilic erythroblasts and inhibits apoptosis of red cell precursors, which increases rbc mass and thus oxygen-carrying capacity in blood. In adults, the major site of EPO synthesis is the kidney, where peritubular interstitial fibroblast-like cells respond to decreases in tissue pO2 with increased EPO synthesis. Abnormal EPO responsiveness in the bone marrow or deregulated renal EPO production can lead either to excessive rbc production and polycythemia, or to hypoproliferative anemia, a condition that is commonly found in patients with chronic kidney disease (CKD) and is primarily due to relative EPO deficiency (1).

HIF-2, the transcription factor responsible for the hypoxic induction of renal EPO, is required for normal erythropoiesis (2), as its deletion from renal tissue results in severe anemia (3). HIFs consist of an oxygen-sensitive α-subunit and a constitutively expressed β-subunit, HIF-β, which is also known as the aryl hydrocarbon receptor nuclear translocator (ARNT). Together with HIF-1, HIF-2 regulates a multitude of hypoxia responses that allow cells to adapt to and survive low-oxygen environments (4). While HIF-α subunits are continuously synthesized, they are rapidly degraded in the presence of molecular oxygen. Under normoxia, oxygen-, iron-, and 2-oxoglutarate–dependent prolyl-4-hydroxylase domain (PHD) proteins, PHD1, PHD2, and PHD3, also known as egl-9 homolog 2 (EGLN2), EGLN1, and EGLN3, respectively, hydroxylate HIF-α at specific proline residues. This hydroxylation reaction is key to targeting HIF-α for proteasomal degradation via ubiquitination by the von Hippel-Lindau (VHL) E3-ubiquitin ligase complex (5). Under hypoxia prolyl-4-hydroxylation of HIF-α is inhibited, resulting in its translocation to the nucleus, where it heterodimerizes with ARNT and transactivates a large number of oxygen-regulated genes (5). In the kidney, activation of HIF-2 by either hypoxia or pharmacologic or genetic PHD inhibition increases serum EPO levels and rbc production (1). However, the role of individual PHDs in the regulation of HIF-2–mediated renal hypoxia responses and EPO gene transcription under physiologic and injury conditions is not well understood.

IHC and in situ hybridization (ISH) studies as well as findings from genetic mouse models have provided strong evidence that fibroblast-like interstitial cells and not epithelial or endothelial cells synthesize EPO in the kidney (6–9). Renal interstitial fibro-
HIF-2 activity, and to determine to what degree pericytes and other perivascular cells contribute to the kidney’s EPO response, we have used the Cre-loxP system to target the PHD/HIF-2/EPO axis in FOXD1 stroma-derived renal interstitium. We found that the ability to synthesize EPO is completely contained within the FOXD1 stroma-derived cell population and that the majority of FOXD1-derived interstitial cells in cortex and outer medulla have EPO-producing capacity. Using multiplex ISH techniques we quantified the number of EPO-producing cells and determined the size of the REPC pool under conditions of systemic hypoxia or individual HIF-PHD inactivation. Our data provide genetic evidence for the existence of distinct subpopulations of renal interstitial cells that differ in responsiveness to Phd2 inactivation and thus regulation of HIF-2 activity and EPO synthesis.

Results

REPCs are derived from FOXD1-expressing stroma. We have previously examined the role of HIF-2α in renal EPO production using P3Pro-Cre transgenic mice, which express Cre-recombinase in both renal tubular epithelium and interstitium (3). To specifically investigate the role of PHD oxygen sensors in Epo regulation in renal interstitium, we used Foxd1cre/cre transgenic mice and targeted the components of the PHD/HIF-2/EPO axis individually or in combination. The Foxd1-Cre transgene encodes an EGFP/Cre-recombinase (EGFP/Cre) fusion protein under transcriptional control of the Foxd1 promoter and was generated by homologous recombination. Foxd1-Cre is expressed during kidney development, but not in the adult kidney (15, 16). To visualize FOXD1 stroma-derived interstitial cells in the kidney, Foxd1cre/cre transgenic mice were intercrossed with Cre-reporter mice (ROSA26-ACTB-tdTomato,EGFP), which expressed fluorescent membrane-bound tdTomato red prior to excision and membrane-bound EGFP following excision of a floxed stop cassette (17). As expected, EGFP expression was observed in the interstitial space of cortex and medulla, as well as in cells of vessel walls and glomeruli (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI83551DS1). IHC analysis revealed a normal pattern of PDGFRβ expression, indicating that kidney development was not affected (Supplemental Figure 1B). EGFP expression was furthermore observed in a subpopulation of tubular epithelial cells, which is consistent with previously published reports (16). The latter is most likely due to FOXD1 expression in a small number of mesenchymal progenitor cells that give rise to both FOXD1-expressing stroma and SIX2-expressing epithelial progenitors cells (16).

In order to study the degree of contribution of FOXD1 stroma-derived interstitial cells to EPO production in the kidney, we generated Foxd1cre/cre Epoβ/β mice, herein referred to as Foxd1-Epoβ/β, by
crossing Foxd1<sup>+/–</sup> transgenics with mice homozygous for a conditional Epo allele (18). These mice were born in mendelian ratios and developed normally. Genomic PCR was used to assess recombination in different tissues. Recombination was detected in multiple tissues including kidney and brain, but not in the liver (Figure 1A). Foxd1-Epo<sup>+/–</sup> mice developed severe anemia (hematocrit [Hct] of 18.9% ± 1.0% vs. 41.1% ± 0.9% in controls; hemoglobin [Hb] of 6.3 ± 0.3 g/dl vs. 14.0 ± 0.4 g/dl and rbc count of 4.4 ± 0.2 M/μl vs. 9.4 ± 0.2 M/μl in controls; n = 3 each; P < 0.001 each; Figure 1B), which was associated with a decreased reticulocyte production index (0.7 ± 0.1 in mutants vs. 3.5 ± 0.3 in controls; n = 3 each; P < 0.01), indicating that Foxd1-Epo<sup>+/–</sup> mice had developed hypoproliferative anemia (Figure 1B). We next used real-time PCR to determine renal Epo transcript levels, which were severely reduced. The absence of significant renal Epo transcription in mutant mice was also reflected in abnormal plasma EPO concentrations, as serum EPO levels were significantly lower in mutant mice (0.047 ± 0.004 ng/ml vs. 0.32 ± 0.059 ng/ml in controls; n < 0.001 each; Figure 1C).

Since our data suggested that the renal EPO response was completely ablated in anemic Foxd1-Epo<sup>+/–</sup> mice, we examined whether exposure to normobaric hypoxia (10% O<sub>2</sub> for 2 days) or treatment with an oral pan-PHD inhibitor (PHI), GSK1002083A, which has been shown to robustly activate HIF-2 in the kidney (3), was capable of stimulating EPO responses in Foxd1-Epo<sup>+/–</sup> kidneys. While PHI or hypoxia treatment increased renal Epo mRNA levels in Cre<sup>–</sup> control mice (~32-fold increase in the PHI group and ~3-fold for the hypoxia group compared with nontreated Cre<sup>–</sup> controls; Figure 2, A and B), significant increases in renal Epo mRNA levels were not detected in PHI- or hypoxia-treated Foxd1-Epo<sup>+/–</sup> mutants. Serum EPO levels in Cre<sup>–</sup> control mice increased from 0.32 ± 0.059 ng/ml to 12.7 ± 0.9 ng/ml with PHI treatment and to 1.9 ± 0.4 ng/ml following exposure to hypoxia (n = 3; P < 0.001 and 0.01, respectively). In Foxd1-Epo<sup>+/–</sup> mutant mice hypoxia treatment did not result in an increase in serum EPO (n = 4), while PHI treatment raised serum EPO concentra-

### Figure 2. Renal EPO-producing cells are derived from FOXD1 stromal progenitors. Shown are the relative renal and liver Epo responses to treatment with an orally administered HIF-PHD inhibitor (PHI) or normobaric hypoxia in control (Co) and Foxd1-Epo<sup>+/–</sup> mutants (Epo<sup>+/–</sup>). (A) PHI treatment with compound GSK1002083A (n = 3 each). (B) Treatment with 2 days of normobaric hypoxia (Hx) at 10% O<sub>2</sub> (n = 3 and 4, respectively). The induction of Epo mRNA is completely suppressed (top panels), and serum EPO responses (bottom panels) are blunted in Foxd1-Epo<sup>+/–</sup> mutants. Data are represented as mean ± SEM; 2-way ANOVA with post hoc Tukey’s test; *P < 0.05, **P < 0.01, ***P < 0.001.
Epo induction in Foxd1-Phd2–/– kidneys is submaximal. Although Foxd1-Cre-mediated inactivation of Phd2 alone resulted in a very strong increase in renal Epo mRNA levels, it was unclear whether Epo was maximally induced. In order to address this question, we generated Foxd1Cre/+ ROSA26-ACTB-tdTomato,-EGFP Phd2fl/fl conditional knockout mice, herein referred to as Foxd1-mT/mG-Phd2–/– mutants. In this knockout line, Phd2-deficient cells were simultaneously tagged with membrane-bound EGFP, which permitted identification of cells having undergone recombination by visual inspection.

We first used high-resolution ISH to visualize EGFP transcripts in formalin-fixed, paraffin-embedded kidney sections. As expected, HIF-2α localized to the renal interstitium as well as glomerular cells (Supplemental Figure 4). In contrast to whole-tissue homogenates, the expression of HIF target gene Vegf was increased in renal interstitium, where the majority of Epo+ cells expressed detectable levels of Vegf (Figure 3E). In summary, our data demonstrate that inactivation of PHD2, but not PHD1 or PHD3, is sufficient for the activation and induction of EPO synthesis in FOXD1 stroma-derived renal interstitial cells.
of tubular epithelial cells (Supplemental Figure 1A) (15, 16, 24). In the absence of the ROSA26-ACTB-tdTomato,EGFP reporter allele, EGFP was not detectable in kidneys from 8-week-old mice, confirming that the Foxd1 promoter is no longer active in adults (Foxd1Flox/+ mice express an EGFP/Cre fusion protein during development; Supplemental Figure 5A).

We next examined to what degree cortical and outer medullary FOXD1 stroma-derived interstitial cells contributed to EPO production in Foxd1-Phd2–/– kidneys by using multiplex ISH using fluorescent probes against EGFP and Epo transcripts. We found that the number of cells that actively transcribed Epo increased from 2.5 ± 1.7 cells/mm² in 8-week-old mice, confirming that the Foxd1 promoter is no longer active in adults (Foxd1Flox/+ mice express an EGFP/Cre fusion protein during development; Supplemental Figure 5A).

We next examined whether the pool of cells that transcribed Epo in Foxd1-Phd2–/– kidneys was expandable, and asked whether the provision of additional hypoxic stimuli would lead to a further increase in EPO production. We phlebotomized Foxd1-mT/mG-Phd2–/– mice to an average Hct of 35.8% ± 4.3% and found that phlebotomy further increased REPC numbers and total renal Epo levels (365.5 ± 25.9 cells/mm² vs. 117.4 ± 25.7 cells/mm²;}

![Figure 4](image_url)
73.0% ± 4.1% of EGFP+ interstitial cells were Epo+ in phlebotomized vs. 39.0% ± 5.5% in nonphlebotomized mutants; n = 5 each; P < 0.001; 3.5-fold increase in Epo levels; P < 0.05; Figure 4). Interestingly, we also observed a significant increase in the absolute number of EGFP+ interstitial cells in kidneys from Phd2–/– mice subjected to phlebotomy (Figure 4). Taken together our data indicate that Phd2 inactivation in FOXD1 stroma-derived interstitial cells resulted in submaximal EPO production, which was associated with Epo induction in only a subset of Phd2-deficient renal interstitial cells. However, Phd2-deficient renal interstitial cells that failed to express Epo retained EPO-producing capacity and converted to Epo-expressing cells upon provision of a hypoxic stimulus.

Different subpopulations of renal interstitial cells give rise to REPCs. To further characterize Phd2-deficient interstitial cells that failed to induce Epo, we analyzed the expression of Pdgfrb, a cellular marker of REPCs, pericytes, and other fibroblast-like perivascular cells. Since only 50% of EPO-producing cells were reported to express transcripts encoding α-smooth muscle actin (ACTA2) and were never associated with vessel walls (Supplemental Figure 5C), we sought to identify EPO expressing renal interstitial cells that fail to induce Epo as a result of Phd2 inactivation, which may lack PDGFRB expression. Taken together our data suggest that renal interstitial cells, which fail to induce Epo in response to Phd2 inactivation, cannot be distinguished based on Pdgfrb expression.

To further define the cellular heterogeneity of Phd2–/– renal interstitial cells, we examined EPO responses in a specific subpopulation of pericytes and perivascular fibroblasts using a Cre-recombinase transgene under the control of the chondroitin sulfate proteoglycan 4 (Cspg4) promoter. CSPG4 is also known as neuro-glial antigen 2 (NG2) and is expressed in a subpopulation of pericytes and perivascular fibroblasts using a Cre-recombinase transgene under the control of the chondroitin sulfate proteoglycan 4 (Cspg4) promoter. CSPG4 is also known as neuro-glial antigen 2 (NG2) and is expressed in a subpopulation of pericytes and perivascular fibroblasts using a Cre-recombinase transgene under the control of the chondroitin sulfate proteoglycan 4 (Cspg4) promoter.
that Ng2-Phd2+/− mice was behaved physiologically as a result of Phd2 inactivation (Supplemental Figure 6). In contrast to Phd2 inactivation, phlebotomy induced Epo in cells labeled with Ng2-Cre (Supplemental Figure 6). These data indicate that renal interstitial cells with a history of Ng2-Cre expression behaved similarly to FOXD1 stroma-derived cells that failed to express Epo in Foxd1-Phd2−/− kidneys, and furthermore raise the possibility that NG2 expression may be associated with renal interstitial cells that differ in their responsiveness to Phd2 inactivation and thus regulation of HIF-2 activity and EPO production.

Differential regulation of HIF-2 activity in FOXD1 stroma-derived renal interstitial cells. Although inactivation of Phd1 and/or Phd3 by itself does not induce renal Epo (Supplemental Figure 3), it is not known to what degree PHD1 and/or PHD3 have a modulatory effect on EPO responses in the kidney. Because we found that inactivation of Phd2 induced Epo transcription in only a limited number of FOXD1 stroma-derived interstitial cells, we asked whether additional pharmacologic inhibition of PHD catalytic activity had any further effect on REPC pool size and/or renal EPO output in Foxd1-Phd2−/− mutant mice. To examine this, we treated Foxd1-mT/mG-Phd2−/− mice with GSK1002083A, a PHI, which reversibly inhibits the catalytic activity of all PHDs (3). Treatment with PHI raised the number of cells that transcribed Epo from 117.4 ± 25.8 to 390.8 ± 70.57 cells/mm² (n = 5 and 3, respectively, P < 0.001), which correlated with an approximately 4.5-fold increase in renal Epo mRNA levels (n = 3 and 6, respectively; Figure 6). To further assess the relative contributions of PHD1 and PHD3 to the regulation of REPC pool size in Foxd1-Phd2−/− mutants, we generated Foxd1-Phd1−/−-Phd2−/−, Foxd1-Phd2−/−-Phd3−/−, and Foxd1-Phd1−/−-Phd2−/−-Phd3−/− triple mutant mice. Whereas Foxd1-Phd2−/− mutants were born in mendelian ratios and developed normally, homozygous inactivation of all 3 Phd alleles or the combined inactivation of Phd2 and Phd3 resulted in juvenile lethality, precluding the analysis of REPC pool size and renal Epo levels in adults that either lacked all copies of Phd1, Phd2, and Phd3 or were double-deficient for Phd2 and Phd3 (data not shown). In contrast, dual targeting of Phd1 and Phd2 resulted in normal development and did not further increase renal Epo mRNA levels compared with Phd2 inactivation alone (Supplemental Figure 8), suggesting that (a) PHD1 is unlikely to play an adjunctive role in regulating REPC pool size in Phd2−/− kidneys and (b) inhibition of PHD3 activity was most likely responsible for the additional increase in REPC numbers in Foxd1-mT/mG-Phd1−/−-Phd2−/−-Phd3−/− mice and PHI-treated Foxd1-Phd2−/− mutants. However, since we were unable to assess renal EPO production in adult Phd2−/− double and Phd1/2/3 triple mutants, we cannot exclude with certainty that PHD1 plays a contributory role in the regulation of REPC pool size.

In anemic mice the increase in REPC numbers is usually associated with their spatial expansion toward the outer cortex (Figure 7). To investigate whether the topography of REPCs in Foxd1-Phd2−/− mutants followed a specific pattern or whether it was similar to that found in anemic mice, we examined the spatial distribution of Epo+ interstitial cells in the outer, mid-, and inner renal cortex (Figure 7). In Phd2−/− or Phd1−/−-Phd2−/−-Phd3−/− knockout mice REPCs were distributed equally throughout the entire cortex, whereas the majority of Epo+ cells in anemic control mice localized to the inner cortex (Fig-
Differential expression of Phd1 and Phd3 in Foxd1-Phd2−/− mutants. Our genetic studies suggested that HIF-2α stabilization and thus induction of Epo transcription were prevented by PHD1/3-mediated HIF prolyl-4-hydroxylation in a subpopulation of Phd2−/− interstitial cells. We therefore hypothesized that Epo Phd2−/− and Epo Phd2−/− renal interstitial cells differed with regard to Phd1 and Phd3 expression and that the fraction of Epo Phd2−/− cells that expressed Phd1 and/or Phd3 might be increased compared with that of Epo Phd2−/− cells. To test this hypothesis we performed multiplex ISH with probes against EGFP, Epo, and either Phd1 or Phd3 transcripts. We found that the majority of Epo-expressing Phd2−/− cells did not express detectable levels of Phd1 (84.7% ± 7.0% of EGFP+Epo− cells; Figure 8A) or Phd3 (90.1% ± 2.2% of EGFP+Epo− cells; Figure 8A). In contrast, a larger proportion of cortical and outer medullary EGFP+Epo− cells expressed Phd1 (42.7% ± 13.8%) or Phd3 (23.5% ± 5.5%), suggesting (a) the possibility that the presence of PHD1 and/or PHD3 prevented HIF-2 activation in Phd2−/− cells that failed to express Epo, and (b) that inactivation of PHD1 and PHD3 by genetic or pharmacologic (PHI treatment) means was required for the conversion of these cells to EPO-producing interstitial cells (Figure 8B). For technical reasons we were unable to simultaneously hybridize against Phd1, Phd3, Epo, and EGFP, and could therefore not directly determine to what degree Phd1− and Phd3−expressing Epo− subpopulations overlapped. However, we estimate from separate ISH studies that up to 45% of either Phd1− or Phd3−expressing cells can express both genes (data not shown). In summary, our data suggest that differences in Phd1 and Phd3 expression modulate HIF-2 and EPO responses in FOXD1 stroma-derived renal interstitial cells.

Discussion
Here we used a genetic approach to investigate the role of the 3 HIF-prolyl-4-hydroxylases, PHD1, PHD2, and PHD3, in the regulation of REPC plasticity and renal EPO production. We...
Renal peritubular interstitial cells begin to synthesize EPO when HIF-2α is stabilized, either under hypoxic conditions or as a result of HIF-PHD inhibition by pharmacologic or genetic means (1). Here we demonstrate by cell-specific gene targeting that cells with EPO-producing capacity are entirely contained within FOXD1 lineage–derived interstitial cells. However, a recent study reported that cells with EPO-producing ability are partly derived from P0 lineage cells, which overlap with FOXD1-expressing progenitor cells that start to surround the cap mesenchyme from E10.5 onward (11, 14). While the exact interrelationships between those two cell types are not known, it has been suggested that neural crest–derived cells migrate into the developing kidney at E13.5, start to express FOXD1, and replenish existing FOXD1 progenitor cells (11, 14). Since previous studies were not able to assign all EPO-producing cells to the P0 lineage (14), it is plausible that non–P0 lineage interstitial cells with EPO-producing capacity are derived from FOXD1-expressing stromal progenitor cells and identify a subpopulation of renal interstitial cells that respond to Phd2 inactivation with the induction of Epo. We also provide evidence that PHD3 plays an adjunctive role in the regulation of plasma EPO levels, as it modulates the number of renal interstitial cells that respond to Phd2 inactivation with EPO synthesis. We propose that EPO-producing renal interstitial cells derive from distinct subpopulation of FOXD1 lineage cells, which vary in their ability to regulate HIF-2α degradation, partly as a result of differences in PHD activity levels.

A clinical hallmark of advanced CKD is anemia, which, although multifactorial, is primarily attributable to relative EPO deficiency (1). As a result of renal injury, renal interstitial cells lose their ability to respond to hypoxia with adequate and sufficient EPO production resulting in anemia. Whether this phenomenon is due to a change in the regulation of HIF-2α production and/or degradation or results from impaired EPO transcription in the...
presence of nuclear HIF-2 is unclear. However, increased NF-κB signaling and transdifferentiation into myofibroblasts have been suggested as potential underlying mechanisms for the development of renal anemia (14, 29). The reduced ability to mount an adequate EPO response appears to be reversible to a certain degree, as treatment with glucocorticoids or removal of fibrotic stimuli in experimental fibrosis models protected the EPO-producing ability of the injured kidney (14, 29). In a recent genetic study in mice, Souma and colleagues showed that PHD inactivation in Epo-expressing cells was capable of reversing the decline of renal EPO production in a unilateral ureteral obstruction model (28). This study made use of a Cre-recombinase transgene that controlled, i.e., inducible by hypoxia. Phd2 inactivation alone or in conjunction with either Phd1 and/or Phd3 using Epo-Cre resulted in the development of polycythemia. Inactivation of Phd1, Phd2, and Phd3 was associated with renal Epo transcript levels that were similar to control, which is in contrast to our study, where large increases in Epo mRNA were detected with Phd2 inactivation alone. This discrepancy is explained by the very small number of renal interstitial cells targeted with the use of the Epo-Cre transgene. The ability of PHD inhibition to protect the kidney’s EPO-producing ability under injury conditions suggests that pharmacologic HIF activation increases EPO synthesis in fibrotic kidneys either by converting non-EPO-producing renal interstitial cells into REPCs, or, as proposed by Souma and colleagues, by maintaining Epo transcription in EPO-producing cells that are undergoing myofibroblast transformation (28). The possibility that even severely diseased kidneys do not completely lose their ability to synthesize EPO in response to HIF-2 activation has been suggested based on studies in CKD patients whose serum EPO levels increased upon ascent to high altitude or following treatment with HIF-2α-stabilizing compounds (30–32). While the tissue source of EPO was not determined in these studies, this notion is furthermore supported by a large retrospective clinical study, which showed that dialysis patients living at high altitude required lower doses of recombinant EPO to maintain Hb (33).

While recombinant forms of EPO have been a mainstay of renal anemia therapy for almost 3 decades, cardiovascular safety concerns have been raised regarding the use of recombinant EPO and prompted the FDA to issue a black-box warning (34, 35). Although the underlying molecular mechanisms for the increase in adverse cardiovascular events are still unclear, they may be related to the repeated administration of recombinant EPO doses that generate very high and supraphysiologic plasma EPO levels (36). Oral therapy with HIF-α-stabilizing PHD-inhibiting compounds is currently being investigated in phase III clinical trials, and has the potential to provide a more physiologic and potentially safer therapeutic approach to renal anemia (1). Our data would argue that renal interstitial cells with EPO-producing potential exhibit differential sensitivity to HIF-prolyl-4-hydroxylase inhibition, especially if compounds that target PHDs with different efficacy are used. Our data furthermore support the concept that compounds that specifically inhibit PHD2 are very effective in stimulating renal EPO synthesis. The efficacy of such PHD2-specific compounds could be enhanced or modulated by hypoxia, as phlebotomy substantially increased the number of Epo-transcribing cells in Phd2+/− kidneys.

Although renal interstitial fibroblast-like cells/pericytes and hepatocytes are the main cellular sources of EPO under most physiologic conditions (1), other cell types, such as osteoblasts and astrocytes, have been shown to synthesize EPO when HIF was activated by genetic means in mice (37, 38). Therefore these cell types have potential to contribute to plasma EPO upon systemic administration of PHIs. In the kidney, genetic studies showed that HIF-2 activation via Vhl gene deletion converted renin-producing cells to EPO-producing cells (39, 40). Whether these cells are also capable of synthesizing EPO in response to PHD2 inactivation is currently not known.

While renal Epo mRNA levels correlated with REPC pool size in our study, we also observed a statistically significant rise in the absolute number of EGFP-labeled FOXD1 stroma-derived interstitial cells when Phd2+/− mice were phlebotomized or PHI-treated, suggesting a physiologic response rather than a developmental abnormality. To what degree this increase in EGFP-labeled cells was transient, or whether it had any adverse consequences on renal function, is currently being investigated in our laboratory. While it is plausible that sustained HIF activation during nephrogenesis affects interstitial cell numbers in the adult kidney, an association between interstitial cell proliferation and increased EPO production has recently been described in rats treated with poly-D-glutamic acid (41); furthermore, hypoxia has been shown to stimulate pericyte proliferation in vitro (42). From genetic fate-tracing studies, it has become evident that FOXD1 stroma-derived interstitial cells and pericytes have the potential to proliferate. While the underlying molecular mechanisms are complex and involve multiple signaling molecules, FOXD1 stroma-derived interstitial cells proliferate and transdifferentiate into myofibroblasts under injury conditions, which leads to excessive extracellular matrix production and the development of fibrosis (11, 15).

While HIF-2 is the main regulator of EPO in kidney and liver (3, 43), renal interstitial cells and hepatocytes differ from each other with regard to PHD-mediated control of HIF-2α degradation. While our study identified at least 2 interstitial cell populations in the kidney that displayed differential sensitivity to Phd2 inactivation, inactivation of at least 2 HIF-PHDs is required for moderate Epo induction in hepatocytes. To achieve very high levels of Epo induction in the liver, inactivation of all 3 HIF-PHDs is required (21–23). The role of individual PHDs in the regulation of EPO in other cell types, however, is not clear. Inactivation of all 3 HIF-PHDs appeared to be necessary for the induction of Epo in osteoblasts (38), while Phd2 deletion alone activated Epo transcription in neurons (44). In our study, Phd3, and not Phd1, was critical for the regulation of REPC pool size in the absence of Phd2. Phd3 itself is HIF-regulated and has been shown to be part of a negative-feedback loop that suppresses HIF activity under hypoxia and reoxygenation conditions (45, 46). Thus it is plausible that interstitial cells that respond to Phd2 inactivation with EPO synthesis are more sensitive to oxygen deprivation and may represent a distinct population of renal interstitial cells that is part of a first-line defense against hypoxia. Our data suggest that failure to activate Epo in Phd2+/− cells does not reflect increased availability of oxygen; the presence of oxygen is predicted to keep PHD1 and PHD3 catalytically active and thus suppress HIF-2 activation, i.e., Epo induction. If this were the case, one would predict that the spa-
tial distribution of Epo-expressing cells followed the intracortical oxygen gradient (47) and would be similar to the distribution of REPCs under hypoxic conditions. However, this was not observed in our studies. Whether cells that respond to Phd2 inactivation with EPO synthesis are functionally distinct in other respects remains to be investigated.

In summary, we have shown that renal interstitial cells with EPO-producing capacity are derived from FOXD1-expressing stromal progenitors and are heterogeneous with regard to their responsiveness to Phd2 inactivation, regulation of HIF-2 activity, and EPO production. Our genetic data identify Phd2 as a main, and PHD3 as an additive, regulator of REPC plasticity. These findings have immediate clinical relevance as HIF-PHIs are currently being evaluated in large clinical trials for the treatment of renal anemia. To what degree different subpopulations of renal interstitial cells with EPO-producing capacity differ in their sensitivity to renal injury and contribution to the pathogenesis of renal anemia warrants further investigation.

Methods

Generation and genotyping of mice and animal procedures. The generation and genotyping of mice with floxed alleles for Epo, Hif2a (Epas1), Phd1 (Egn1), Phd2 (Egn1), and Phd3 (Egn3) have been described previously (18, 20, 48–50). To target FOXD1 stroma-derived cells, floxed alleles were bred to mice that expressed Foxd1-Cre (16). Foxd1-Cre/+; Epo(fl/fl), Foxd1-Cre/+; Hf2a(fl/fl); Foxd1-Cre/+; Phd1(fl/fl), Foxd1-Cre/+; Phd2(fl/fl), Foxd1-Cre/+; Phd3(fl/fl), and Foxd1-Cre/+; Phd3(fl/fl) are referred to as Foxd1-Epo−/−, Foxd1-Hf2a−/−, Foxd1-Phd1−/−, Foxd1-Phd2−/−, Foxd1-Phd3−/−, and Foxd1-Phd1−/−/Phd3−/−, respectively. For the inactivation of Phd2 alone we initially used 2 different mouse lines, which produced identical results (20, 48). For the inactivation of multiple Phd alleles and for ISH studies, the floxed Phd2 allele described in ref. 48 was used. To visualize cells that had undergone Foxd1-Cre-mediated recombination, mice were bred to mice that carried the ROSA26-ACTB-tdTomato-EGFP double-fluorescent Cre-reporter strain, generating Foxd1-mT/mG mice (17). For the targeting of Phd2 in PDGFRB-expressing and NG2-expressing renal interstitial cells, Pdgfrb-Cre and Cspg4-Cre transgenic mice were used (51, 52). Anemia was induced by retro-orbital phlebotomy, and hypoxia experiments were performed in an animal hypoxia chamber (BioSpherix) as previously described (3).

Analysis of blood samples and renal interstitial cell isolation. Hct, rbc, Hb, serum EPO level, and reticulocyte counts were determined as described previously (50). For the isolation of multiple Phd alleles and for ISH studies, the floxed Phd2 allele described in ref. 48 was used. To visualize cells that had undergone Foxd1-Cre-mediated recombination, mice were bred to mice that carried the ROSA26-ACTB-tdTomato-EGFP double-fluorescent Cre-reporter strain, generating Foxd1-mT/mG mice (17). For the targeting of Phd2 in PDGFRB-expressing and NG2-expressing renal interstitial cells, Pdgfrb-Cre and Cspg4-Cre transgenic mice were used (51, 52). Anemia was induced by retro-orbital phlebotomy, and hypoxia experiments were performed in an animal hypoxia chamber (BioSpherix) as previously described (3).

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Statistics. Data are reported as mean ± SEM. Statistical analyses were performed with Prism 6 software (GraphPad Software Inc.) using Student’s t-test and 1- or 2-way ANOVA with Tukey’s post hoc analysis. P values less than 0.05 were considered statistically significant.

Study approval. All studies involving human tissues were approved by the Institutional Review Boards of Vanderbilt University and Karolinska University Hospital. All procedures involving mice were performed in accordance with NIH guidelines for the use and care of live animals and were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Author contributions

HK and VHH conceived and designed the research studies, analyzed and interpreted data, wrote the manuscript, and made the figures. HK, QL, AAU, TCB, OD, PPK, and ASP performed experiments and acquired and analyzed data. HO, AW, ABF, GHF, and KWG contributed either mouse reagents or human tissues. KWG provided conceptual input.
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