Integrity of the Prolyl Hydroxylase Domain Protein 2:Erythropoietin pathway in aging mice

Xiping Li[a], **Scott Sutherland**[a], **Kotaro Takeda**[b], **Guo-Hua Fong**[b], and **Frank S. Lee**[a],[*]

[a]Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

[b]Center for Vascular Biology, Department of Cell Biology, University of Connecticut Health Center, Farmington, CT 06030, USA

Abstract

The central transcriptional response to hypoxia is mediated by the Prolyl Hydroxylase Domain protein (PHD):Hypoxia Inducible Factor (HIF) pathway. In this pathway, PHD prolyl hydroxylates and thereby negatively regulates the α-subunit of the transcription factor HIF (HIF-α). An important HIF target gene is that for Erythropoietin (EPO), which controls red cell mass. Recent studies have identified PHD2 as the critical PHD isoform regulating the *EPO* gene. Other studies have shown that the inducibility of the HIF pathway diminishes as a function of age. Thus, an important question is whether the PHD2:EPO pathway is altered in the aging. Here, we employed a mouse line with a globally-inducible *Phd2* conditional knockout allele to examine the integrity of the Phd2:Epo axis in young (six to eight month old) and aging (sixteen to twenty month old) mice. We find that acute global deletion of *Phd2* results in a robust erythrocytosis in both young and aging mice, with both age groups showing marked extramedullary hematopoiesis in the spleen. *Epo* mRNA is dramatically upregulated in the kidney, but not in the liver, in both age groups. Conversely, other *Hif* targets, including *Vegf*, *Pgk1*, and *Phd3* are upregulated in the liver but not in the kidney in both age groups. These findings have implications for targeting this pathway in the aging.

Keywords

Erythropoietin; Prolyl Hydroxylase Domain protein; Hypoxia Inducible Factor; Prolyl hydroxylation; Gene regulation

Introduction

The HIF pathway plays a central role in coordinating cellular, local, and systemic responses to hypoxia [1;2]. In this pathway, HIF-α (of which there are two main isoforms) is site-specifically prolyl hydroxylated by a family of three PHDs (also known as HIF Prolyl Hydroxylases and Egg Laying Defective Nine proteins) [3]. The three PHD isoforms are PHD1, PHD2, and PHD3. This modification provides a recognition motif for the von Hippel Lindau

[*]Corresponding Author: Frank S. Lee, Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, 605 Stellar Chance Labs, 422 Curie Blvd., Philadelphia, PA, USA 19104. Tel: 215-898-4701; Fax: 215-573-2272; franklee@mail.med.upenn.edu.

tumor suppressor protein (VHL), a component of an E3 ubiquitin ligase complex that specifically targets hydroxylated HIF for degradation by the ubiquitin-proteasome pathway [4]. Under normoxic conditions, this modification occurs constitutively and hence, HIF-α is maintained at very low steady state levels. This modification is inherently oxygen dependent [5] and is inhibited by hypoxia-generated reactive oxygen species [6;7;8]. Therefore, under hypoxic conditions the modification is inhibited, allowing the stabilization of HIF-α. HIF-α then heterodimerizes with a distinct protein, HIF-β, and binds to hypoxia response elements (HREs) in the promoters and enhancers of HIF target genes, allowing the transactivation of these genes.

The prototypical HIF target gene is that encoding for EPO [9;10;11;12]. EPO is a glycoprotein that is the central growth factor controlling red cell mass. In situations such as anemia, *EPO* gene transcription is induced, leading to increased circulating levels of EPO. EPO then binds to the EPO receptor on red cell progenitors in the bone marrow, initiating signaling through the JAK:STAT pathway, and leading to expansion of red cell mass. The expanded red cell mass increases delivery of oxygen to the tissues of the body, and this in turn downregulates HIF and hence *EPO* gene transcription. This negative feedback loop thus ensures that HIF maintains EPO at a level that is appropriate for a given tissue oxygenation. *EPO* gene transcription is also regulated in a developmental and tissue specific manner. In the mammalian fetus, the liver is the predominant source of EPO [13]; postpartum, the site of EPO production shifts from the liver to the kidney.

The multiplicity of PHD isoforms raises the question of which, if any, are involved in EPO regulation. Studies of patients with idiopathic erythrocytosis, an uncommon condition characterized by elevated red cell mass, have revealed that a subset of these patients harbor heterozygous mutations in the *PHD2* gene [14;15;16;17]. These mutations comprise missense, nonsense, and frameshift mutations. PHD2 consists of an N-terminal non-catalytic domain, and a C-terminal catalytic domain that is homologous to a larger family of 2-oxoglutarate dependent prolyl hydroxylases that include the collagen 4-prolyl hydroxylases [3]. All reported missense mutations studied to date affect the catalytic domain and severely impair enzymatic activity [16;17;18]. The frameshift mutations occur within the N-terminal domain and thereby delete the catalytic domain, and the single nonsense mutation reported is predicted to delete the C-terminal 50 amino acids [14].

Studies of genetically engineered mice have demonstrated a critical role for Phd2 in red blood cell control. Conventional knockout of *Phd2* leads to embryonic lethality between day E12.5 to E14.5 due to placental and cardiac defects [19], necessitating a conditional knockout (CKO) approach to studying its role in adult red cell control. Employing a global, tamoxifen-inducible system, we and others have found that acute deletion of *Phd2* in the mouse results in marked erythrocytosis [20;21]. In contrast, neither *Phd1* −/− nor *Phd3* −/− mice display erythrocytosis, but interestingly, *Phd1* −/− ; *Phd3* −/− double knockout mice exhibit a mild erythrocytosis [20]. Collectively, the human and mouse studies point to PHD2 as being the central PHD isoform regulating red cell mass.

These and other findings have stimulated great interest in targeting this pathway for therapeutic benefit [22]. Based on available evidence, inhibition of PHD2 would be predicted to increase EPO and thereby stimulate erythropoiesis, which could be of benefit in situations such as anemia. However, an important caveat to the mouse studies mentioned above is that they were performed on young mice (two to three months of age). Therapeutic targeting of this pathway, in contrast, would likely have its greatest impact in the aging population, which is the population that is preferentially affected by anemia due to causes that include end stage renal disease and chemotherapy. It should also be noted that a substantial segment of the aging

population has anemia of unknown cause [23;24]. Anemia in the aging is associated with increased mortality risk, with the degree of risk correlated with the degree of anemia [25].

This issue is all the more relevant because there is substantial evidence that there are aging-associated changes in the HIF pathway. Thus, there is impaired hypoxia-induced HRE binding activity in senescent mice [26], a defect in hypoxia-induced HIF-1 α activation in aging aortic smooth muscle cells from rabbits [27], decreased hypoxia-induced activation of HIF-1 α in rat carotid bodies as a function of age [28], decreased hypoxia-induced HRE-binding activity in aging rat lungs [29], impaired induction of HIF-1 α protein by ischemia in hindlimb skeletal muscle of aging mice [30], decreased hypoxia-induced HIF-1 α expression in the aging mouse brain [31], and diminished ischemia-induced HIF-1 α activation in tissue flaps of aging mice [32]. Collectively, these studies suggest an impairment of the HIF activation pathway as a function of aging.

This then raises the important question of whether there is a fundamental defect in the HIF pathway in aging mammals. The PHD2:EPO pathway, with its high level of inducibility and its ability to be assessed quantitatively by indices that include hematocrit (Hct) and hemoglobin (Hb), provides an ideal and clinically relevant system for examining this. To pursue this, we employed a genetic model in which *Phd2* can be temporally knocked out in order to examine young and aging mice. Contrary to what might have been expected based on previous published reports on aging and the HIF pathway, we find that loss of *Phd2* in both age groups induces dramatic induction of erythrocytosis. The kidney continues to be the source of Epo in these aging mice. Furthermore, other *Hif* target genes are inducibly upregulated in the liver in both age groups. These findings indicate that the PHD2:EPO pathway is robustly inducible in aging mammals.

Materials and Methods

Mice

The generation of mice in which exon 2 of the *Phd2* gene has been flanked by loxP sites (floxed) has been described previously [19]. These mice were maintained in a mixed CD1/129/BL6 background. Mice expressing either a constitutively active Cre recombinase or a tamoxifen-inducible CreER^{T2} fusion protein from the Rosa26 locus were obtained from Taconic [the strain designations are C57BL/6-*Gt(ROSA)26Sor$^{tm16(cre)Arte}$* (which we hereafter refer to as *Rosa26*-Cre) and C57BL/6-*Gt(ROSA)26Sor$^{tm9(cre/Esr1)Arte}$* (which we hereafter refer to as *Rosa26*-CreER^{T2}), respectively]. *Phd2* +/− mice were generated by crossing *Phd2* f/f (where f = flox) mice with the constitutive active *Rosa26*-Cre deleter mouse. *Phd2* f/−; *Rosa26*-CreER^{T2} and *Phd2* f/+ mice were generated by crossing *Phd2* f/f mice with *Phd2* +/−; *Rosa26*-CreER^{T2} mice. For genotyping or determination of *Phd2* exon 2 deletion efficiency, DNA was isolated from tails or various organs using a DNeasy tissue kit (Qiagen). For genotyping the *Phd2* allele, we employed the following three primers: PHD2rec55: 5'-AGG GCT TCT GGC ATT AGT TGA CC-3'; PHD2mouseR: 5-TCA ACT CGA GCT GGA AAC C-3'; and Pint 1–4 5': 5'-ATG AAT CAG AGT TCC CCG TG-3'. The wild type *Phd2* allele produces a 1.09 kb band with the PHD2rec55 and PHD2mouseR primers, the floxed *Phd2* allele produces a 0.95 kb band with the Pint1–4 5' and PHD2mouseR primers, and the knockout *Phd2* allele produces a 0.8 kb band with the PHD2rec55 and PHD2mouseR primers. For genotyping the Cre allele, we employed the following three primers: Rosa26-1: 5'-TGG AGG CAG GAA GCA CTT GCT CTC-3'; Rosa26-2: 5'-CAT ACT GTA GTA AGG ATC TCA AGC-3'; Cre-1(R): 5'-GCA TGT TTA GCT GGC CCA AAT G-3'. The wild type *Rosa26* locus produces a 0.55 kb band with the Rosa26-1 and Rosa26-2 primers. The *Rosa26* locus containing either the Cre or CreER^{T2} transgene produces a 0.69 kb band with the Rosa26-1 and Cre-1(R) primers. All animal procedures were approved by the Institutional Animal Care

and Use Committees at the University of Pennsylvania in compliance with Animal Welfare Assurance.

Tamoxifen administration

A 10 mg/ml stock solution of tamoxifen free base (MP Biomedicals) was prepared in corn oil with shaking at 37 °C, and then aliquoted and stored at −20 °C. Tamoxifen was administered by oral gavage for five consecutive days (2 mg doses for young mice; 1 mg doses for aging mice).

Hematologic analysis

Peripheral blood samples were obtained from the retroorbital cavity and collected in Microvette 100 lithium heparin tubes (Sarstedt). Hematocrit was measured using a Critspin hematocrit reader (Iris). Hemoglobin measurements and complete blood counts were determined using a Hemavet FS950 hematology analyzer (Drew Scientific).

Epo assay

Plasma was obtained by centrifuging the retroorbital blood samples at 20 min at $2,000 \times g$ for 20 min at room temperature. Plasma Epo levels were then determined using a rodent Quantikine Epo Immunoassay kit (MEP00, R&D Systems). Enzyme linked immunoabsorbent assay (ELISA) readings were made on a Tecan Sunrise microplate reader.

Erythroid Burst Forming Unit (BFU-E) assay

Cells were obtained from either bone marrow or spleen, and red blood cells were lysed in a solution containing 150 mM NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA for 5 min at room temperature. The remaining cells were suspended in Iscove's modified Dulbecco's medium. Cells (1×10^5) were plated in 35 mm dishes containing methylcellulose media and varying concentrations of EPO (prepared by mixing appropriate amounts of Methocult M3436, which contains 3 U/ml EPO, and Methocult M3236, which is the EPO-deficient base media for M3436). After fourteen days of culture at 37 °C in a humidified chamber with 5% CO_2, BFU-E colonies were counted using an Olympus CK2 phase contrast microscope.

Real Time PCR analysis (RT-PCR)

Total RNA was isolated from kidney, liver, and bone marrow using TRIzol reagent (Invitrogen). Reverse transcription of 0.8 µg of total RNA was then performed using an ABI High Capacity cDNA Synthesis Kit (Applied Biosciences). RT-PCR was then performed on 20 ng cDNA equivalents using a SYBR green PCR master mix (Applied Biosciences) and an ABI 7300 Real Time PCR System. Relative quantification was performed employing the ΔΔCt method and β-actin as the endogenous control.

The primers employed for RT-PCR were as follows. *Phd2*, 5'-AGG CAA CGG AAC AGG CTA TG-3' and 5'-CGC ATC TTC CAT CTC CAT TTG-3'; *Epo*, 5'-CAT CTG CGA CAG TCG AGT TCT G-3' and 5'-CAC AAC CCA TCG TGA CAT TTT C-3'; *Vegf* (A isoform), 5'-TGT CAC CAC CAT GCC ATC AT-3' and 5'-GAC CCA AGT GCT CCT CGA A-3'; *Pgk1*, 5'-GGA AGC GGG TCG TGA TGA-3' and 5'-GCC TTG ATC CTT TGG TTG TTT G-3'; *Tfr1*, 5'-TGG AGA CAG ATG CTC CCT CC-3' and 5'-TTT GTG CTC TGT GTA TGT GGT AAG G-3'; *Phd3*, 5'-CTC TGC CCA CGT CGT TCA G-3' and 5'-CCC GGC AAG AAA ACA TGA AG-3'; *Hepcidin*, 5'-TGT CTC CTG CTT CTC CTC CT-3' and 5'-CTC TGT AGT CTG TCT CAT CTG TTG-3'; *Alas2*, 5'-TCC AAG GCA TTC GCA ACA-3' and 5'-CCT GGG TCA TTG TGT CTG AAG A-3'; *Dmt1*, 5'-GGC TTT CTT ATG AGC ATT GCC TA-3' and 5'-GGA GCA CCC AGA GCA GCT TA-3'; *EpoR*, 5'-CGG CTC CGT GCG TTT CT-3' and 5'-CGG CAC AAA ACT CGA TGT GT-3'; *Tf*, 5'-CCT GGC CCA AGC TCC

AA-3' and 5'-CCG GGC TGC CTT CTC TTT-3'; β-*actin*, 5'-GGC CAA CCG TGA AAA GAT GA-3' and 5'-CAC AGC CTG GAT GGC TAC GT-3'.

Histologic analysis

Mouse liver and spleen were fixed in 10% buffered formalin and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin. Photomicrographs were obtained with a Leica DM2500 upright light microscope fitted with a DFC420 digital camera. The eyepiece lens was 10×, and the objective lens was 10× (total magnification = 100×).

Statistical analysis

Data are presented as the mean ± standard deviation. Groups were compared by unpaired Student's *t* test. *p* values below 0.05 were considered to be significant.

Results

We and others have previously shown that acute global deletion of *Phd2* in young mice results in marked erythrocytosis [20;21]. In the studies that we conducted, the mice were two to three months of age. This leaves open a number of important questions, namely (1) whether acute global deletion in aging mice also results in erythrocytosis, (2) whether the kidney continues to be the source of Epo, and (3) whether other Hif target genes are inducible to a comparable degree in the aging mice.

Towards this end, we examined the *Phd2* conditional knockout mouse model employed previously [19;20]. In this strain, *Phd2* exon 2 is flanked by loxP sites. Exon 2 contains two of the three essential iron-chelating residues of *Phd2*; hence, its deletion will abolish enzymatic activity. We generated two groups of mice with the following genotypes: *Phd2* f/−; *Rosa26*-CreER^{T2} (the experimental group) and *Phd2* f/+ (the control group). We examined them at six to eight months (which we hereafter refer to as the young group) and at sixteen to twenty months (which we hereafter refer to as the aging group). This strategy allowed us to examine both the effect of *Phd2* heterozygosity and the effect of acute global deletion of *Phd2* as a function of age. We first obtained baseline hematocrit and complete blood counts. We find that the *Phd2* heterozygotes display a mild erythrocytosis in both the young and aging groups (Fig. 1A and B). In the young mice, the Hct was 46.8 ± 1.8% (mean ± S.D.) in the controls, and 48.8 ± 1.4% in the heterozygotes ($p < 0.05$). In the aging mice the corresponding values were 43.9 ± 1.6% and 47.4 ± 2.3%, respectively ($p < 0.05$). Similar results were obtained with hemoglobin (Hb) measurements (Fig. 1B). No significant differences were seen in white blood cell or platelet counts in either age group (Fig. 1C and D, respectively).

We next treated both the experimental group and the control group with tamoxifen by oral gavage (the experimental group treated with tamoxifen is hereafter referred to as CKO). RNA was isolated from kidneys and livers of animals from both the young and aging groups. We examined, by Real Time PCR, the levels of *Phd2* mRNA. As shown in Fig. 2A and B, we find that tamoxifen induces a marked decrease of *Phd2* message in the kidneys and livers of both the young and the aging mice, respectively, indicative of effective Cre-mediated recombination. We also employed a PCR reaction that can distinguish the floxed from knockout *Phd2* allele to survey other tissues for recombination efficiency. For this purpose, we examined the conditional knockout mice at both age groups. Consistent with the Real Time PCR results, we find that tamoxifen treatment induces efficient recombination in both liver and kidney, with perhaps more variability in the kidney than in the liver (Fig. 2C, top two panels). Examination of other tissues, including heart, lung, spleen, and muscle reveals effective recombination in these tissues as well (Fig. 2C, bottom four panels), and is consistent with the known activity of the *Rosa26*-CreER^{T2} mouse line [33].

We next examined hematologic parameters in these mice. We find, as before [20], that acute global deletion of *Phd2* in young mice induces a dramatic erythrocytosis, as reflected by both an increase in hematocrit and hemoglobin (compare first two columns in Figs. 1E and 1F, respectively). The Hct was $45.9 \pm 1.7\%$ in the control group and $69.4 \pm 8.5\%$ in the *Phd2* conditional knockout (Fig. 1E). Importantly, we find that it also induces a comparable increase in these parameters in the aging mice. Thus, for the aging mice, the Hct was $42.3 \pm 1.3\%$ in the control group, and $74.7 \pm 4.9\%$ in the *Phd2* conditional knockout, representing a 77% increase (compare last two columns in Fig. 1E). Interestingly, we find that white blood cell counts (WBC) are increased in both young and aging mice upon *Phd2* deletion (Fig. 1G). For example, in the aging group, the WBC was $15.2 \pm 5.34 \times 10^3/\mu L$ in the control group and $25.2 \pm 5.85 \times 10^3/\mu L$ in the *Phd2* conditional knockout group. *Phd2* deletion also induces an increase in platelet counts in the young mice but not in the aging mice (Fig. 1H).

Further analysis of white blood cells indicates that *Phd2* deletion in aging mice results in increases in neutrophils, eosinophils, basophils, monocytes, and lymphocytes (Table 1). In the young mice, increases were observed in the first three of these.

Liver weights were not increased upon *Phd2* deletion in either young or aging mice (Fig. 3A). In contrast, spleens increased dramatically in size in both age groups upon *Phd2* deletion (Fig. 3B). The increase was 5.8-fold in young mice, 3.7-fold in aging mice. Histologic examination of spleens revealed marked extramedullary hematopoiesis in the *Phd2* conditional knockout mice in both the young and aging groups (Fig. 3C–F). Extramedullary hematopoiesis was not observed in histologic exam of the livers (data not shown). We examined erythroid progenitors from the spleens by isolating splenic cells from young and aging mice, and assessing their capacity to form BFU-E colonies. We find that *Phd2* loss induces a marked increase in splenic BFU-E activity in both age groups (Fig. 4A), consistent with the histologic findings in the spleen. These BFU-E assays were performed at an EPO concentration 3U/ml. We next treated two month-old control or *Phd2* CKO mice with tamoxifen, isolated splenic cells, and performed BFU-E assays at different EPO concentrations (Fig. 4B). A dose response curve for EPO indicates increased BFU-E activity not only at an EPO concentration of 3U/ml, but also at one of 0.3 U/ml.

Parallel assays were performed on bone marrow cells, and these also demonstrate increased BFU-E activity at both 0.3 U/ml and 3 U/ml EPO concentrations (Fig. 4C). We examined bone marrow for the expression of genes involved in erythropoiesis and iron metabolism, including *Alas2*, *EpoR*, *Tf*, *Tfr1*, and *Dmt1* (Fig. 5). Among these, we find upregulation of *Alas2* mRNA in *Phd2* CKO mice. While there was also a trend towards an increase in the *EpoR* and *Tf* mRNA, it was not statistically significant.

We measured plasma Epo levels and found a dramatic increase in both young and aging mice upon *Phd2* deletion (Fig. 6A). It increased 62-fold in the young mice, and 46-fold in the aging mice. We did not find a statistically significant difference in *Phd2* deletion-induced Epo levels between young and aging mice. To examine the tissue source of the Epo, we measured *Epo* mRNA levels by Real Time PCR, and observed a large increase in *Epo* mRNA in the kidneys of both the young and aging mice (Fig. 6B). We did not observe an increase in the *Epo* message from liver, thus indicating that kidney is the source of Epo upon *Phd2* deletion in both the young and aging mice. Erythropoiesis is tightly coupled to iron homeostasis since approximately two-thirds of total body iron is contained within erythrocytes. Iron homeostasis, in turn, is regulated by the circulating hormone, hepcidin, which is produced by the liver [34]. Low hepcidin levels promote iron mobilization for use in cells such as erythroid progenitors, while high hepcidin concentrations inhibit this mobilization. We find that *Phd2* knockout induces a marked decrease in hepcidin mRNA levels in the liver in both young and aging mice (Fig. 6C). The hepcidin message was also found to be the most highly

downregulated of genes in microarray analysis of mRNA from liver upon *Phd2* knockout (data not shown).

To examine whether other Hif target genes other than *Epo* are upregulated upon *Phd2* knockout, we examined mRNA levels of the known Hif target genes *Vegf*, *Pgk1*, *Tfr1*, and *Phd3*. In both the young and aging groups, we find increases in all four of these genes upon *Phd2* knockout in the liver, though not in the kidney. The degrees of induction varied depending on both the gene and on the age group. For example, the degree of induction of *Vegf* and *Tfr1* was greater in the aging mice than in the young mice, whereas the degree of induction of *Pgk1* was greater in the young mice than in the aging mice. Hence, global *Phd2* knockout upregulates not only the *Epo* gene but also other Hif target genes in both young and aging mice.

Discussion

Recent studies have pointed to a central role for the PHD2 in regulating red cell mass in adult mammals [14;16;17;20;21]. In this study, we compared the role of Phd2 in regulating red cell mass in young versus aging mice. Our studies allow us to make the following observations.

First, *Phd2* heterozygosity results in a mild erythrocytosis. This is in contrast to our earlier findings [20], in which no differences were observed. In the previous study, we examined mice at two to three months of age, while in the present study, we examined them at six to eight months of age. It is possible that this may be the explanation. In any case, we observe here that *Phd2* heterozygosity continues to result in a mild erythrocytosis in the aging mice (Figs. 1A and B). This therefore supports *Phd2* haploinsufficiency leading to erythrocytosis in the mouse. Clinically, all reported human erythrocytosis-associated *PHD2* mutations are heterozygous and include missense, frameshift, and nonsense mutations [35;36]. Based on these findings, it seems plausible that *PHD2* haploinsufficiency is the underlying mechanism in at least some of these cases, such as the frameshift and nonsense mutations. However, it still leaves open the possibility that other mutations, such as the missense mutations, may operate by other mechanisms, such as dominant negative ones.

Second, the oxygen-sensitive Epo pathway is intact and robustly induced in the aging mice, since the acute global deletion of *Phd2* leads to (i) the induction of *Epo* mRNA (Fig. 6B), (ii) an increase in circulating Epo in the plasma (Fig. 6A), (iii) extramedullary hematopoeisis and splenomegaly (Fig. 3), (iv) marked splenic BFU-E activity (Fig. 4A), and (v) dramatic increases in hematocrit and hemoglobin (Fig. 1E–F). Previous studies have shown aging-associated increases in PHD3 protein levels in tissues including heart, skeletal muscle, liver, and brain [37] [31;38]. The present studies show that such a mechanism, if it were to occur in the Epo-producing cells of the kidney, is not sufficient to prevent robust induction of Epo upon loss of Phd2. It may also be noted that a clinical study on human patients showed that there was an appropriate increase in endogenous EPO in response to venesection in aging individuals, indicating that the EPO pathway is functional in these individuals [39].

A third observation from these studies is that the kidney continues to be the source of Epo in aging mice. Following fetal development, there is a shift in Epo production from the liver to the kidney. We do not find evidence for a shift of Epo production back to the liver in the aging mice. Indeed, the degree of induction of kidney *Epo* mRNA upon *Phd2* deletion in aging mice is comparable to that seen in the young mice.

Fourth, *Phd2* loss is associated not only with induction of the Epo pathway, but also with other effects, which include increases in Hif target genes in the liver as well as increases in white blood cell count. These changes are observed in both young and aging mice. With regard to the white blood cell count changes, it may be noted that Hif-2α has been implicated in maintaining the proper bone marrow microenvironment for hematopoeisis in the mouse [40;

41]. Hence, the PHD:HIF pathway may play a more general role in hematopoiesis beyond erythropoiesis. With regard to the induction of other Hif target genes, the upregulation of such genes in the liver is consistent with *Phd2* playing a role, non-redundant with other Phd isoforms, in the control of the Hif pathway in the liver. We observed that *Hepcidin* mRNA is dramatically decreased upon *Phd2* deletion. Further investigations will be required to determine if this decrease is due to *Phd2* deletion-induced upregulation of Hif activity, which in turn has the capacity to directly affect *Hepcidin* gene transcription [42], or alternatively, if it is a consequence of the erythropoiesis itself—which through an unidentified factor—is known to result in potent inhibition of *Hepcidin* gene transcription [43].

The finding of robust induction of the Epo pathway upon *Phd2* deletion would appear to contrast with a substantial body of evidence indicating impaired inducibility of the HIF pathway as a function of aging [26;27;28;29;30;31;32]. One possibility is that there is a shift in the dose-response curve relating HIF activation to Phd2 activity with aging such that age-dependent differences are apparent with partial loss of Phd2 activity (as with hypoxia) but not with complete loss of activity (as with genetic ablation). Arguing against this possibility is the observation that mice that are *Phd2* haploinsufficient display erythrocytosis in both young and aging populations (Fig. 1A–B). Other factors that should be taken into consideration include the following. (i) Some of the other studies examined species other than mice, including rabbit and rat [27;28;29;31]. (ii) The findings presented here primarily pertain to the Epo-producing interstitial cells of the renal cortex; these cells were not specifically examined in the previous studies. (iii) The present studies do not rule out the possibility that there may be impaired hypoxia-induced activation of the pathway as opposed to a defect in the integrity of the components of the pathway. For example, there may be an aging-associated defect in hypoxia-induced generation of reactive oxygen species that serve to inhibit Phd2 activity. In this scenario, the defect is not in the Phd2 protein *per se*, but in the signal transduction pathway that inhibits its activity under hypoxic conditions.

A clinical implication of these studies is that PHD2 continues to be a tenable target for manipulating the EPO pathway in the aging population because *Phd2* loss of function in these experiments induces a robust erythropoiesis in aging mice. This, however, must be tempered by the fact that PHD2 loss of function will have systemic effects, and at least some of these effects are comparable in the young and aging. While some consequences, such as the decrease in *Hepcidin* message, may be desirable in the setting of augmenting erythropoiesis, others, such as the induction of *Vegf* mRNA, may warrant caution in other disease contexts. These studies reinforce the notion that PHD2 is not only a potent regulator of EPO, but also plays a broader role in regulating the hypoxic response.

Acknowledgments

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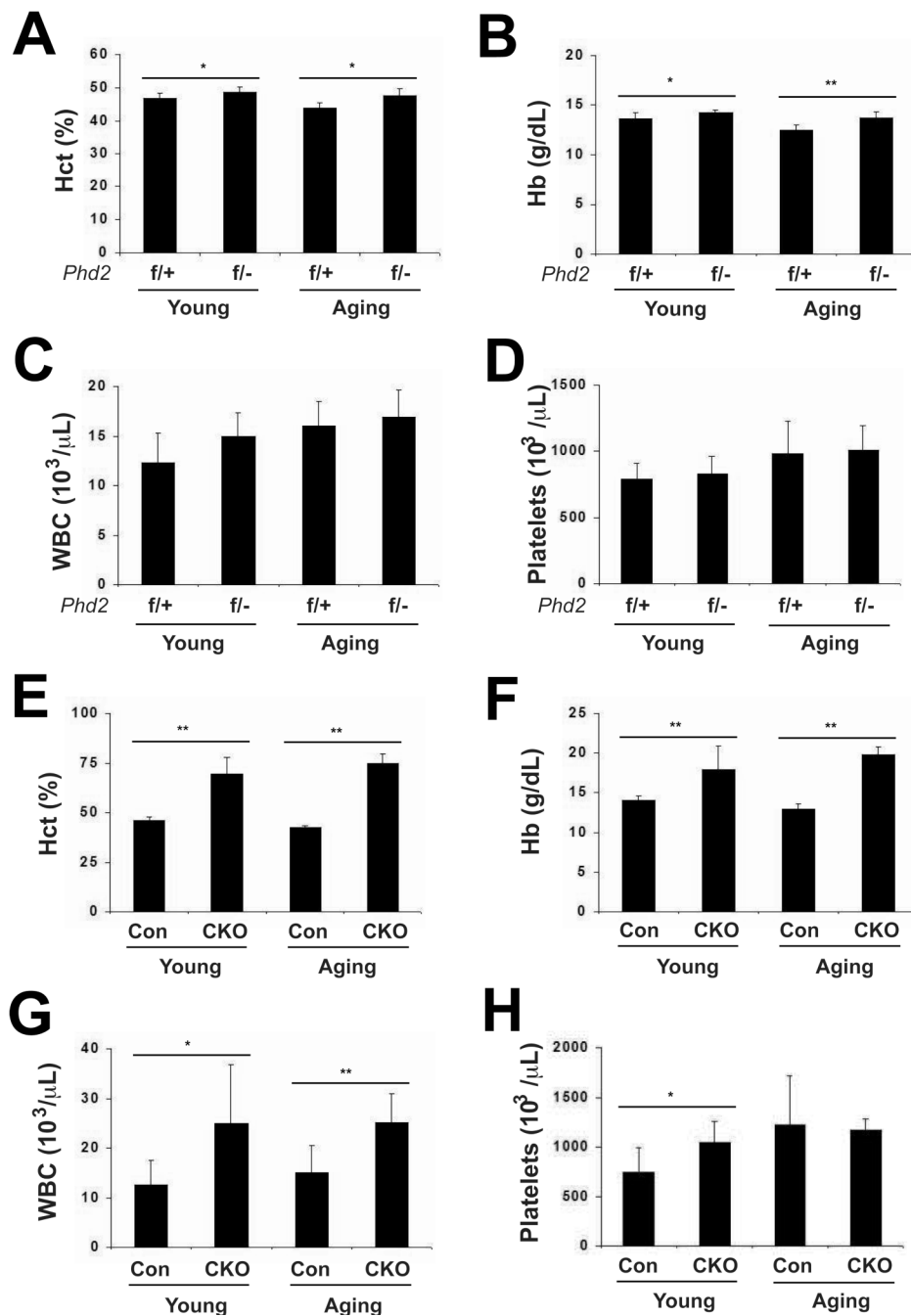
Fig. 1.
Analysis of peripheral blood in young and aging mice. (A, E) hematocrit (Hct), (B, F)
hemoglobin (Hb), (C, G) white blood cell count (WBC), and (D, H) Platelets were measured.
For all panels, n = 6–8. For (A–D), genotypes are as follows. f/+ = *Phd2* f/+; f/− = *Phd2* f/−;
Rosa26-CreER[T2]. * indicates $p < 0.05$ and ** indicates $p < 0.01$ in comparing f/+ and f/−
groups at a given age. For (E–H), genotypes are as follows. Controls (Con): *Phd2* f/+; CKO:
Phd2 f/−; *Rosa26*-CreER[T2]. Control and *Phd2* CKO mice were administered tamoxifen for
five consecutive days. Four weeks after the initial tamoxifen dose, peripheral blood was
collected. * indicates $p < 0.05$ and ** indicates $p < 0.01$ in comparing control and CKO groups
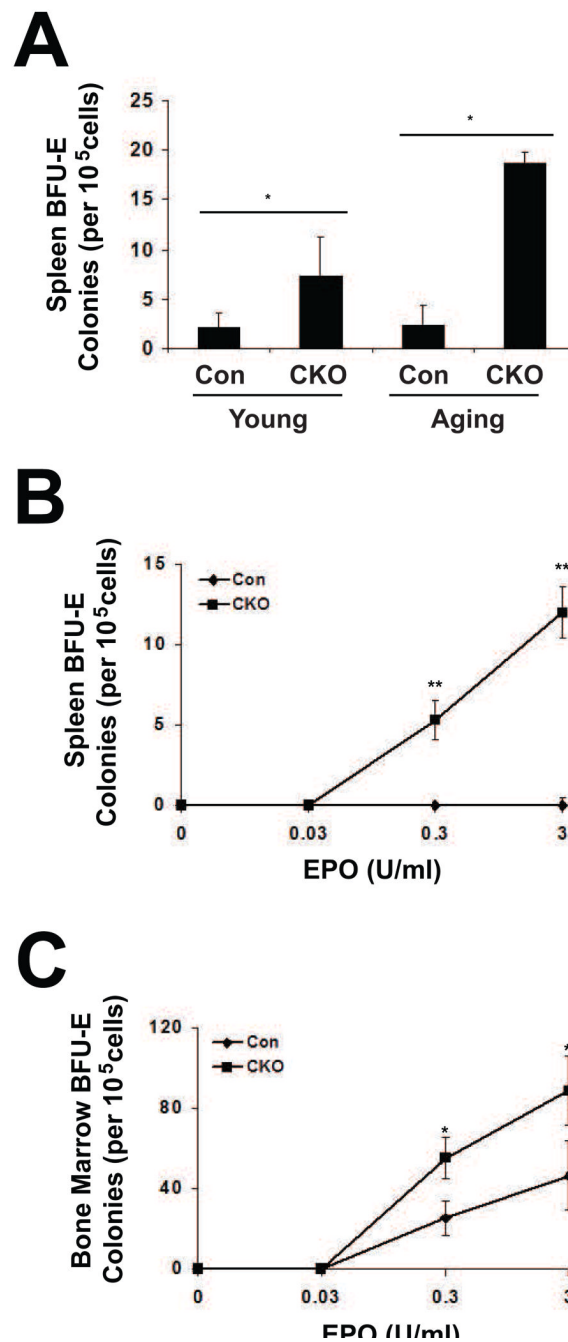at a given age.
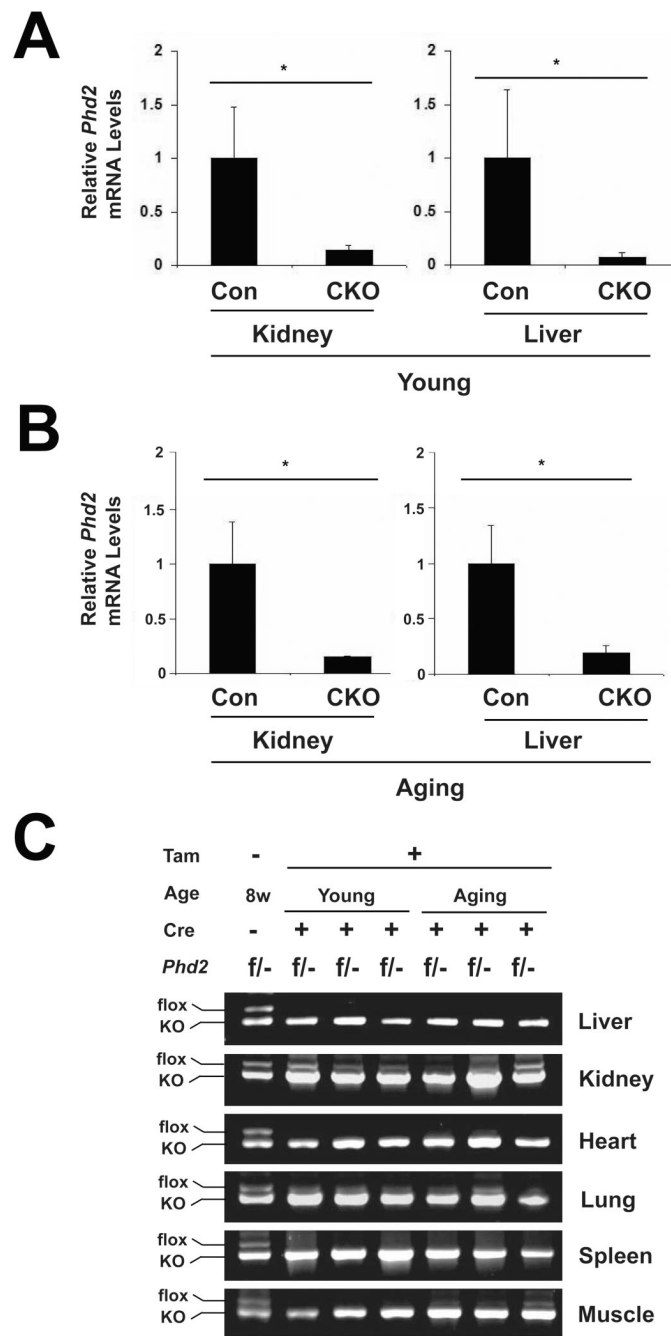
Fig. 2.
Phd2 exon 2 deletion efficiency in mice after tamoxifen induction. Both young and aging mice were treated with tamoxifen for five consecutive days. Four weeks after the initial tamoxifen dose, mice were sacrificed. RNA and DNA were then isolated from select mouse tissues. (A and B) *Phd2* mRNA levels in liver and kidney were measured by RT-PCR in (A) young and (B) aging mice. n = 4. * indicates $p < 0.05$ in comparing control (Con, *Phd2* f/+) and CKO (*Phd2* f/−; *Rosa26*-CreER[T2]) groups at a given age. (C) *Phd2* exon 2 recombination efficiency was surveyed in *Phd2* CKO mice in both the young and aging groups. A PCR reaction employing three primers was performed on genomic DNA obtained from the indicated tissues. The floxed *Phd2* allele without recombination produces a 0.95 kb band, while the knockout

Phd2 allele produces a 0.8 kb band. In each panel, the first lane shows results obtained with a control DNA from a *Phd2* f/− mouse.
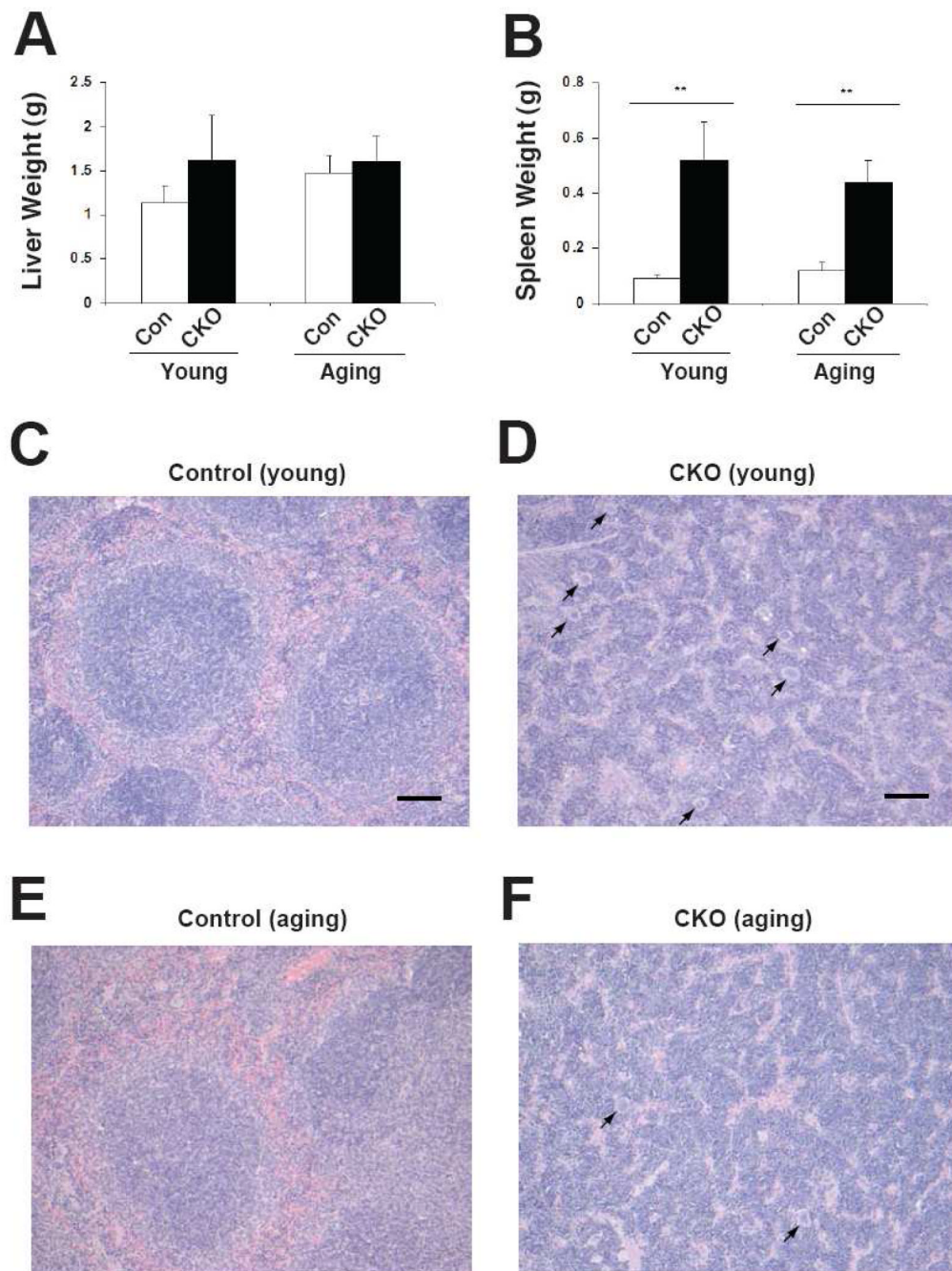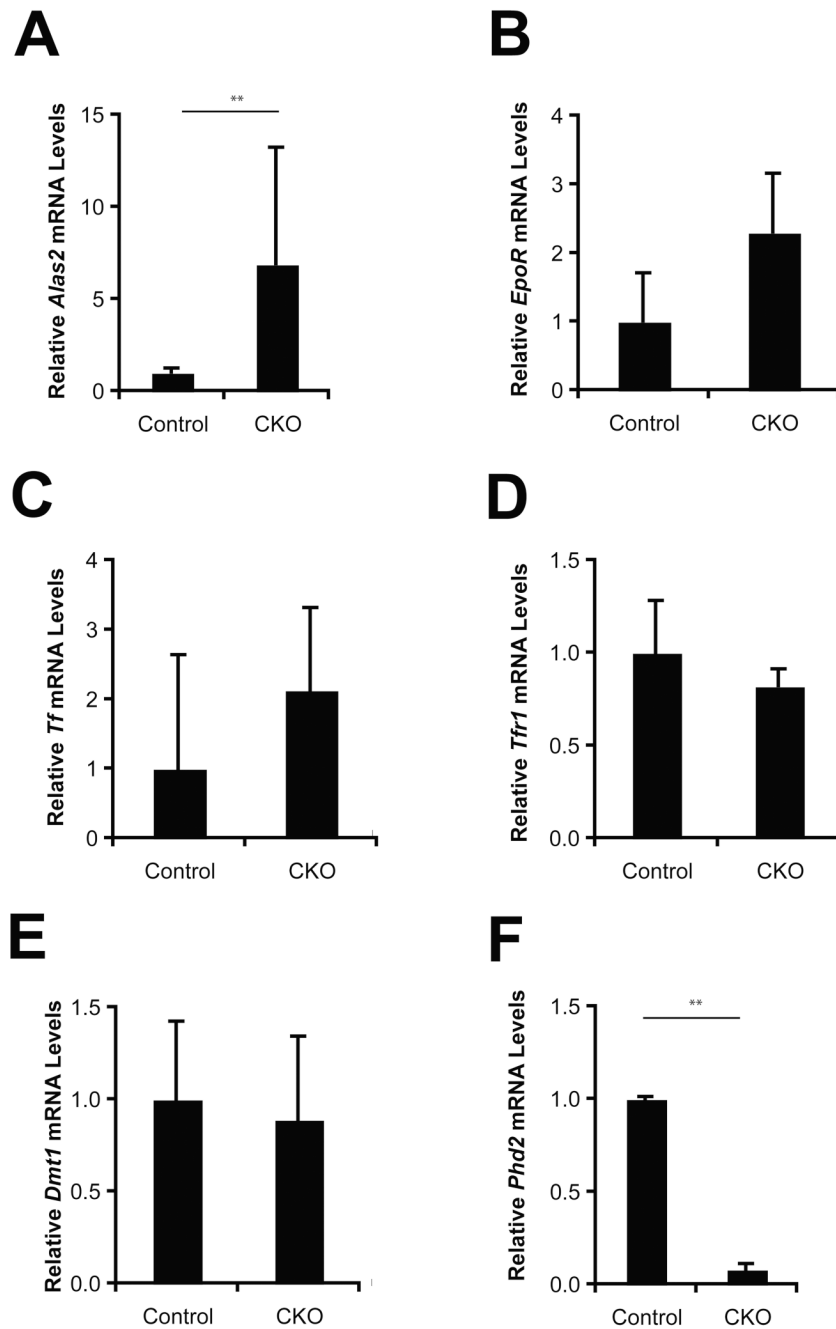
Fig. 3.
Extramedullary hematopoiesis in *Phd2* CKO mice. Both young and aging mice were treated with tamoxifen for five consecutive days. Four weeks after the initial tamoxifen dose, mice were sacrificed. Genotypes are as follows. Controls: *Phd2* f/+; CKO: *Phd2* f/−; *Rosa26-*CreER^{T2}. (A) Liver and (B) spleen weights were measured. For (A) and (B), n=6–8. ** indicates $p < 0.01$. (C–F) Photomicrographs of hematoxylin and eosin stained sections of spleen from young (C,D) and aging (E,F) mice. (Leica DM2500 microscope equipped with Leica FireCam digital capture software, magnification 100×). Bars in (C) and (D) indicate 100 µm. Megakaryocytes are present in the spleens of the *Phd2* CKO mice (indicated by arrows). Examination of ten high power fields from two mice in each age group reveals a higher number

of megakaryocytes in the spleens of young *Phd2* CKO mice (6.3 ± 2.0) as compared to aging Phd2 CKO mice (3.5 ± 1.5; *p* < 0.01).
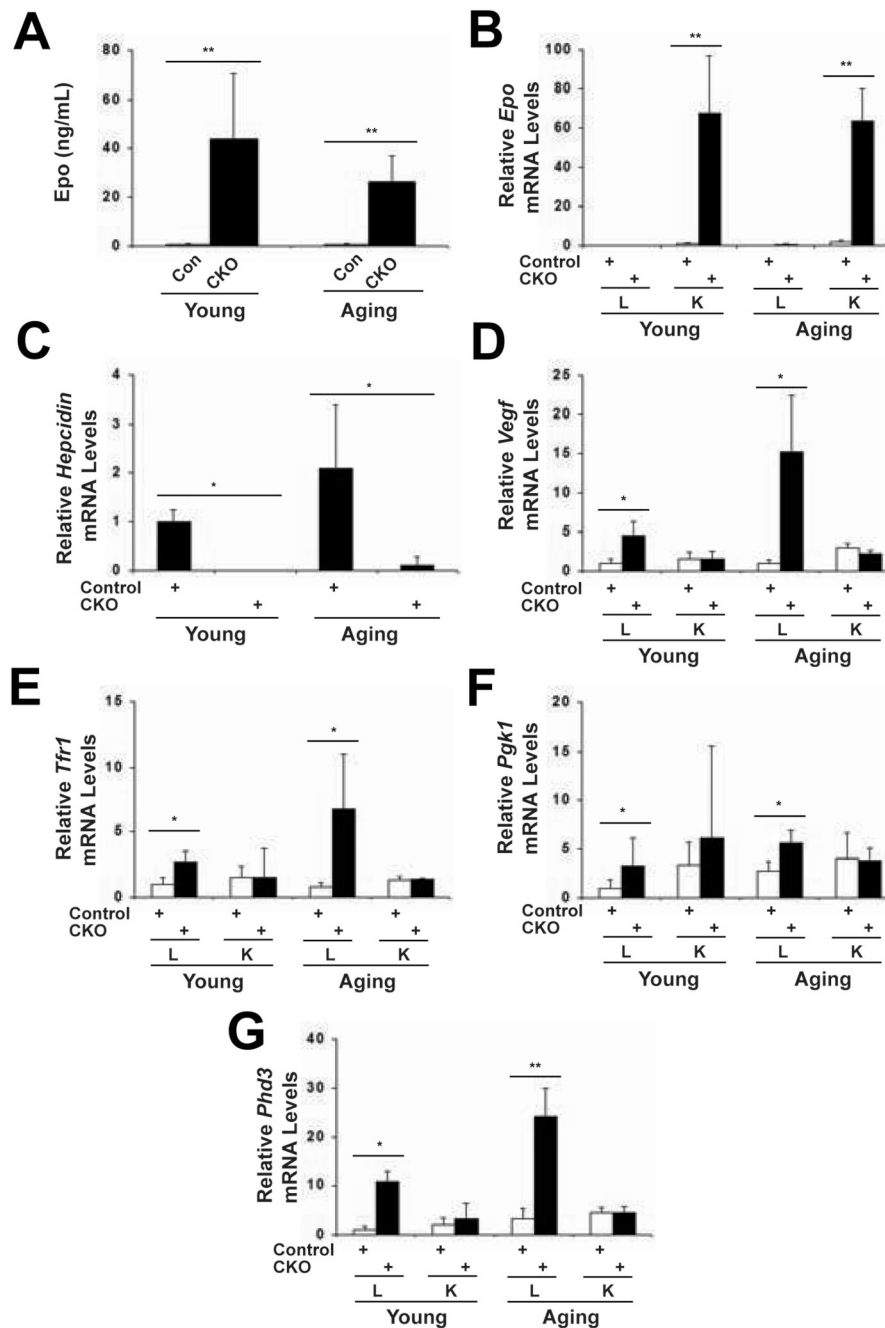
Fig. 4.
Erythroid burst forming unit assays. (A) Young and aging mice were treated with tamoxifen for five consecutive days. Four weeks after the initial tamoxifen dose, cells were isolated from the spleen and BFU-E assays were performed in the presence of 3 U/ml EPO. Genotypes are as follows. Controls: *Phd2* f/+; CKO: *Phd2* f/−; *Rosa26*-CreER[T2]. (B and C) Two month old mice were treated with tamoxifen for five consecutive days. Three weeks after the initial treatment, cells were harvested from the (B) spleen and (C) bone marrow and BFU-E assays were performed in the absence or presence of the indicated EPO concentrations.

Fig. 5.
Aging mice were treated with tamoxifen for five consecutive days. Three weeks after the initial treatment, RNA was extracted from bone marrow. The mRNA levels of select genes were determined by Real Time PCR and normalized to that of β-actin. Abbreviations are as follows. Controls: *Phd2* f/+ mice; CKO: *Phd2* f/−; *Rosa26*-CreER^{T2}. For both groups, n = 3. ** indicates *p* < 0.01 in comparing control and CKO groups.

Fig. 6.
Upregulation of Hif target genes in *Phd2* CKO mice. Both young and aging mice were treated with tamoxifen for five consecutive days. Four weeks after the initial dose, the mice were sacrificed. (A) Plasma Epo levels were measured by ELISA. Epo levels in young and aging CKO mice were compared by unpaired Student's t test and the difference was not found to be statistically significant ($p > 0.05$). (B to G) RNA was extracted from kidney and liver. The mRNA levels of select genes were determined by Real Time PCR and normalized to that of β-actin. Abbreviations are as follows. Controls: *Phd2* f/+ mice; CKO: *Phd2* f/−; *Rosa26*-CreER[T2]; L: Liver; K: Kidney. For (A) n = 6–9; for (B to G) n = 4. * indicates $p < 0.05$ and ** indicates $p < 0.01$ in comparing control and CKO groups.

Table 1

WBC differential in control and *Phd2* conditional knockout mice[a]

	Young			Aging		
	Control	*Phd2* CKO	*p* value[b]	Control	*Phd2* CKO	*p* value[b]
Neutrophils	2.7 ± 1.7	13.1 ± 10	< 0.05	3.9 ± 1.8	8.5 ± 3.3	< 0.01
Monocytes	0.96 ± 0.33	4.3 ± 4.8		1.5 ± 0.26	2.1 ± 0.33	< 0.01
Eosinophils	0.54 ± 0.4	2 ± 1.5	< 0.05	0.78 ± 0.52	1.7 ± 0.45	< 0.01
Basophils	0.17 ± 0.12	0.49 ± 0.3	< 0.05	0.21 ± 0.19	0.5 ± 0.2	< 0.01
Lymphocytes	8.1 ± 2.8	10.9 ± 2.5		8.9 ± 3.3	12.3 ± 2.3	< 0.05

[a] Values for all cell types are in $10^6/\mu$L. Measurements were made four weeks after the initial tamoxifen dose. For each group, n = 6–8. Young = 6 to 8 months, aging = 16 to 20 months.

[b] *p* values are for the comparison between Controls and *Phd2* CKO mice in a given age group