Renal CD133$^+$/CD73$^+$ Progenitors Produce Erythropoietin under Hypoxia and Prolyl Hydroxylase Inhibition

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ABSTRACT

The identity of the peritubular population of cells with mesenchymal phenotype thought responsible for producing erythropoietin in humans remains unclear. Here, renal CD133$^+$/CD73$^+$ progenitor cells, isolated from the human renal inner medulla and described as a population of mesenchymal progenitors, released erythropoietin under hypoxic conditions. CD133$^-$ cells did not synthesize erythropoietin, and CD133$^+$ progenitor cells stopped producing erythropoietin when they differentiated and acquired an epithelial phenotype. Inhibition of prolyl hydroxylases, using either dimethyloxalylglycine or a small hairpin RNA against prolyl hydroxylase-2, increased both hypoxia-inducible factor-2α (HIF-2α) expression and erythropoietin transcription. Moreover, under hypoxic conditions, inhibition of prolyl hydroxylase significantly increased erythropoietin release by CD133$^+$ progenitors. Finally, blockade of HIF-2α impaired erythropoietin synthesis by CD133$^+$ progenitors. Taken together, these results suggest that it is the renal CD133$^+$ progenitor cells that synthesize and release erythropoietin under hypoxia, *via* the prolyl hydroxylase-HIF-2α axis, in the human kidney. In addition, this study provides rationale for the therapeutic use of prolyl hydroxylase inhibitors in the setting of acute or chronic renal injury.

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The glycoprotein hormone erythropoietin (EPO) regulates blood red cell production, linking decreased tissue oxygenation to an adequate erythropoietic response. In adults, the kidney is responsible for >90% of EPO production. Many efforts have been made to identify renal EPO-producing cells. In the rodent anemic kidney, EPO production is restricted to interstitial peritubular fibroblast-like cells localized in the deep cortex and outer medulla and coexpressing EPO mRNA and the mesenchymal marker CD73.[1–6] In the human kidney, the precise localization of EPO-producing cells is unknown. It is conceivable that, similarly to rodents, a peritubular population of mesenchymal cells/fibroblasts is responsible for EPO production. Data obtained from a human EPO-producing cell line isolated from human kidney showed that these cells possess mesenchymal characteristics and the ability to synthesize EPO in response to hypoxia-dependent hypoxia-inducible factor-2α (HIF-2α) stabilization and activation.[7] However, in human renal tissue, *in situ* hybridization studies showed EPO production in cells of renal tubules. In particular, EPO mRNA was expressed by epithelial distal tubular cells, collecting tubules and additionally by glomerular cells.[8] High EPO levels are also released

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by tumor cells of renal carcinomas,[9] considered to derive from transformed tubular or progenitor/stem cells. In addition, murine embryonic renal stem cells organized in an organoid and implanted *in vivo* in rats produced murine EPO, suggesting that EPO-producing cells derive from renal stem cells.[10]

Using CD133 as a marker, a population of renal resident progenitors has been localized in different segments of the nephron.[11–14] In particular, $CD133^+$ progenitors are enriched in the Henle's loop and thin segments of the papillary region of medulla, which is characterized by a very low oxygen tension.[15] *In vitro*, hypoxia was shown to be a key factor in the maintenance of the progenitor phenotype and stem properties of these cells.[13]

In this study, we investigated whether $CD133^+$ renal progenitors could be a source of EPO within the kidney.

RESULTS

EPO Production by Renal $CD133^+$ Cells in Normoxia and Hypoxia

$CD133^+$ cells located within the Henle's loop in the human inner medulla coexpressed the hypoxic marker carbonic anhydrase IX (CAIX) (Figure 1), indicating their continuous exposure to an hypoxic environment. Isolated $CD133^+$ progenitors from renal inner medulla displayed a mesenchymal-like phenotype,[11–13] including expression of CD73, CD29, CD44, CD90, and vimentin (Figure 2, A and F). All 16 different $CD133^+$ cell preparations used in the study, isolated by magnetic sorting from human specimens of renal medulla, showed coexpression of CD133, vimentin, and CD73 (Table 1). CD73 was also coexpressed by $CD133^+$ cells *in situ* within the renal tissue (Figure 1), suggesting their mesenchymal phenotype *in vivo*. This is in agreement with a previous study showing vimentin expression by $CD133^+$ cells in the cortex.[16]

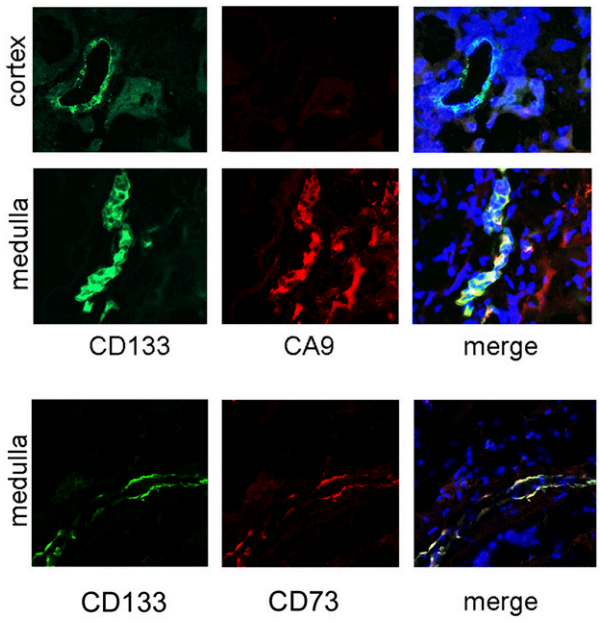

Figure 1. $CD133^+$ cells in human inner medulla express CAIX and CD73. (Upper panels) Representative confocal immunofluorescence micrographs showing staining of different regions of the normal human kidney with CD133 (clone AC133–1; green) and the hypoxic marker CAIX (red). In cortical tissue, $CD133^+$ tubules do not express CAIX. In inner medulla, $CD133^+$ structures morphologically corresponding to the Henle's loop express CAIX. (Lower panels) Representative confocal immunofluorescence micrographs showing costaining of CD133 and CD73. Nuclear staining is performed with Hoechst dye 33342. Original magnification, ×400.

The production of EPO by $CD133^+/CD73^+$ isolates and clones was tested in normoxia and hypoxia, as schematically depicted in Figure 3. Basal EPO production was detected in all isolates (Figure 2B and Table 1). After 24–48 hours in hypoxia (1% oxygen, corresponding roughly to 7.6 mmHg), EPO release into the cell supernatant of $CD133^+$ progenitors, but not of $CD133^-$ tubular epithelial cells, was significantly increased (Figure 2B). Concomitantly, $CD133^+/CD73^+$ progenitors undergoing hypoxia significantly upregulated EPO mRNA (Figure 2C). To verify the cell selectivity of EPO release, three different $CD133^+$ cell isolates were subjected to clone generation by limiting dilution technique in 96-well plates. A total of 91 clones were collected (cloning efficiency equal to 35.1%). Ten clones, showing CD133/CD73 expression >95% cells, were expanded and tested for EPO release. All these clones released EPO in the normoxic or hypoxic condition (Figure 2D). The levels of EPO release were comparable with those previously reported in renal EPO releasing human cells *in vitro*[7] and were roughly half of those obtained by HepG2 cells (Figure 2E).[17] The selective EPO expression by $CD133^+$ progenitors was further confirmed by the reduction of EPO mRNA in $CD133^+$ progenitors undergoing differentiation into renal epithelial cells. EPO mRNA downregulation was observed after 7 days and reached significance after 14 days of differentiation (Figure 2G). In parallel, $CD133^+$ cells underwent mesenchymal to epithelial differentiation, as shown by loss of the CD133 marker and of the mesenchymal marker vimentin, reduction of the mesenchymal transcription factors Snail, Slug, and Twist and acquirement of epithelial nephron markers (Figure 2, F and H, and Table 1), as previously reported.[13]

EPO Regulation by Prolyl Hydroxylase-2 Inhibition

HIF stabilization required for EPO production is modulated through prolyl hydroxylases (PHDs), the enzymes that target the α subunit of HIF-1 and HIF-2 for proteasomal degradation.[18] $CD133^+/CD73^+$ progenitors expressed PHD2 and upregulated its levels under hypoxic conditions (Supplemental Figure 1). We therefore evaluated the effect of a nonspecific PHD inhibitor dimethyloxalylglycine (DMOG)[19] on EPO synthesis. No significant cytotoxic effect was induced by DMOG at 100–500 μM of on $CD133^+$ progenitors after 24-hour incubation (cell vitality evaluated by Annexin V/propidium

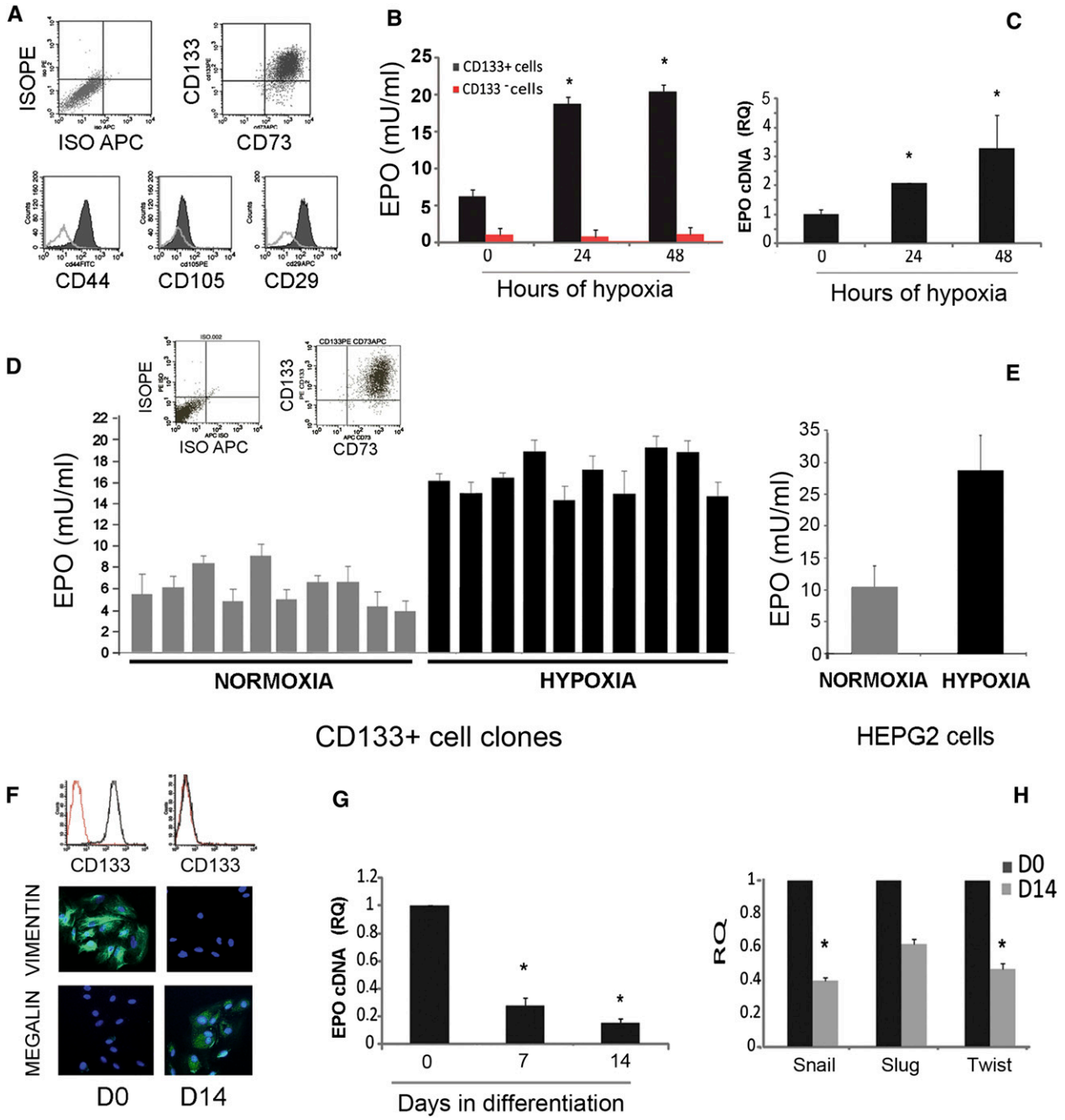

Figure 2. EPO synthesis by renal CD133$^+$/CD73$^+$ cells under hypoxia and during differentiation. (A) FACS analysis showing expression of CD133 and of mesenchymal markers CD73, CD44, and CD29, but not CD105 by isolated cells. Data are representative of all cell lines in the study. (B) Increase in EPO release by CD133$^+$ but not CD133$^-$ cells after 24 or 48 hours of hypoxia. Data are mean ± SD of different experiments performed in triplicate using six different cell isolates. (C) Quantitative RT-PCR analysis showing increase in mRNA encoding for EPO after 24 or 48 hours of hypoxia. Data are normalized to TBP mRNA and to 1 for time 0 and are the mean ± SD of three different experiments performed using three different cell isolates. ANOVA with Dunnett's comparison test was performed for B and C. *$P<0.05$ versus time 0. (D and E) EPO release by 10 different CD133$^+$/CD73$^+$ cell clones or by HepG2 cells in normoxia and after 24 hours of hypoxia. EPO release is tested in triplicate by cells plated in 24-well plates (250,000 cells per well). A typical cytofluorimetric analysis of CD133/CD73 expression is also shown. (F) Representative cytofluorimetric histogram of CD133 expression and representative micrographs of the immunofluorescence staining of cells at day 0 and 14 after differentiation. Nuclei are stained in blue with Hoechst dye 33342. Data are representative of all cell lines in the study. (G and H) Quantitative RT-PCR analysis showing decrease in mRNAs encoding for EPO (E) and mesenchymal transcription factors (F) after 7 or 14 days of epithelial differentiation. Data are normalized to TBP mRNA and to 1 for time 0 and are the mean ± SD of three different experiments performed using three different cell isolates. ANOVA with Dunnett's comparison test (G) or *t* test (H). *$P<0.05$. TBP, TATA binding protein. Original magnification, ×400 in F.

Table 1. Phenotypic and functional characteristics of the cell isolates in the study

Isolate No.	CD133 (% Expression)	CD73 (% Expression)	Vimentin (% Positive Cells)	Epithelial Difference (% Megalin/THP Positive Cells)	EPO (mU/ml)
1	97.5	95.5	>95	>95/>95	6.4
2	95.7	97.3	>95	>90/>95	7.0
3	92.7	96.5	>95	>95/>95	5.1
4	90.0	96.5	>95	>95/>95	6.2
5	94.2	98.9	>95	>95/>95	4.8
6	97.0	95.7	>95	>95/>95	7.4
7	98.5	91.9	>95	>95/>95	5.9
8	94.2	95.7	>95	N.D.	6.2
9	87.7	98.5	>95	>90/>95	4.2
10	96.7	99.5	>95	>95/>95	5.5
11	94.0	94.4	>95	>95/>95	7.5
12	93.3	96.3	>95	>95/>95	8.3
13	94.6	98.0	>95	>95/>95	8.0
14	91.5	92.7	>95	>95/>95	5.4
15	95.9	96.8	>95	N.D.	6.8
16	96.4	98.7	>95	N.D.	5.1

Expression of CD133 and of the mesenchymal markers CD73 and vimentin by the cell isolates in the study was evaluated by cytofluorimetric analysis or immunofluorescence staining at the first culture passage. Epithelial differentiation was determined by the ability to acquire the nephron segment-specific markers megalin, expressed by proximal tubular epithelial cells, and Tamm-Horsfall protein (THP), expressed by the ascending limb of the loop of Henle and by distal convolute tubules. Cells were cultured in epithelial differentiative medium for 14 days, and differentiation evaluated by immunofluorescence staining. EPO release into the cell supernatant was assessed in normoxia.

iodide staining using cytofluorimetric analysis, was >95%). In addition, we specifically inhibited PHD2, the PHD isoform known to be involved in EPO synthesis,[20] using two different small hairpin RNA (shRNA). PHD inhibition with 100 μM DMOG induced HIF-2α protein increase in $CD133^+/CD73^+$ cells (Figure 4A). An increase in HIF-1α protein levels was also observed (Supplemental Figure 2A). In parallel, PHD2 was upregulated in $CD133^+/CD73^+$ cells treated with DMOG (Figure 4C), as expected in virtue of the rapid induction of its synthesis by HIFs.[18] Indeed, DMOG does not affect the PHD protein levels because it acts as a competitive inhibitor.[19] The specific PHD2 inhibition by lentiviral shRNA for PHD2 (shPHD2 cells) enhanced HIF-2α protein levels in $CD133^+$ cells and downregulated PHD2 expression (Figure 4, B and D). HIF-1α protein levels were also increased (Supplemental Figure 2B). In $CD133^+/CD73^+$ cells, PHD2 inhibition, using either shPHD2 delivery with two different lentiviral vectors or DMOG administration, induced an upregulation of EPO mRNA (Figure 4E and Supplemental Figure 3A). In particular, DMOG treatment upregulated the expression of EPO mRNA >9-fold. The evaluation of EPO release into the cell supernatant, however, did not show a significant increase in normoxia (Figure 4F and Supplemental Figure 3B). At variance, PHD2 inhibition significantly enhanced the EPO release at 24 hours when cells were submitted to hypoxia (1% oxygen) (Figure 4F and Supplemental Figure 3B). In normoxia, increasing concentrations of the nonspecific PHD inhibitor DMOG were required to induce EPO release (Figure 4G), whereas its release in hypoxia was already at plateau at the lower DMOG concentration. Indeed, in shPHD2 cells, EPO increased in normoxia at an intracellular level (Figure 4H), indicating a possible hypoxia-based PHD2 independent control of EPO release.

Role of HIF-2α in EPO Production

EPO production is known to be modulated by hypoxia through HIF-2α activation.[7,21,22] The involvement of HIF-2α in EPO production by $CD133^+/CD73^+$ progenitors was investigated by generation of negative progenitors for HIF-2α (shHIF-2α) by infection with two different specific shRNA lentiviruses (Figure 5A). shHIF-2α cells almost completely lost the ability to synthesize EPO (Figure 5B and Supplemental Figure 3, A and B) but not vascular endothelial growth factor, used as control for maintenance of cellular activity (Figure 5C).

DISCUSSION

Taken together, the results of this study identify $CD133^+/CD73^+$ renal progenitors as a possible source of EPO and show that EPO release in hypoxic conditions can be enhanced by PHD2 inhibition. $CD133^+/CD73^+$ progenitors therefore appear as a new source of EPO production within the human kidney. Similarly to EPO-producing renal cells described in murine studies or isolated from human kidney, $CD133^+/CD73^+$ cells present a mesenchymal phenotype. The ability to synthesize EPO was restricted to $CD133^+$ progenitors because it was lost after epithelial differentiation and it was absent in $CD133^-$ cells. Indeed, in epithelial cells, the mechanisms underlying the repression of EPO gene expression were recently shown to depend on the GATA promoter motif,[23] underlying a cell type– dependent inhibitory mechanism that could be relevant

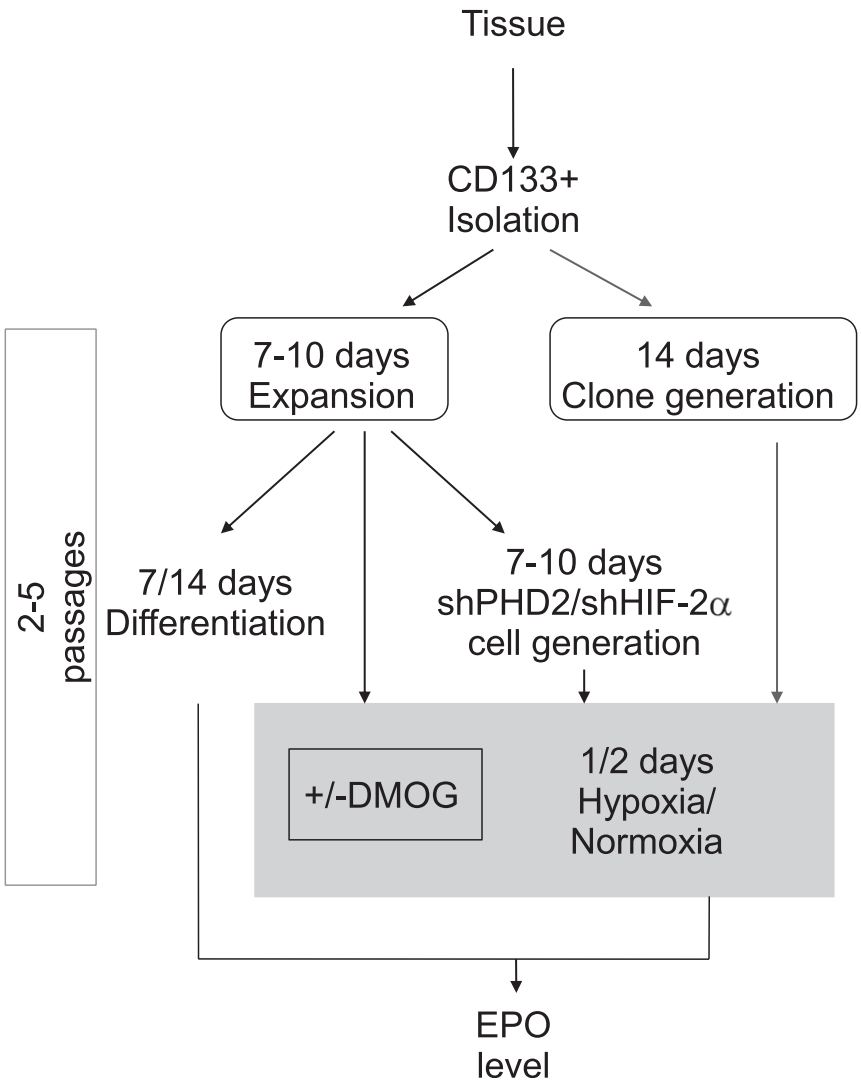

Figure 3. Schematic representation of the experimental procedures used in the study, illustrating the temporal relationship between the $CD133^+$ cell isolation, expansion/cloning and experimental treatment, and the final EPO analysis.

in the epithelial differentiation of renal cells. In contrast with the described localization of EPO-producing cells in the interstitium of rat and mouse kidney,[1–6,21] $CD133^+$ progenitors have been identified along the renal nephron.[11–14] This observation overlaps the described localization of EPO-producing cells in different segments of the human nephron by *in situ* hybridization studies.[8,9] These dissimilar results may depend on differences in species. Indeed, a population of cells corresponding to the human $CD133^+$ cells has not been identified in mice and rats because the AC133 antibodies recognize a glycosylation-dependent stem cell–specific isoform of CD133 only in human cells.[24] Alternatively, it can be speculated that $CD133^+/CD73^+$ cells within the nephron in the inner medulla represent an additional source of EPO within the kidney, together with a fibroblast-like interstitial population. Indeed, nonclassic sites of EPO production have also been described in different organs such as brain, lung, heart, and bone marrow.[25] In this context, EPO may play tissue-specific physiologic roles possibly unrelated to erythropoiesis such as modulation of angiogenesis and cell survival.[26,27]

The molecular pathways involved in the control of oxygen sensing and leading to EPO synthesis have been fully elucidated in recent years.[27] *In vivo* studies in rats as well as *in vitro* studies on EPO-producing cell lines clearly showed that EPO production depends on HIF-2α activation,[7,21,22] which, in turn, is modulated by the hydroxylation of its proline residues by PHD2.[27] This was supported both in genetic murine studies as well as in human clinical settings. In fact, the loss of PHD2 function, either by inherited mutation or by genetic deletions of *Egln1* (the gene codifying for PHD2), or the increase in HIF-2α function in patients with *Hif2a* inherited mutation is associated with excessive EPO and polycythemia.[28–30] We here confirmed the involvement of PHD2–HIF-2α axis in hypoxia-induced EPO synthesis by renal $CD133^+/CD73^+$ progenitor cells. However, as PHD2 inhibition in normoxia increased intracellular EPO production, but not its release, at variance with the nonspecific PHD inhibitor DMOG, the presence of additional PHD2-independent hypoxia-related mechanisms controlling EPO release can be envisaged. Indeed, hypoxia is known to control exocytosis trafficking[31] and to modulate specific receptor turnover independently by PHD2.[32] Moreover, a previous work in a renal carcinoma cell line showed the presence of an intracellular pool of preformed EPO that could be rapidly released in response to an increase of cAMP.[33] Although these mechanisms still require elucidation, the hypoxic environment within the renal medulla appears instrumental for EPO release by $CD133^+/CD73^+$ progenitors.

Pharmacologic modulation of the local renal EPO production is a major goal in nephrology and several pharmaceutical companies are believed to have an interest in PHD-based drug discovery.[34] Indeed, PHD inhibitors have been shown to induce EPO production in mouse and rhesus macaque models.[35,36] Besides EPO production, PHD inhibitors were also shown to display renoprotective activity in AKI,[37] possibly due to the effect of EPO on survival of endothelial and renal tubular cells. In this context, the finding that pharmacologic inhibition of PHD, and particularly of PHD2,

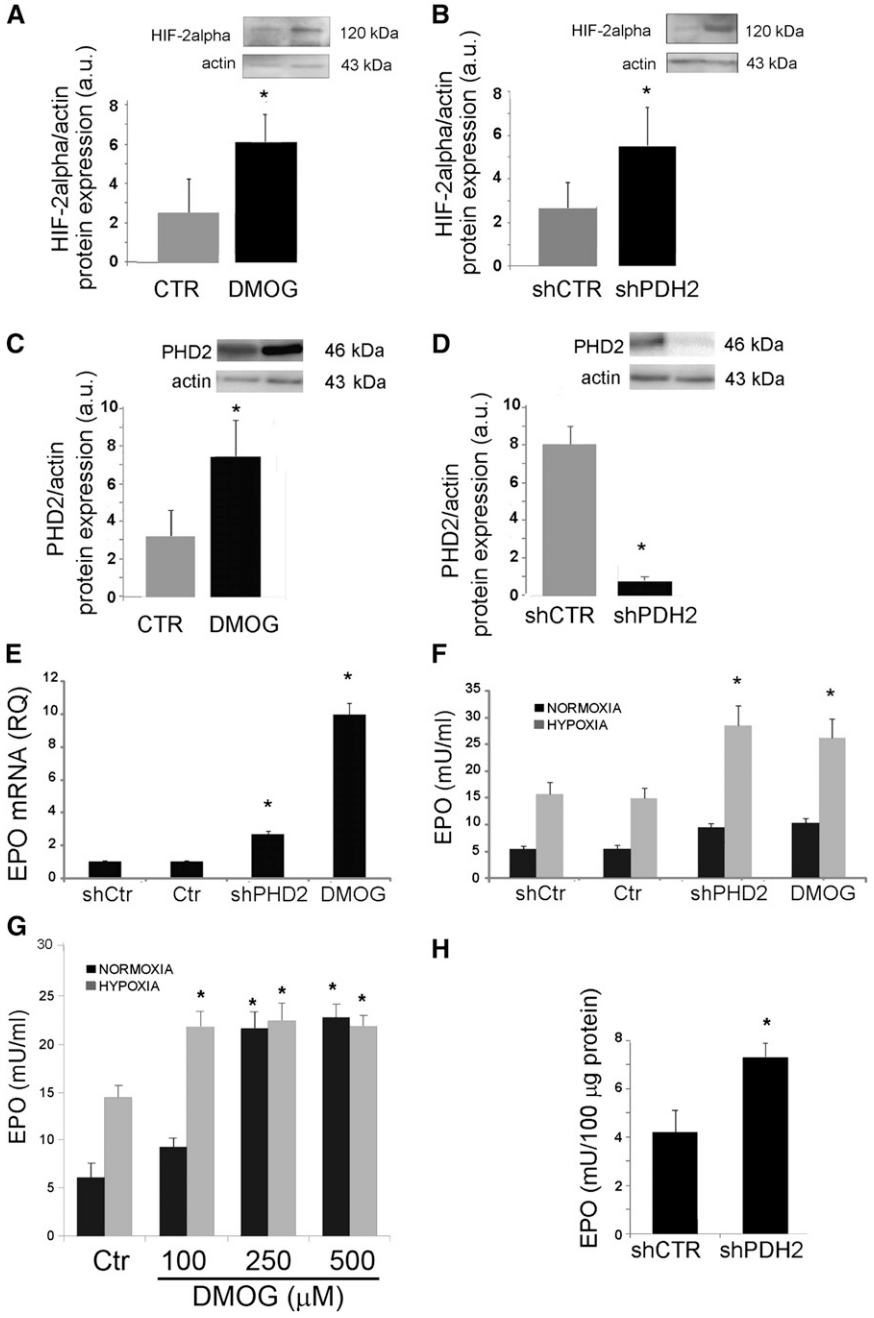

Figure 4. Increase in EPO released by $CD133^+/CD73^+$ cells after PHD2 inhibition. $CD133^+/CD73^+$ cells are infected with shRNA for PHD2 (shPHD2) or with scrambled vector (shCtr) or treated with DMOG (100 μM for 24 hours). (A–D) Western Blot micrographs and densitometric analysis of HIF-2α (A and B) and PHD2 (B and D) expression. Data, shown as arbitrary units, are representative of three different experiments performed using three different cell isolates and are normalized to actin expression. (E) Quantitative RT-PCR analysis showing increase in mRNA encoding for EPO in shPHD2 and DMOG treated $CD133^+/CD73^+$ cells. (F and G) EPO release by shPHD2 and DMOG treated $CD133^+$ cells in normoxia or hypoxia. Data are the mean ± SD of experiments performed in triplicate using three (shPHD2) or six (DMOG) different cell isolates. (H) Detection of EPO in the cell lysates of shCtr and shPHD2 cells in normoxia or hypoxia. Data are the mean ± SD of three different experiments. The *t* test is performed. *$P<0.05$ DMOG versus Ctr or shPHD2 versus shCtr.

enhances EPO release by $CD133^+/CD73^+$ progenitors under hypoxia may support and provide a new rationale for the use of PHD inhibitors in clinical settings of acute or chronic renal injury. Moreover, isolated $CD133^+/CD73^+$ cells could represent a useful model to dissect the mechanisms of EPO synthesis and release from human renal cells.

CONCISE METHODS

Isolation and Culture Conditions

Renal progenitor cells were obtained from the normal portion of the inner medulla obtained from surgically removed kidneys, after approval by the Ethical Committee for the Use of Human Tissue of the University of Torino, as described,[13] and cultured in endothelial basal medium medium plus supplement kit (Cambrex BioScience, East Rutherford, NJ) without serum addition. Cell isolates, obtained from different renal specimens (n=16), were used between passages 2 and 5 (Figure 3 and Table 1). The $CD133^-$ cell population obtained after magnetic sorting was plated in RPMI plus 10% FCS and used after 1–2 culture passages. To generate clones, $CD133^+$ cells were seeded using a limiting dilution technique in 96-well plates. After 12 hours, wells not containing single cells were discarded by microscopical visualization and clones derived from a single cell were expanded in expansion medium. CD133 expression was evaluated by FACS analysis.

Epithelial differentiation was obtained by culturing cells for 14 days in expansion medium with 10 ng/ml human hepatocyte growth factor (Sigma Aldrich, St. Louis, MO) and 10 ng/ml human fibroblast growth factor-4 (Sigma Aldrich). When cultured in hypoxic conditions, cells were placed in hypoxic chambers with 1% O_2. DMOG (100 μM; Frontier Scientific, Logan, UT) dissolved in PBS was added to the cells for the indicated amount of time.

Cell Infection and Transfection

The Block-it Pol II miR RNAi Expression Vector Kit (Life Technologies, Grand Island, NY) was used to construct pcDNA6.2-GW/EmGFP-miR vectors (Life Technologies) expressing the target miRNA (miR-PHD2) according to the manufacturer's instructions. The Rapid BP/LR Recombination Reaction (Block-it Lentiviral Pol II miR RNAi Expression System; Life

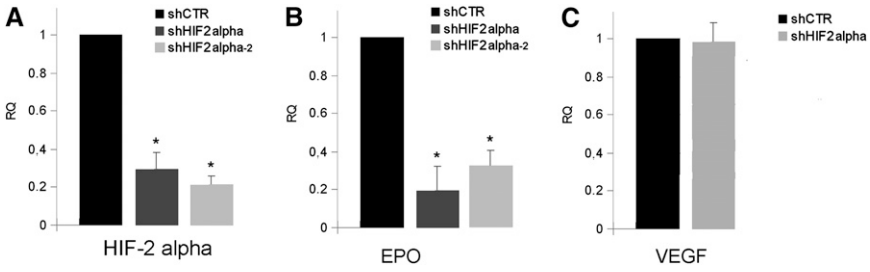

Figure 5. HIF-2α is required for EPO but not VEGF synthesis by CD133$^+$/CD73$^+$ cells. Quantitative RT-PCR analysis showing reduction in mRNA encoding for HIF-2α (A) and EPO (B), but not for VEGF (C) in CD133$^+$ cells infected with two different shRNA for HIF-2α (shHIF2 alpha) in respect to cells infected with a scrambled vector (shCtr). Data are normalized to TBP mRNA and to 1 for shCtr and are the mean ± SD of three experiments performed using three different cell isolates. The *t* test is performed. *$P<0.05$ shHIF2 alpha versus shCtr. VEGF, vascular endothelial growth factor.

Technologies) between pDONR 221, pcDNA6.2-GW/EmGFP-miR, and pLenti6/V5-DEST was performed to generate the pLenti6/V5-GW/EmGFP-miR expression construct. For knockdown of HIF-2α, a pGIPZ lentiviral vector (Open Biosystems, Lafayette, CO) carrying shHIF-2a was used. The constructs were then transfected with the 293T cell line using the ViraPower Packaging Mix (Life Technologies) for lentivirus production. To confirm the results, the following lentiviral particles carrying other hairpin RNA were obtained from Sigma Aldrich: scrambled PLKO.1 and shRNA for HIF-2α (HIF-2α 2, TPXN0000082303) and scrambled PLVX.1 and shRNA for PHD2 (PHD2 2, TRCN0000001042). After titering the lentiviral stock, CD133$^+$ progenitors were transduced with lentiviral particles following the manufacturer's instructions.

Immunofluorescence and Western Blot Analyses

Immunofluorescence and immunohistochemistry were performed using the following antibodies: anti-CD133/1 (clone AC133, 293C3; Miltenyi Biotec, Auburn, CA), anti-CD73 (Becton Dickinson, San Jose, CA), anti-megalin and anti-Tamm-Horsfall protein (Santa Cruz Biotechnology, Santa Cruz, CA), anti-vimentin (Sigma), anti-PHD2 (Abcam, Cambridge, UK), and anti-HIF-1α, anti-HIF-2α, and CA IX (Novus Biologicals, Littleton, CO). Details are reported in the Supplemental Materials and Methods.

RNA Preparation and RT-PCR

Total RNA was isolated from different cell preparations using the RNAqueos-Micro isolation kit (Ambion, Life Technologies) according to the manufacturer's protocol. RNA was then quantified spectrophotometrically (NanoDrop ND-1000; NanoDrop Products, Wilmington, DE). For gene expression analysis, quantitative real-time PCR was performed in 20 μl of reaction mixture containing 5 ng of cDNA template, sequence-specific oligonucleotide primers (purchased from MWG-Biotech AG, Ebersberg, Germany), and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). TATA binding protein mRNA is used to normalize RNA inputs. Fold-change expression with respect to control was calculated for all samples. Sequence-specific oligonucleotide primers are reported in the Supplemental Materials and Methods.

ELISA for EPO

EPO protein in cell culture supernatants and in cell lysates was measured by Platinum ELISA (eBioscience, San Diego, CA) according to the manufacturer's recommendations.

Statistical Analyses

Statistical analysis was performed using the *t* test, or ANOVA with Dunnett's multiple comparison tests, as appropriate. A P value of <0.05 was considered significant.

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DISCLOSURES

None.

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ERYTHROPOIETIN IS PRODUCED BY RENAL CD133$^+$ PROGENITORS UNDER HYPOXIA AND PROLYL HYDROXYLASE INHIBITION

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SUPPLEMENTARY DATA

SUPPLEMENTARY MATERIALS AND METHODS

Isolation and culture conditions

Renal progenitor cells were obtained from the normal portion of the papillary region of the inner medulla obtained from surgically removed kidneys, after approval of the Ethical committee for the use of human tissue of the University. Briefly, tissue samples of approximately 3-5 mm^3 were obtained at the papillary region of a renal pyramid. Tissue was rinsed with Hank's Balanced Salt Solution (Sigma-Aldrich, St. Louis, MO) and, after cutting, digested in 0,1% Collagenase type I (Sigma-Aldrich) for 45 minutes at 37°C. Tissue was subsequently forced through a graded series of meshes to separate the cell components from stroma and aggregates. The filtrate was pelleted by centrifugation. CD133$^+$ cells were isolated by magnetic cell sorting, using the MACS system (Miltenyi Biotec, Auburn, CA), and cells re-suspended in expansion medium (EBM medium plus supplement kit; Cambrex BioScience, East Rutherford, NJ) without serum addition at a density of 1.0×10^5 viable cells per cm^2. Colonies were observed after 2-3 days and confluence was achieved 7 days after plating. Cell isolates, obtained from different renal specimens (n=16) were used between passage 2 and 5. The CD133$^-$ cell population obtained after magnetic sorting was plated in RPMI plus 10% FCS and used after 1-2 culture passages.

To generate clones, CD133$^+$ cells were seeded using a limiting dilution technique in a 96 well plates. After 12 hours, wells not containing single cells were discarded by microscopical

visualization and clones derived from a single cell were routinely observed and expanded in expansion medium. CD133 expression was evaluated by FACS analysis.

Cell treatment

Hypoxic conditions were achieved by placing the culture dishes of $CD133^+$ cells and HepG2 control cells cultured in expansion medium in a hypoxia chamber (Stem Cell Technologies, Vancouver, BC) flushed for 2 minutes at 2 psi with a hypoxic gas mixture (1% O_2, 5% CO_2, 94% N_2,). Afterwards, the chamber was sealed and incubated at 37°C for 24-72 hours. Non-hypoxic cultures (20% O_2,) were kept in a standard tissue culture incubator (95% air and 5% CO_2,). Dimethyloxalylglycine (100-500 μM; DMOG; Frontier Scientific, Logan, UT) dissolved in phosphate-buffered saline was added for the indicated amount of time. Cell vitality in DMOG-treated cells was evaluated by Annexin V/Propidium Iodide staining using cytofluorimetric analysis.

In Vitro Differentiation

Epithelial differentiation was done by culturing cells for 10 days in expansion medium with 10 ng/ml human Hepatocyte Growth Factor (HGF; Sigma-Aldrich) and 10 ng/ml human Fibroblast Growth Factor-4 (FGF-4; Sigma-Aldrich), as described[13].

Immunofluorescence studies

For flow cytometry, cultured cells were detached with a non-enzymatic cell dissociation solution (Sigma-Aldrich) and blocked with RPMI containing 10% of heat-inactivated FCS. Cells were then incubated in PBS containing 0,1% Bovine Serum Albumin (BSA; Sigma-Aldrich) for 30 minutes with fluorescein isothiocyanate or phycoerythrin conjugated Abs. In each experimental point, 10,000 cells were analyzed on a FACScan (Becton Dickinson, San Jose, California).

Indirect immunofluorescence was performed on cells cultured on chamber slides. Cells, cultured until confluence on chamberslides coated with fibronectin (Sigma-Aldrich), were fixed in 4% paraformaldehyde containing 2% sucrose for 15 minutes at 4°C and, when needed, permeabilized with 0,1% Triton X-100 (Sigma-Aldrich) for 8 minutes at 4°C and then incubated overnight at 4°C

with the appropriate antibodies. Immunofluorescence on 10-µm cryostatic sections of human kidney was performed as described.[13] Briefly, sections were incubated with 0.005% SDS in 0.2% gelatin-PBS for 30 min. The samples were then rinsed with 0.15% saponin/0.2% gelatin in PBS (S/G solution) for 30 min each and labeled overnight at 4°C with the selected antibodies. When required, unlabelled primary antibodies were detected using appropriate secondary antibodies. Hoescht 33258 dye (Sigma) was added for nuclear staining and imaging was performed using a Zeiss SM 5 Pascal Model Confocal Microscope (Carl Zeiss International, Germany).

The following monoclonal antibodies (mAb) were used, all fluorescein isothiocyanate, phycoerythrin or allophycocyanin-conjugated: anti-CD133/1 (clone AC133 293C3; Miltenyi Biotech, Auburn, CA); anti-CD73, anti-CD44 and anti-CD105 (Becton Dickinson). Fluorescein isothiocyanate, phycoerythrin or allophycocyanin-conjugated mouse nonimmune isotypic IgG (Miltenyi Biotech) were used as negative controls. In addition, the following unconjugated Abs were used: anti-megalin Ab, anti-Tamm Horfall Ab (Santa Cruz Biotechnology, Santa Cruz, CA), anti-vimentin mAb (Sigma) and carbonic anhydrase IX (Novus Biologicals, Littleton, CO, USA). Recognition of primary antibodies was done using Alexa Fluor 488 or Texas Red conjugated anti-rabbit, anti-mouse or anti-goat antibodies (Molecular Probes, Leiden, Netherlands).

RNA and protein analysis

Total RNA was isolated from different cell preparations using the RNAqueos-Micro RNA isolation kit (Ambion, Life Technologies, NY, USA) according to the manufacturer's protocol. RNA was then quantified spectrophotometrically (Nanodrop ND-1000, Wilmington DE). For gene expression analysis, quantitative real-time PCR was performed. Briefly, first-strand cDNA was produced from 200 ng of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time PCR experiments were performed in 20 µl reaction mixture containing 5 ng of cDNA template, the sequence-specific oligonucleotide primers (purchased from MWG-Biotech AG, Ebersberg, Germany, www.mwg-biotech.com) and the Power SYBR® Green PCR Master Mix (Applied Biosystems). β-actin or TATA binding protein (TBP)

mRNA were used to normalize RNA inputs. Fold change expression with respect to control was calculated for all samples.

Sequence-specific oligonucleotide primers are human EPO, Snail, Slug, Twist, PHD2, HIF-1α, HIF-2α.

h-EPO: forward, 5'-AGC CCA GAA GGA AGC CAT CT-3' (20 bp) and reverse, 5'-GGA AAG TGT CAG CAG TGA TTG TTC-3'

h-TWIST: forward, 5'-TCA CGA GCG GCT CAG CTA C-3' and reverse, 5'-TCT CTG GAA ACA ATG ACA TCT AGG TC-3'

h-SNAIL: forward, 5'-ATG CCG CGC TCT TTC CT-3' and reverse, 5'-GCT GGA AGG TAA ACT CTG GAT TAG A-3'

h-SLUG: forward, 5'AAA CTA CAG CGA ACT GGA CAC ACA-3' and reverse,5'-GAG CAG CGG TAG TCC ACA CAG

h-PHD2: forward, 5'-GGA CGA AAG CCA TGG TTG C-3' and reverse,5'-CCA CTT ACC TTG GCA TCC CA-3'

h-HIF-1α: forward, 5'-TGC ACT CAA TCA AGA AGT TGC A-3' and reverse, 5'-GGA CTA TTA GGC TCA GGT GAA CTT TG-3'

h-HIF-2 α: forward, 5'-TGA CCC AAG ATG GCG ACA T-3' and reverse, 5'-CTC CAC CTG TGT AAG TCC CAT GA-3'

h-VEGF-A: forward, 5′-TCA TCA CGA AGT GGT GAA GTT CA-3′ and reverse, 5′-TCA GGG TAC TCC TGG AAG ATG TC-3′

TBP: forward, 5′-TGT GCA CAG GAG CCA AGA GT-3′ and reverse, 5′-ATT TTC TTG CTG CCA GTC TGG-3′

For protein analysis, cells were lysed at 4°C for 30 min in RIPA Buffer (20 nM Tris-Hcl, 150 nM NaCl, 1% Deoxycholate, 0.1%SDS, 1% Triton X-100, pH7.8) supplemented with protease and phosphatase inhibitors cocktail and PMSF (Sigma). Aliquots of the cell lysates containing 40 μg protein, as determined by the Bradford method, were run on 8% sodium dodecyl sulphate

polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and electroblotted onto PVDF membrane filters. For Western Blot analysis, the following antibodies were used: anti-actin Ab (Santa Cruz Biotechnology), anti-PHD2 (Abcam, Cambridge, UK) and anti-HIF-1 α and anti-HIF-2 α (Novus Biologicals).

Cell infection

Knockdown of PHD2 was achieved by transduction with lentiviral vectors carrying shRNAs against PHD2 (GATCCCCGTACAGCCAGCATACGCCATTCAAGAGATGGCGTATGCTGGCTGTACTTTTTA); or scramble (GATCCCCAGATCTCAAGTTCCTCACATTCAAGAGATGTGAGGAACTTGAGATCTTTTTTAAGCT).

Knockdown of HIF-2α was achieved by transduction with lentiviral vectors carrying shRNAs against HIF-2α. HIF-2α: (ACCACCTTCAATGACTTCTAATTAGTGAAGCCACAGATGTAATTAGAAGTCATTGAAGGTGGG) or scramble (ATCTCGCTTGGGCGAGAGTAAGTAGTGAAGCCACAGATGTACTTACTCTCGCCCAAGCGAGAG);

The Block-it Pol II miR RNAi Expression Vector Kit (Life Technologies, Grand Island, NY) was used to construct pcDNA6.2-GW/EmGFP-miR vectors (Life Technologies) expressing the target miRNA (miR-PHD2) according to the manufacturer‘s directions. The Rapid BP/LR recombination reaction (Block-it Lentiviral Pol II miR RNAi Expression System, Life Technologies) between pDONR 221, pcDNA6.2-GW/EmGFP-miR and pLenti6/V5-DEST was performed to generate the pLenti6/V5-GW/EmGFP-miR expression construct. For knock down of HIF-2α, a pGIPZ lentiviral Vector (Open Biosystems, Lafayette, CO) carrying shHIF-2a was used. The constructs were then transfected with the 293T cell line using the ViraPower Packaging Mix (Life Technologies) for lentiviruses production. To confirm the results, lentiviral particles carrying other hairpin RNA were

obtained from Sigma: scrambled PLKO.1 and shRNA for HIF-2α (HIF-2α 2, TPXN0000082303) ; scrambled PLVX.1 and shRNA for PHD2 (PHD2 2, TRCN0000001042) (Sigma). After titering the lentiviral stock, $CD133^+$ progenitors were transduced with lentiviral particles following the manufacturer's instructions. Cells were selected by specific antibiotics and, after 6 days, antibiotic-resistant cells were expanded. Cell infection was evaluated by GFP^+ >90%, as assessed by FACS analysis, and by downregulation of the target gene >70% by quantitative RT-PCR.

Cytokine and EPO detection

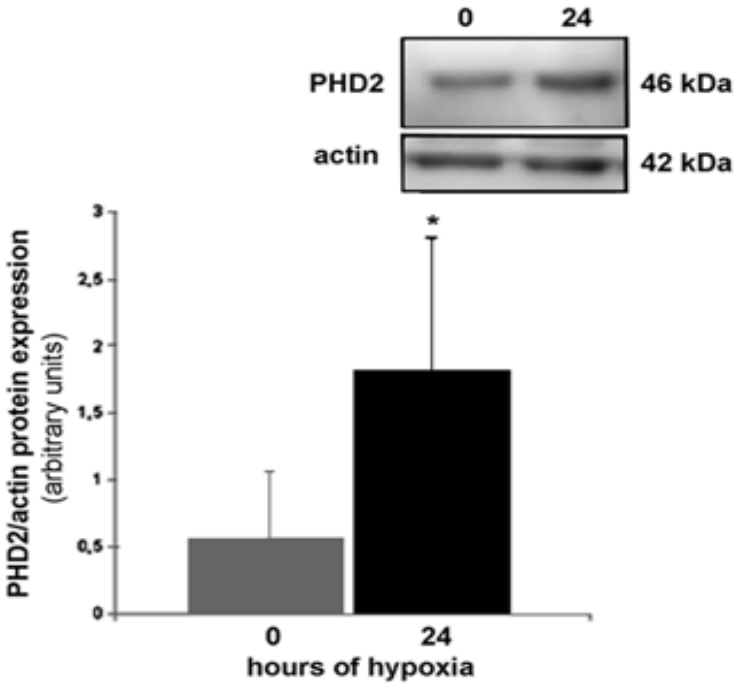
For EPO detection, cells were plated in 24 well plates in expansion medium until confluence (typically 250.000 cells). Wells were then washed and 1 ml RPMI medium containing 0.25% bovine serum albumin (BSA) was added and cells subjected to the experimental treatments. EPO protein in cell culture supernatants or in the cell lysates (extracted with the Paris extraction kit, Life Technologies) was measured by Platinum ELISA (eBioscience, San Diego, CA) according to the manufacturer's recommendations.

Statistical Analysis

Statistical analysis was performed by using the Student *t* test, or ANOVA with Dunnet's multicomparison tests, as appropriate. A p value of <0.05 was considered significant.

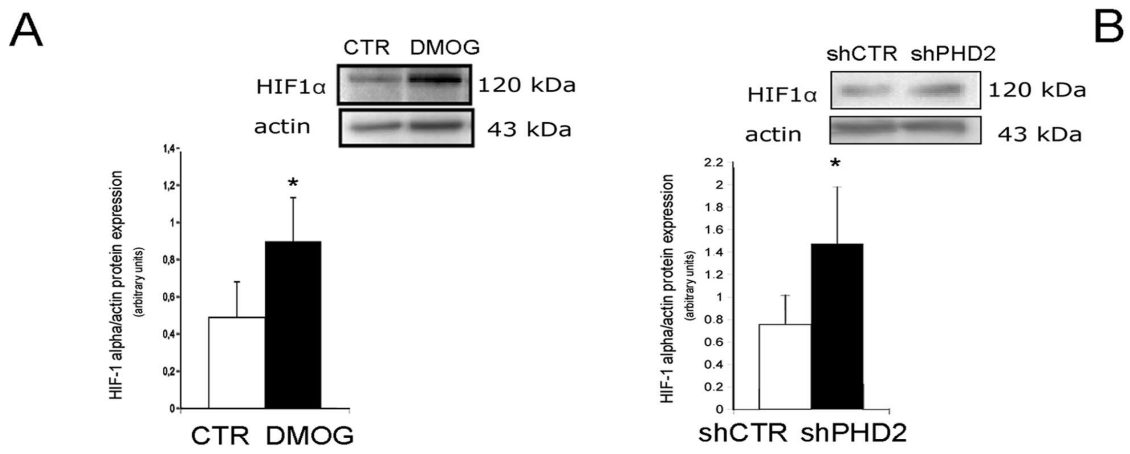
SUPPLEMENTARY FIGURES

Supplementary Figure 1



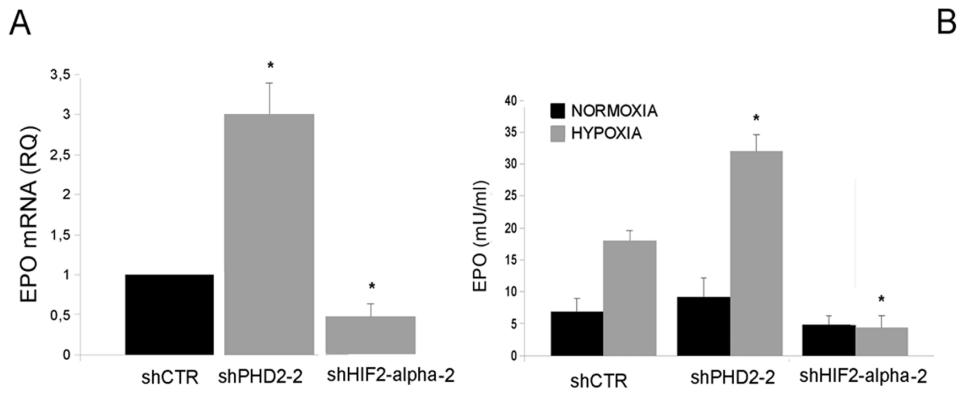
Suppl. Fig. 1. *Increase in PHD2 by hypoxia*. Western Blot micrographs and densitometric analysis of PHD2 expression by renal $CD133^+$ cells at time 0 and 24 hours after hypoxic treatment (1% O_2). Data, shown as arbitrary units, are representative of three different experiments and were normalized to actin expression. Student's t test was performed: *=$p<0.05$.

Supplementary Figure 2



Suppl. Fig. 2. *Increase in HIF-1α by PHD inhibition.* Western Blot micrographs and densitometric analysis of HIF-1α expression by renal CD133+ cells treated with DMOG (100 μM for 24 hours, **A**) or transduced with a lentiviral shRNA for PHD2 (shPHD2 cells, **B**). Data, shown as arbitrary units, are representative of three different experiments and were normalized to actin expression. Student's t test was performed: *=$p<0.05$.

Supplementary Figure 3



Suppl. Fig. 3. *EPO synthesis via the PHD2-HIF-2α axis.* (**A**) Quantitative RT-PCR analysis showing increase in mRNA encoding for EPO in $CD133^+$ cells infected with shRNA for shPHD (shPHD-2) and its inhibition in $CD133^+$ cells infected with shRNA for HIF-2α (shHIF2 alpha-2) in respect to cells infected with the respective scrambled vector (shCtr). Data were normalized to TBP mRNA and to 1 for shCtr. (B) EPO release by shPHD2-2 and shHIF2 alpha-2 cells in normoxia or hypoxia. Data are mean ± SD of three experiments performed using three different cell isolates. Student's t test was performed: *=$p<0.05$ vs Ctr.