Renal epithelium regulates erythropoiesis via HIF-dependent suppression of erythropoietin

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Introduction
Renal oxygen sensing mechanisms play a key role in the regulation of erythropoiesis in adults, as the kidney is the main source of erythropoietin (EPO), an oxygen-sensitive glycoprotein that is essential for red blood cell production. Decreases of renal pO2, promote hypoxia-inducible factor 2-mediated (HIF-2-mediated) induction of EPO in peritubular interstitial fibroblast-like cells, which serve as the cellular site of EPO synthesis in the kidney. It is not clear whether HIF signaling in other renal cell types also contributes to the regulation of EPO production. Here, we used a genetic approach in mice to investigate the role of renal epithelial HIF in erythropoiesis. Specifically, we found that HIF activation in the proximal nephron via induced inactivation of the von Hippel–Lindau tumor suppressor, which targets the HIF-α subunit for proteasomal degradation, led to rapid development of hypoproliferative anemia that was associated with a reduction in the number of EPO-producing renal interstitial cells. Moreover, suppression of renal EPO production was associated with increased glucose uptake, enhanced glycolysis, reduced mitochondrial mass, diminished O2 consumption, and elevated renal tissue pO2. Our genetic analysis suggests that tubulointerstitial cellular crosstalk modulates renal EPO production under conditions of epithelial HIF activation in the kidney.

The adult kidney plays a central role in erythropoiesis and is the main source of erythropoietin (EPO), an oxygen-sensitive glycoprotein that is essential for red blood cell production. Decreases of renal pO2 promote hypoxia-inducible factor 2-mediated (HIF-2-mediated) induction of EPO in peritubular interstitial fibroblast-like cells, which serve as the cellular site of EPO synthesis in the kidney. It is not clear whether HIF signaling in other renal cell types also contributes to the regulation of EPO production. Here, we used a genetic approach in mice to investigate the role of renal epithelial HIF in erythropoiesis. Specifically, we found that HIF activation in the proximal nephron via induced inactivation of the von Hippel–Lindau tumor suppressor, which targets the HIF-α subunit for proteasomal degradation, led to rapid development of hypoproliferative anemia that was associated with a reduction in the number of EPO-producing renal interstitial cells. Moreover, suppression of renal EPO production was associated with increased glucose uptake, enhanced glycolysis, reduced mitochondrial mass, diminished O2 consumption, and elevated renal tissue pO2. Our genetic analysis suggests that tubulointerstitial cellular crosstalk modulates renal EPO production under conditions of epithelial HIF activation in the kidney.

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Results

Pan-epithelial Vhl inactivation in the kidney results in rapid development of renal anemia. In order to study the effects of constitutive HIF stabilization in renal epithelial cells, we generated a mouse model of conditional Vhl inactivation in which a luciferase and Cre-recombinase transgene (LC-1) is under control of a tetracycline-sensitive bidirectional promoter, which is activated by a reverse tetracycline-responsive transactivator (rtTA) under control of paired box 8 (Pax8) gene regulatory elements (14). As previously reported, Pax8–rtTA–based Cre/loxP-mediated recombination targets renal epithelial cells along the entire nephron (Figure 1A) as well as a subset of hepatocytes (14). To achieve efficient and reproducible Vhl inactivation, 4-week-old Vhl<sup>−/−</sup> Pax8–rtTA LC-1 mice and their Cre-negative littermates were treated with doxycycline for 2 weeks (hereafter referred to as P8;Vhl<sup>−/−</sup> mice and control mice). Phenotypic analysis was carried out within 2 weeks after completion of doxycycline treatment. Genomic PCR was used to confirm efficient recombination in the kidney and liver; recombination in other organs was not detected (Figure 1B and data not shown). Pax8–rtTA–mediated inactivation of Vhl resulted in HIF-1α and HIF-2α stabilization in the kidney (Figure 1B), and immunohistochemical analysis revealed strong nuclear HIF-1α staining in both cortical and medullary tubular epithelial cells, whereas HIF-2α was predominantly found in cortical nephron segments (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI74997DS1). The nuclei of renal interstitial cells and glomeruli remained negative for both HIF-1α and HIF-2α, which is in line with the published expression pattern of Pax8–rtTA (14). In P8;Vhl<sup>−/−</sup> mice, HIF-2α was detected in a subpopulation of hepatocytes (approximately 5% of all hepatocytes) (Supplemental Figure 1A). HIF-2α stabilization in hepatocytes was associated with polycythemia (hematocrit [Hct]: 65.7% ± 0.7% in P8;Vhl<sup>−/−</sup> mutants vs. 47.7% ± 1.2% in littermate controls; n = 3 each) (Figure 1C), which is consistent with findings from our other laboratories (15, 16). Hepatic Epo transcript levels were strongly increased in P8;Vhl<sup>−/−</sup> mutant mice, while renal Epo mRNA levels were suppressed, indicating that the kidneys of P8;Vhl<sup>−/−</sup> mutant mice responded physiologically to the presence of polycythemia and that REPCs were not targeted by Pax8–rtTA–mediated Cre recombination (Figure 1C). In contrast to the Pax8–rtTA model, Vhl inactivation in renal epithelium as well as in nonendothelial interstitial cells using a Cre transgene driven by the Pax3 promoter (4) increased renal Epo mRNA levels substantially (Supplemental Figure 1B). Taken together, our genetic data support the concept that renal epithelial cells do not have the capacity to synthesize EPO under conditions of constitutive HIF activation (Figure 1D).

In order to understand the polycythemia-independent effects of renal epithelial Vhl inactivation on the kidney, we established a Pax8–rtTA–based conditional Vhl knockout model, in which the supraphysiologic EPO production in the liver was ablated. For this, we generated Vhl/<sup>Epo</sup> double-knockout mice (P8;Vhl<sup>−/−</sup>Epo<sup>−/−</sup>), which were homozygous for both the Vhl- and the Epo–2-lox alleles. In this model, both alleles recombined with equal efficiency in kidney and liver (Figure 2A). Because REPCs were not targeted and because EPO synthesis in Vhl-deficient hepatocytes was ablated, we hypothesized that erythropoiesis in P8;Vhl<sup>−/−</sup>Epo<sup>−/−</sup> mutant mice would be normal. Surprisingly, P8;Vhl<sup>−/−</sup>Epo<sup>−/−</sup> mutant mice developed significant anemia rapidly, within 2 weeks after doxycycline treatment was initiated (Hct: 28.8% ± 0.8% vs. 43.4% ± 0.6%; Hb: 8.7 ± 0.4 g/dl vs. 13.0 ± 0.1 g/dl; rbc: 6.2 ± 0.2 M/l vs. 9.2 ± 0.1 M/l; n = 5 each) (Figure 2B). Reticulocyte counts and reticulocyte production index (RPI) were reduced, which indicated that anemia was hypoproliferative (Figure 2C). In contrast, erythropoiesis in P8;Epo<sup>−/−</sup> single-knockout mice was not impaired, which is in line with our observation that REPCs were not targeted in the Pax8–rtTA–based genetic model and which established that anemia was the result of Vhl inactivation and not Epo inactivation in epithelial cells (Figure 2B). Renal Epo transcript levels were significantly reduced in P8;Vhl<sup>−/−</sup>Epo<sup>−/−</sup> mutant mice, indicating that P8;Vhl<sup>−/−</sup>Epo<sup>−/−</sup> mutants developed anemia due to insufficient renal Epo transcription, whereas nonanemic P8;Epo<sup>−/−</sup> mutants produced normal renal Epo transcript levels at baseline (Figure 2D) and had normal sEPO responses to phlebotomy (Supplemental Figure 4B). Taken together, our findings demonstrate that acute ablation of Vhl in renal epithelial cells suppresses EPO production in the kidney and results in the rapid development of renal anemia.

Since anemia in patients with CKD develops when renal function declines by at least 50% and glomerular filtration rate (GFR) is less than 60 ml/min (3), we examined whether rapid anemia development in P8;Vhl<sup>−/−</sup>Epo<sup>−/−</sup> mice was accompanied by decreased renal function. We measured blood urea nitrogen (BUN) levels and determined GFR by measuring FITC-inulin clearance. We furthermore evaluated renal blood flow (RBF) by 99mTc-MAG3 scintigraphy 2 weeks after completion of doxycycline treatment. We did not observe significant differences in either BUN levels (43.25 ± 1.8 mg/dl in P8;Vhl<sup>−/−</sup>Epo<sup>−/−</sup> mutants vs. 36.75 ± 4.6 mg/dl in controls, n = 4 each), GFR values (234.5 ± 38.4 μl/min in P8;Vhl<sup>−/−</sup>Epo<sup>−/−</sup> mutants vs. 218.0 ± 13.0 μl/min in controls, n = 3 each), or RBF (0.36 ± 0.01 relative activity/s in P8;Vhl<sup>−/−</sup>Epo<sup>−/−</sup> mice vs. 0.32 ± 0.04 relative activity/s in controls, n = 4 each) between P8;Vhl<sup>−/−</sup>Epo<sup>−/−</sup> and control mice (Supplemental Figure 2A). Plasma Na⁺ and K⁺ levels were normal, indicating that sodi-
anemia in P8;Vhl fl/fl Epo fl/fl mice did not result from a loss of renal function or structural changes in the kidney and was not associated with renal inflammation.

Inactivation of epithelial Vhl reduces the number of REPCs. Previous studies have shown that the number of REPCs, which is dependent on the degree of renal hypoxia, controls renal EPO production (for a detailed overview see ref. 3). To examine whether acute Vhl ablation in renal epithelium affected the number of REPCs, we used high-resolution in situ hybridization to visualize REPCs in nonphlebotomized and phlebotomized P8;Vhl fl/fl Epo fl/fl and Cre–Cre control mice. We found that the number of REPCs at baseline was significantly reduced in P8;Vhl fl/fl Epo fl/fl mice compared with Cre–Cre control animals (1.7 ± 0.8 cells/mm² in mutants and 9.8 ± 0.5 cells/mm² in control mice). To examine EPO production in P8;Vhl fl/fl Epo fl/fl mice (Hct: 20.1% ± 1.5%, n = 7) (Supplemental Figure 3C). Despite the predicted increase of REPC number was severely blunted in P8;Vhl fl/fl Epo fl/fl mice (37.1 ± 12.4 cells/mm²) compared with control mutant and control mice (n = 3 each). (D) Schematic depicting the cellular contributions to sEPO under physiological conditions in control (left panel) and P8;Vhl fl/fl Epo fl/fl mice (right panel). The red X indicates Cre-mediated gene inactivation. The black x indicates that the Epo gene in tubular epithelial cells (TEC) is not transcribed. The tilde indicates that Epo is nondetectable in hepatocytes at baseline and does not contribute to plasma EPO levels under normoxic conditions. In P8;Vhl fl/fl Epo fl/fl mutants, EPO is produced in hepatocytes, while renal EPO output is physiologically suppressed. ***P < 0.001, 2-tailed Student’s t test. Shown are mean values ± SEM. Scale bars: 200 μm. 2-lox, nonrecombined conditional allele; 1-lox, recombined allele. See Supplemental Figure 1 for additional information.

Renal cortical PtO₂ is elevated in P8;Vhl fl/fl Epo fl/fl mice. Because the size of the REPC pool is PtO₂ dependent, we next asked whether the reduction in REPC number in P8;Vhl fl/fl Epo fl/fl mice was associated with changes in renal PtO₂. Cortical PtO₂ was determined with a Clark electrode in 3 groups of mice: P8;Vhl fl/fl Epo fl/fl mutants (Hct: 33.0% ± 0.8%, obtained in 6 of 10 experimental mice), normoxic control mice (Hct: 44.8% ± 1.1%, obtained in 6 of 10 experimental mice), and phlebotomized anemic control mice (Hct: 20.1% ± 1.5%, n = 7) (Supplemental Figure 3C). Despite of anemia in P8;Vhl fl/fl Epo fl/fl and control mice by phlebotomy (Hct: 14.4% ± 0.6% and 16.7% ± 1.4%, respectively). We found that the predicted increase of REPC number was severely blunted in P8;Vhl fl/fl Epo fl/fl mice (210.2 ± 19.1 cells/mm²) (Figure 3A). The decrease in REPC number was associated with a significant reduction in sEPO levels as well as a reduction in renal Epo transcript levels (Supplemental Figure 3A). In contrast with P8;Vhl fl/fl Epo fl/fl mutants, the level of renal Epo induction was similar between phlebotomized P8;Vhl fl/fl Epo fl/fl and control mice, demonstrating that Pax8–rtTA–mediated inactivation of Epo alone in renal epithelium did not affect the physiologic behavior of renal interstitial cells with EPO-producing potential (Supplemental Figure 3B).
A prolyl-4-hydroxylase inhibitor (PHI) would result in equally efficient HIF activation in kidneys from mutant and control mice and lead to comparable Epo transcription if the PHD/HIF/EPO axis was functionally intact in interstitial fibroblast-like cells. For the pharmacologic inhibition of HIF prolyl-4-hydroxylation, we utilized tool compound GSK1002083A (GlaxoSmithKline). We have previously shown that GSK1002083A resulted in robust activation of the HIF/EPO axis regardless of PtO2 levels (4). While PHI treatment led to an approximately 16-fold increase in renal Epo mRNA levels in control mice, Epo induction in PHI-treated P8;Vhlfl/fl Epofl/fl mutants was blunted (~7-fold increase compared with control; n = 8 each; P < 0.001) (Figure 3C). This finding suggested that the PHD/HIF/EPO axis in P8;Vhlfl/fl Epofl/fl mutants was less sensitive to PHD inhibition and raised the possibility that anemia development in these mutants was not exclusively due to increased cortical PtO2 and may also have involved PtO2-independent mechanisms.

In the presence of anemia, cortical PtO2 in P8;Vhlfl/fl Epofl/fl mice was significantly elevated compared with that in normemic controls (39.26 ± 2.2 mmHg vs. 30.8 ± 1.3 mmHg; n = 10 for both groups; P < 0.01). PtO2 values in anemic control mouse averaged 14.2 ± 2.6 mmHg, which was expected, as oxygen-carrying capacity in these mice was significantly reduced (n = 7) (Figure 3B). Arterial O2 saturation (SaO2) or PaO2 was found to be indistinguishable between mutants and control mice, indicating that differences in arterial oxygenation did not account for the observed differences in cortical PtO2 (Supplemental Figure 3C).

To gain additional insights into the regulation of EPO in P8;Vhlfl/fl Epofl/fl mutants, we asked whether EPO suppression in P8;Vhlfl/fl Epofl/fl mutants was exclusively dependent on PtO2. Assuming equal bioavailability, we predicted that systemic administration of a potent pan-HIF prolyl-4-hydroxylase inhibitor (PHI) would result in equally efficient HIF activation in kidneys from mutant and control mice and lead to comparable Epo transcription if the PHD/HIF/EPO axis was functionally intact in interstitial fibroblast-like cells. For the pharmacologic inhibition of HIF prolyl-4-hydroxylation, we utilized tool compound GSK1002083A (GlaxoSmithKline). We have previously shown that GSK1002083A resulted in robust activation of the HIF/EPO axis regardless of PtO2 levels (4). While PHI treatment led to an approximately 16-fold increase in renal Epo mRNA levels in control mice, Epo induction in PHI-treated P8;Vhlfl/fl Epofl/fl mutants was blunted (~7-fold increase compared with control; n = 8 each; P < 0.001) (Figure 3C). This finding suggested that the PHD/HIF/EPO axis in P8;Vhlfl/fl Epofl/fl mutants was less sensitive to PHD inhibition and raised the possibility that anemia development in these mutants was not exclusively due to increased cortical PtO2 and may also have involved PtO2-independent mechanisms.

Figure 2. Renal epithelial Vhl inactivation results in anemia. Hematologic effects of Pax8-rtTA–mediated inactivation of Vhl in the absence of hepatic EPO induction. (A) Genomic PCR analysis of DNA isolated from kidney, liver, and tail of P8;Vhlfl/fl Epofl/fl and Cre control mice. (B) Hct, Hb, and rbc counts in P8;Vhlfl/fl Epofl/fl and control (n = 5 each). (C) Corresponding reticulocyte counts (Retic.) and RPI in P8;Vhlfl/fl Epofl/fl and control mice (n = 5 each). (D) Relative renal and hepatic Epo mRNA levels in P8;Vhlfl/fl Epofl/fl and control mice (top panel; n = 5 each) and P8;Epofl/fl (bottom panel; n = 6 kidney samples and n = 3 liver samples) in comparison with control mice (n = 8 kidney samples and n = 3 liver samples). (E) Schematic depicting cellular contributions to sEPO levels in P8;Epofl/fl single mutants (left panel) and P8;Vhlfl/fl Epofl/fl double mutants (right panel). Red x indicates Cre-mediated gene inactivation; black x indicates that the Epo gene is not transcribed in tubular epithelial cells. The tildes indicate that Epo is not detectable in hepatocytes under baseline conditions. EPO production in the liver is no longer increased in P8;Vhlfl/fl Epofl/fl double mutants (right panel). **P < 0.01; ***P < 0.001, 1-way ANOVA followed by Dunnett’s post hoc analysis (B); unpaired 2-tailed Student’s t test (C and D). Shown are mean values ± SEM. 2-lox, nonrecombined conditional Vhl or Epo allele; 1-lox, recombined allele. See Supplemental Figure 2 for additional information.
**Vhl ablation in renal epithelium shifts renal metabolism toward glycolysis.** We next asked whether changes in renal metabolism could be responsible for the increased cortical PtO₂, as HIF regulates cellular energy metabolism by increasing glucose utilization and glycolysis and shifts oxidative phosphorylation toward glycolysis (17) by blocking the conversion of pyruvate to acetyl-CoA via inhibition of pyruvate dehydrogenase (PDH) (18, 19). To assess the effects of pan-tubular HIF activation on renal energy metabolism in vivo, we first performed microarray-based genome-wide mRNA expression analysis in whole-kidney renal energy metabolism in vivo, we first performed microarray hybridization (red spots) in kidney sections from P8;Vhlfl/fl Epofl/fl and Cre control mice at baseline and following phlebotomy. DAPI (blue) was used for nuclear staining. Number of Epo-expressing cells was counted in each renal cross-section and expressed as number of cells/mm² (n = 3 each). (B) Shown are the results from cortical tissue, where partial pressure (PtO₂) measurements in P8;Vhlfl/fl Epofl/fl and control mice. Each data point represents the average mean value of at least 3 measurements per individual mouse (n = 10, 10, and 7, respectively). (C) Relative Epo mRNA levels in whole-kidney homogenates from P8;Vhlfl/fl Epofl/fl and control mice treated with PHI GSK1002083A 1 week after completion of doxycycline treatment (n = 8, 8, and 3, respectively). *P < 0.05; **P < 0.01; ***P < 0.001, unpaired 2-tailed Student’s t test (A); 1-way ANOVA followed by Dunnett’s post-hoc analysis (B and C). Shown are mean values ± SEM. Scale bar: 100 μm. See Supplemental Figure 3 for additional information.

**Vhl ablation in renal epithelium decreases O₂ consumption.** To assess renal O₂ consumption in Vhl-deficient kidneys, we performed high-resolution respirometry in mechanically permeabilized cortical and medullary tissue samples from P8;Vhlfl/fl and P8;Vhlfl/fl Epofl/fl mutant kidneys. We assessed respiratory activity...
at complex I (cI), complex II (cII), and complex IV (cIV), utilizing complex-specific substrates and inhibitors. In cortical and medullary tissue preparations, state 2 respiration (lack of ADP in the presence of substrate) and the activities of cI, cII, and cIV were markedly reduced in both P8;Vhlfl/fl and P8;Vhlfl/fl Epofl/fl kidneys (shown here are data for P8;Vhlfl/fl Epofl/fl and control kidneys; n = 5, respectively) (Figure 5A). Because HIF regulates mitochondrial biogenesis (20), we next assessed mitochondrial mass in Vhl-deficient mice. For this, we measured the activity of mitochondrial citrate synthase (CS) in cortical and medullary tissue samples (21). Both cortical and medullary CS activity, which typically correlate with mitochondrial mass, were significantly reduced, by approximately 30% and 50%, respectively. This finding was furthermore reflected in a comparable reduction of mitochondrial DNA (mtDNA) (Figure 5, B and C). Single mitochondrial respiration in cortex, which was assessed by normalizing O2 consumption to CS activity, was not different between P8;Vhlfl/fl Epofl/fl and control mice (n = 5 each). Normalized O2 consumption in medullary preparations was also unchanged for state 2, cI, and cII respiration, while single mitochondrial O2 consumption through cIV was increased (Figure 5D). In summary, our results demonstrate that reduced renal O2 consumption in Vhl-deficient mice was associated with a reduction in mitochondrial mass.

Cocontribution of epithelial HIF-1α and HIF-2α to anemia development in P8;Vhlfl/fl Epofl/fl mice. Because both HIF-1α and HIF-2α were detected in renal tissue from Vhl-deficient mice (Figure 1), we generated P8;Vhlfl/fl Epofl/fl Hif1afl/fl and P8;Vhlfl/fl Epofl/fl Hif2afl/fl triple-knockout mice to characterize the individual contributions of HIF-1α

Figure 4. Epithelial Vhl ablation alters renal metabolism. Differential regulation of genes involved in glycolysis (A), fatty acid metabolism (B), and renal transport function (C) by genome-scale RNA expression profiling in kidneys from P8;Vhlfl/fl and Cre− control mice (n = 4 each). Shown are differentially regulated metabolic genes that were either significantly up- or downregulated (≥ 1.5-fold). Color intensity is scaled within each row so that the highest expression value corresponds to bright red and the lowest to bright green. Each column represents expression levels in an individual kidney. (D) Verification of gene-expression analysis by quantitative PCR for key target genes from P8;Vhlfl/fl and Cre− control mice (n = 5 each). (E) Assessment of renal glucose metabolism by quantification of 2-Deoxyglucose uptake in renal medulla from P8;Vhlfl/fl Epofl/fl and control mice (n = 3 each). (F) Renal M3 pyruvate and M3 lactate concentrations in total kidney extracts from P8;Vhlfl/fl Epofl/fl and control mice (n = 3 each). *P < 0.05; **P < 0.01; ***P < 0.001, unpaired 2-tailed Student’s t test. Shown are mean values ± SEM.
and HIF-2α to the development of anemia and metabolic reprogramming in P8;Vhlfl/fl Epofl/fl double mutants. Genomic PCR analysis was used to confirm that efficient recombination of all conditional alleles (Vhlfl/fl, Epofl/fl, and Hif1afl/fl or Hif2afl/fl) was achieved, and only mice with efficient recombination of the respective alleles in renal tissue were included in the analysis (Supplemental Figure 4A). Complete blood count analysis showed that erythropoiesis remained impaired in both P8;Vhlfl/fl Epofl/fl Hif1afl/fl and P8;Vhlfl/fl Epofl/fl Hif2afl/fl mutants (Hct: 30.0% ± 1.3%; Hb: 9.2 ± 0.3 g/dl; rbc: 6.4 ± 0.2 M/l; n = 6 and Hct: 34.3% ± 0.9%; Hb: 10.7 ± 0.2 g/dl; rbc: 7.9 ± 0.2 M/l; n = 3) (Figure 6A), suggesting that both HIF-α homologs contributed to the development of anemia in P8;Vhlfl/fl Epofl/fl mice.

Although renal Epo transcript levels in P8;Vhlfl/fl Epofl/fl Hif1afl/fl and P8;Vhlfl/fl Epofl/fl Hif2afl/fl mice were statistically not significantly different from those of control animals (Figure 6A), they were inadequate for the degree of anemia found in triple-mutant mice. Anemia of nonrenal etiology is usually characterized by an inverse linear relationship between Hct and the decadic logarithm of the hemoglobin concentration (Hct: 30.0% ± 1.3%; Hb: 9.2 ± 0.3 g/dl; rbc: 6.4 ± 0.2 M/l; n = 6 and Hct: 34.3% ± 0.9%; Hb: 10.7 ± 0.2 g/dl; rbc: 7.9 ± 0.2 M/l; n = 3) (Figure 6A), suggesting that both HIF-α homologs contributed to the development of anemia in P8;Vhlfl/fl Epofl/fl mice.

To investigate whether HIF-1 and HIF-2 served redundant functions with regard to the observed changes in metabolic gene expression in P8;Vhlfl/fl Epofl/fl mice, we measured expression levels of genes involved in glycolysis (Pgkd1 and pyruvate dehydrogenase kinase 1 [Pdk1]), lipid metabolism (angiopoietin like 3 [Angptl3] and apolipoprotein C-III [Apoc3]), and transport functions (Scl13a4 and serum/glucocorticoid regulated kinase 2 [Sgk2]). As shown in Figure 6B, upregulation of glycolytic gene expression appeared to be exclusively HIF-1 dependent, whereas the expression of Angptl3 and Apoc3 appeared to be HIF-2 dependent. While we could not attribute the suppression of transport genes Scl13a4 and Sgk2 to a specific HIF-α homolog, coinactivation of either Hif1a or Hif2a in P8;Vhlfl/fl Epofl/fl mice was sufficient to increase mitochondrial mass (Figure 6C). Taken together, our genetic data suggest that the development of anemia in P8;Vhlfl/fl Epofl/fl mice required both HIF-1 and HIF-2 activation and could not be attributed to one HIF homolog alone.

Regulation of EPO synthesis by tubular epithelial cells is HIF dependent and localizes to the proximal nephron. Because we dissected the role of HIF-1 and HIF-2 in the development of anemia in Vhl-deficient mice and found that inactivation of either homolog did not restore normal erythropoiesis, we investigated to what degree anemia development in P8;Vhlfl/fl Epofl/fl mice required intact signaling through both HIF-α homologs. For this, we bred mice that carried a floxed Hif1b (Arnt) allele to P8;Vhlfl/fl Epofl/fl mice, generating P8;Vhlfl/fl Epofl/fl Arntfl/fl triple-knockout mice. ARNT serves as the β-subunit for both HIF-1 and HIF-2 heterodimers, and its inactivation abolishes canonical HIF signaling in renal epithelium (23). Concomitant ablation of Arnt restored hematologic parameters to normal values, as no significant differ-
ences in Hct, Hb, or rbc were found between P8;Vhlfl/fl Epofl/fl Arntfl/fl and control mice (Figure 7A). This finding is consistent with renal Epo transcript levels in P8;Vhlfl/fl Epofl/fl Arntfl/fl mice that were comparable to those of control mice (Figure 7A). These genetic data establish that the suppression of renal EPO production in P8;Vhlfl/fl Epofl/fl mice required intact HIF signaling.

To investigate which specific segment of the nephron is involved in the suppression of renal EPO production, we examined several conditional Vhl knockout lines in which different nephron segments were targeted (Pepck-Cre, renal proximal tubule; Thp-Cre, medullary thick ascending limb of Henle and early distal tubule; Hoxb7-Cre, collecting duct). Since Pepck-Cre–driven conditional ablation of Vhl leads to polycythemia due to induction of hepatic EPO production (23), we generated Pepck-Cre Vhlfl/fl Epofl/fl double-knockout mice. Similar to the P8;Vhlfl/fl Epofl/fl mutants, Pepck-Cre Vhlfl/fl Epofl/fl double-knockout mice were anemic (6 to 8 weeks old, Hct: 29.4% ± 1.5%; Hb: 6.8 ± 0.4 g/dl; rbc: 7.1 ± 0.3 × 10⁶/μl; n = 6), whereas mice with heterozygous deletion of Vhl and homozygous deletion of Epo (Pepck-Cre Vhlfl/+ Epofl/fl) displayed normal erythropoiesis (Hct: 39.0% ± 0.7%; Hb: 12.9 ± 0.2 g/dl; rbc: 8.8 ± 0.1 × 10⁶/μl; n = 4), (Figure 7B). Hematological analysis of 6- to 8-week-old Thp-Cre Vhlfl/fl (n = 5) and Hoxb7-Cre Vhlfl/fl...
The renal proximal tubule regulates EPO production in an HIF-dependent manner. (A) Upper panels show normal Hct, Hb, and rbc counts in P8;Vhlfl/fl Arntfl/fl mutant mice and Cre–control mice (n = 4–5). Lower panel shows relative Epo mRNA expression levels in kidney homogenates from P8;Vhlfl/fl Arntfl/fl, P8;Vhlfl/fl Arntfl/fl, and control mice (n = 4, 4, and 7, respectively). (B) Shown are Hct, Hb, and rbc counts for individual 6- to 8-week-old Pepck-Cre Vhlfl/fl, Pepck-Cre Vhlfl/fl, and Cre–control mice (n = 4, 6, and 7, respectively). (C) Hct, Hb, and rbc counts in 6- to 8-week-old Thp-Cre Vhlfl/fl, Hoxb7-Cre Vhlfl/fl, and Cre–control mice (n = 5, 7, and 5, respectively). **P < 0.01; ***P < 0.001, unpaired 2-tailed Student’s t test (A, upper panel); 1-way ANOVA followed by Dunnett’s post hoc analysis (A, lower panel, B, and C). Shown are mean values ± SEM.

Discussion

In the current study, we utilized inducible and cell type–specific Cre recombinase–mediated gene targeting to investigate the role of non–EPO-synthesizing cell types in the regulation of EPO production in the kidney. We demonstrate that HIF activation in renal epithelial cells via acute Vhl gene deletion suppressed erythropoiesis by restricting the number of interstitial fibroblast-like cells that are able to convert to REPCs. This effect localized to the proximal nephron and was associated with renal gene expression and functional changes that indicated a shift from oxidative phosphorylation toward glycolysis as well as decreased O2 consumption due to reduced mitochondrial mass. As a result of epithelial HIF activation, renal cortical PO2 was elevated despite the presence of marked anemia. Our data suggest that HIF-regulated tubulointerstitial intercellular crosstalk suppresses EPO production under conditions of sustained HIF activation in the kidney. We propose a model whereby HIF activation in a renal cell type that does not synthesize EPO modulates renal EPO production by regulating the number of REPCs.

Ascent to high altitude triggers a pronounced systemic hypoxia response, which results in increased renal EPO synthesis and rbc production. Interestingly, sEPO levels decline rapidly before significant changes in Hct are noted (12, 13). While the reasons for this phenomenon are not clear, increased sequestration of EPO from serum by EPO-expressing cells in the bone marrow has been proposed as a possible underlying mechanism (2). The decline in sEPO levels is, however, paralleled by a reduction in renal Epo mRNA expression pointing toward intercellular crosstalk that is directed at local EPO synthesis (24). While it is possible that the hypoxic induction of PHD3 might reduce HIF levels and suppress Epo transcription in REPCs, there is currently no experimental evidence that PHD3 alone regulates renal EPO synthesis (25–29). Findings from our genetic studies, however, would lend experimental sup-
port to the notion that HIF-regulated metabolic adaptation of renal tubular epithelial cells to hypoxia suppresses EPO synthesis in the kidney partly via modulation of renal O2 consumption.

In our model, both epithelial HIF-1 and HIF-2 were involved in modulating renal EPO production at baseline and under conditions of anemic hypoxia, as concomitant deletion of either Hif1α or Hif2α in P8;Vhlfl/fl Epofl/fl mice did not restore erythropoiesis to control levels and resulted in inadequate sEPO levels for the degree of anemia observed in these mutants. While epithelial HIF activation in P8;Vhlfl/fl Epofl/fl mice was associated with anemia, we did not find that inactivation of either epithelial HIF-1α or HIF-2α alone (P8;Hif1αfl/fl, P8;Hif2αfl/fl) or together (P8;Hif1αfl/fl Hif2αfl/fl) enhanced renal EPO responses when mice were exposed to prolonged hypoxia (8% O2 for 6 days) or phlebotomy (data not shown). This may be due to the degree of hypoxia generated in this model, i.e., levels of HIF-α stabilization, hypoxic adaptation over time, or timing of the analysis. Our model of nonhypoxic HIF activation is based on Vhl gene deletion in renal epithelial cells alone, which leads to the complete loss of the ability to degrade HIF-α and would mimic severe hypoxia. Under severe hypoxia, factor inhibiting HIF (FIH), an HIF asparagine hydroxylase that hydroxylates an asparaginase residue in the C-terminal transactivation domain of HIF-α, would further modify HIF-mediated hypoxia responses, as it operates as a second hypoxic switch that regulates recruitment of coactivators to the HIF transcriptional complex (5). Nevertheless, our model provides genetic evidence for HIF-regulated tubulointerstitial cell interaction affecting the conversion of interstitial cells into EPO-producing cells. It is plausible that in the hypoxic kidney, additional nonepithelial cell–derived paracrine signals that also modulate the renal EPO response may come into play.

EPO synthesis occurs in peritubular interstitial fibroblast-like cells, which are normally found in the inner cortex and corticome-

Figure 8. Renal epithelial cells regulate erythropoiesis. Model depicting the role of renal tubular epithelial cells in the regulation of EPO production at baseline (upper panel) and under conditions of constitutive HIF activation as a result of Vhl inactivation (lower panel). Constitutive HIF stabilization in renal tubular epithelial cells inhibits the conversion of non–EPO–producing peritubular interstitial cells to REPCs.
human RCC patients, as tumorigenesis occurs in a localized fashion and involves only a small fraction of renal epithelial cells.

Renal EPO output correlates with the number of REPCs (6, 7), i.e. the degree of decrease in PTIO, determines the number of peritubular interstitial fibroblast-like cells that switch from a non-EPO-producing to an EPO-producing state. This kind of functional plasticity is not uncommon in the kidney and has been observed in other renal cell types. For example, afferent arteriolar perivascular cells change their functional phenotype and begin to produce renin in response to sodium depletion (35). While PTIO is a major determinant of REPC pool size, the regulation of REPC plasticity, especially the quality of intra- and extracellular signals that control the ability of interstitial fibroblast-like cells to switch to an EPO-producing state, is only incompletely understood. Myofibroblast transdifferentiation of renal interstitial fibroblast-like cells, which is associated with the development of renal fibrosis and increased extracellular matrix production, has been suggested as suppressing their EPO-producing potential, possibly through activation of NF-kB signaling (36). Here, we demonstrate that HIF activation in non-EPO-producing renal epithelial cells modulates REPC pool size and therefore may affect erythropoiesis under kidney injury conditions. This may be particularly important in chronic injury conditions where epithelial HIF stabilization has been shown to associate with the development of fibrosis (37).

Aside from renal interstitial cells, multiple other cell types have been shown to be capable of synthesizing EPO or of modulating erythropoiesis under certain experimental conditions (3). HIF stabilization induced by conditional ablation of Vhl or Phds in hepatocytes, astrocytes, renin-producing cells, osteoblasts, and other cell types led to increased sEPO levels and polycythemia through direct activation of tissue-specific Epo transcription (3). In contrast, HIF stabilization in non-EPO-producing keratinocytes causes polycythemia indirectly by redirecting blood flow to the skin, away from kidney and liver, which induced hypoxia and stimulated renal and hepatic EPO production (38). Our data indicate that renal epithelial HIF modulates EPO synthesis in peritubular interstitial fibroblast-like cells, thus providing evidence for kidney-specific non-cell autonomous control of EPO synthesis and suppression of EPO production in the context of HIF activation.

In summary, our genetic studies demonstrate that HIF activation in proximal tubular epithelial cells, which do not synthesize EPO, negatively modulates erythropoiesis. While more experimental studies are needed to further explore the functional relationships between epithelial cells and interstitial fibroblast-like cells in the kidney, our data provide a molecular basis for the participation of renal epithelial cells in the regulation of interstitial cell plasticity and pathogenesis of renal anemia.

Methods
Generation of mice and genotyping. The generation and genotyping of Vhl, Epo, Arnt, Hif1α, and Hif2α conditional alleles and the lacZ ROSA26 reporter (R26R) mouse line as well as LC-1, Pax8-rtTA, Pepck-Cre, Thp-Cre, Hoxb7-Cre, and Pax3-Cre transgenic lines have been described elsewhere (14–39–47).

Inducible Cre-mediated renal tubular ablation of Vhl, Arnt, Hif1α, Hif2α, and/or Epo was achieved by generating mice that carried both the rtTA-dependent Cre-recombinase (LC-1) transgene and expressed rtTA under control of the Pax8 promoter (Pax8-rtTA). The following genotypes were generated: (a) Vhlfl/fl Pax8-rtTA LC-1, (b) Epofl/fl Pax8-rtTA LC-1, (c) Vhlfl/fl Epofl/fl Pax8-rtTA LC-1, (d) Vhlfl/fl Epofl/fl Arntfl/fl Pax8-rtTA LC-1, (e) Vhlfl/fl Epofl/fl Hif1αfl/fl Pax8-rtTA LC-1, and (f) Vhlfl/fl Epofl/fl Hif2αfl/fl Pax8-rtTA LC-1. After completion of doxycycline treatment, these were referred to as P8;Vhlfl/fl, P8;Epofl/fl, P8;Vhlfl/fl Epofl/fl, P8;Vhlfl/fl Epofl/fl Arntfl/fl, P8;Vhlfl/fl Epofl/fl Hif1αfl/fl, and P8;Vhlfl/fl Epofl/fl Hif2αfl/fl, respectively. For the activation of the Pax8-rtTA/LC1 conditional knockout system, 4-week-old mice were treated for 2 weeks with doxycycline (Sigma-Aldrich) contained in drinking water at a concentration of 0.2 mg/ml in 5% sucrose water.

Constitutive conditional inactivation of Vhl and/or Epo in specific nephron segments was achieved by generating mice that were homozygous for the Vhl and/or Epo conditional alleles and expressed either a proximal tubule–specific Cre transgene (Pepck-Cre), a distal tubule–specific Cre transgene (Thp-Cre), or a collecting duct–specific Cre transgene (Hoxb7-Cre). Analysis of nephron segment-specific knockout mice was performed at age 6 to 8 weeks. Renal interstitial fibroblast-like cells and tubular epithelial cells were targeted with the Pax8-Cre transgene. The following genotypes were generated: (a) Pepck-Cre Vhlfl/fl, (b) Pepck-Cre Vhlfl/fl Epofl/fl, (c) Pepck-Cre Vhlfl/fl Hif1αfl/fl, (d) Thp-Cre Vhlfl/fl, (e) Hoxb7-Cre Vhlfl/fl, and (f) Pax3-Cre Vhlfl/fl, respectively.

DNA, RNA, and protein analysis. DNA and RNA isolation were performed as described previously (48). For real-time PCR analysis, 1 μl of cDNA was subjected to PCR amplification on an ABI StepOnePlus platform using either SYBR green or TaqMan Universal PCR Master Mix (Applied Biosystems). Relative mRNA expression levels were quantified with the relative standard curve method according to the manufacturer’s instructions (Applied Biosystems). Primer sequences for the analysis of Epo, Kim-1, Lcn2, F4/80, and Tnfa expression have been published elsewhere (48, 49). For the analysis of Il6b expression, the following primers were used: fwd, 5′-GGTCAAGGTTTG-GAAAGCAG-3′; rev, 5′-TGTGAAATGCCACCTTGG-3′. All genes were normalized to 18S levels using the 18S TaqMan set from Applied Biosystems. Microarray analysis was carried out using an Affymetrix Exon/Gene (WT) microarray chip. Intensity values from each hybridization were scaled versus a global average signal from the same array and normalized by robust multichip average (RMA) analysis. Statistical analysis was performed using a moderated t test and Bonferroni’s familywise error rate (FWER) multiple testing. Differentially regulated genes were defined by a 1.5-fold difference in mean expression values and an adjusted P value of less than 0.05 between groups. Heat maps were created using Heatmap Builder software (http://ashleylab.stanford.edu/tools/tools-scripts.html). All original microarray data were deposited in the NCBI’s Gene Expression Omnibus (GEO GSE54172). For RNA in situ detection using the RNAscope Multiplex Fluorescent Assay Kit, paraffin sections of mouse kidneys (5-μm thickness) were processed according to the manufacturer’s protocol (Advanced Cell Diagnostics). The hybridized sections were imaged with the Arios SL-50 automated slide scanner. Nuclear protein extracts for Western blot analysis were prepared as previously described; HIF-1α was detected with a polyclonal antibody from Novus Biologicals (catalog NB100-449), and HIF-2α was detected with a custom-made polyclonal antibody from Invitrogen, as previously described (15).

Pharmacological HIF activation. For pharmacological HIF activation, prolyl-4-hydroxylase inhibitor GSK1002083A (GlaxoSmithK...
Kline) was dissolved in 1% methylcellulose and administered by oral gavage at a dose of 60 mg/kg twice, 24 and 4 hours prior to tissue harvest (4). Methylcellulose without compound served as control.

**Blood work and assessment of renal function.** Hcts were determined by microcapillary tube centrifugation in a Micro Hematocrit Centrifuge (International Equipment Co.) or with a Hemavet 950 analyzer (Drew Scientific). sEPO levels were determined by ELISA (R&D Systems). Reticulocyte counts were measured by FACS analysis of whole blood stained with thiazole orange (Sigma-Aldrich). BUN levels were determined using the QuantiChrom Urea Assay Kit (BioAssay Systems), and serum Na+ and K+ concentrations were determined with an iStat analyzer and EC8+ cartridges (Abaxis). For determination of SaO2 and PaO2, blood samples were analyzed with iStat analyzer and CG8+ cartridges (Abaxis). GFR was assessed using the FITT-iculin single-injection method as previously described (50). GFR was calculated based on a 2-compartment model using GraphPad Prism (GraphPad Software). RBF was estimated by 99mTc-MAG3 dynamic scintigraphy as previously described (51).

**Histological analysis.** For HIF-1α and HIF-2α immunohistochemistry, polyclonal rabbit anti–HIF-1α antibody (Cayman Chemical, catalog 10006421) and polyclonal rabbit anti–HIF-2α anti-serum PM8 (52) were used at dilutions of 1:10,000 and 1:20,000, respectively, and detected with the CSA-II High Signal Amplification Kit and the Rabbit Link Reagent (Dako). For detection of CD45+ leukocytes, anti-mouse monoclonal anti-CD45 antibody (BD Biosciences — Pharmingen, catalog 550539) was used and detected with the Vectastain Elite Kit (Vector). X-gal staining was performed on frozen tissues sections according to MacGregor (53).

**High-resolution respirometry.** Respiratory activities in tissue samples from cortex and medulla of mutant and control mice were analyzed with the O2k Oxygraph System (Oroboros). Tissue samples from the cortex and medulla were dissected, minced on ice, and placed on ice in mitochondrial respiration buffer (final concentration: 110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 1 g/l BSA essentially fatty acid free, 3 mM MgCl2, 20 mM tauroine, 10 mM KH2PO4, 20 mM Hepes, adjusted to pH 7.1 with KOH) at a concentration of 1 mg/ml. Samples from P8;Vhlfl/fl Epofl/fl mice and (f) TMPD/ascorbate (final concentration: 0.5/2 mM). Measurements were live recorded using DatLab software (Oroboros).

**Metabolic analysis and cortical PtO2 measurements.** CS activity was measured as previously described (21). Briefly, aliquots from samples used for high-resolution respirometry were homogenized, and 20 μl of homogenate was added to 180 μl medium containing 0.1 mM 5,5′-dithio-bis-(2-nitrobenzoic) acid (DTNB) (Sigma-Aldrich), 0.5 mM oxaloacetate (Sigma-Aldrich), 50 μM EDTA (Sigma-Aldrich), 0.31 mM acetyl coenzyme A (Sigma-Aldrich), 5 mM triethanolamine hydrochloride (Fluka), and 0.1 M Tris–HCl, pH 8.1 (Sigma-Aldrich). The activity of CS was measured spectrophotometrically at 412 nm. Glucose uptake was measured in renal medulla with 2- DG at 13 μCi/mouse and normalized to brain as previously described (54). Polar metabolites were analyzed as previously described (55). Mice were fasted for 4 hours prior to intravenous injection of U-13C glucose (20 mg/mouse). At 25 minutes following the injection, mice were sacrificed and kidneys were harvested. 13C isotopic enrichment was analyzed in total kidneys using Agilent 6890/5975N GC-MS equipped with 25 m DB-5ms capillary column for analysis of isotopic enrichment.

For cortical PtO2 measurements, mice were subjected to inhalation anesthesia with isoflurane. Core body temperature was monitored with a rectal probe and maintained between 36.5°C and 37.5°C. The left kidney was exposed through flank incision and was stabilized without causing excessive strain on renal vessels. The kidney capsule was removed, and the left kidney was covered with mineral oil. Renal PtO2 was measured with a modified Clark-type microelectrode (β = 8–10 μm, Unisense). Two-point calibration was carried out in water saturated with either N2 gas or ambient air at 37°C. For every mouse, a minimum of 3 independent measurements were obtained per kidney 1 mm below the renal surface after a linear correlation between O2 tension and electrical current was established. For statistical analysis, only average mean values from individual mice were used.

**Statistics.** Data reported represent mean values ± SEM. Statistical analyses were performed with Prism 5.0b software (GraphPad Software). When 2 groups of mice were compared, the unpaired, 2-tailed Student’s t test was used; for comparison of 3 and more groups, 1-way ANOVA followed by Dunnett’s post hoc analysis was used. P < 0.05 was considered statistically significant.

**Study approval.** All procedures involving mice were performed in accordance with NIH guidelines for the use and care of live animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of Vanderbilt University.

**Author contributions**

NMF and VHH conceived and designed the research studies, analyzed and interpreted data, wrote the manuscript, and created the figures. NMF, QL, HK, FS, and OD performed experiments and acquired and analyzed data. TAI, PMO, and JF helped with design and interpretation of research studies or contributed reagents or technical expertise.

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