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Increased EPO Levels Are Associated With Bone Loss in Mice Lacking PHD2 in EPO-Producing Cells

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ABSTRACT

The main oxygen sensor hypoxia inducible factor (HIF) prolyl hydroxylase 2 (PHD2) is a critical regulator of tissue homeostasis during erythropoiesis, hematopoietic stem cell maintenance, and wound healing. Recent studies point toward a role for the PHD2-erythropoietin (EPO) axis in the modulation of bone remodeling, even though the studies produced conflicting results. Here, we used a number of mouse strains deficient of PHD2 in different cell types to address the role of PHD2 and its downstream targets HIF-1α and HIF-2α in bone remodeling. Mice deficient for PHD2 in several cell lineages, including EPO-producing cells, osteoblasts, and hematopoietic cells (CD68:cre-PHD2$^{f/f}$) displayed a severe reduction of bone density at the distal femur as well as the vertebral body due to impaired bone formation but not bone resorption. Importantly, using osteoblast-specific (Osx:cre-PHD2$^{f/f}$) and osteoclast-specific PHD2 knock-out mice (Vav:cre- PHD2$^{f/f}$), we show that this effect is independent of the loss of PHD2 in osteoblast and osteoclasts. Using different in vivo and in vitro approaches, we show here that this bone phenotype, including the suppression of bone formation, is directly linked to the stabilization of the α-subunit of HIF-2, and possibly to the subsequent moderate induction of serum EPO, which directly influenced the differentiation and mineralization of osteoblast progenitors resulting in lower bone density. Taken together, our data identify the PHD2:HIF-2α:EPO axis as a so far unknown regulator of osteohematology by controlling bone homeostasis. Further, these data suggest that patients treated with PHD inhibitors or EPO should be monitored with respect to their bone status. © 2016 American Society for Bone and Mineral Research.

KEY WORDS: PHD2; ERYTHROPOIETIN; BONE LOSS; OSTEOBLAST; OSTEOCLAST

Introduction

Bone homeostasis is maintained through a balance between bone formation by osteoblasts and bone resorption by osteoclasts.[1,2] Dysregulation in the activity of these bone cells can be detrimental and lead to osteoporosis characterized by low bone mass and an increase in bone fragility. Currently, antiresorptive drugs (eg, amino-bisphosphonates and the RANKL neutralizing antibodies, denosumab) are the main therapies because they reduce further bone resorption. Bone anabolic therapies, however, are very limited and there is still a great demand for drugs that target osteoblastic cells to increase bone formation and improve bone strength.[3,4]

The bone cells are located in a hypoxic microenvironment during normal development as well as regeneration.[5] The master regulators of the adaptive response to major alterations in oxygen supply are family members of transcription factors known as the hypoxia inducible factors (HIFs). Active HIF is a

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heterodimeric complex composed of an oxygen-sensitive HIFα and a constitutive HIFβ subunit. Of the most intensively studied HIFα genes, HIF-1α has been suggested to represent the response to acute hypoxia, whereas HIF-2α is the predominant subunit to chronic exposure to low oxygen as occurring at high altitude.[6] Moreover, both isoforms have overlapping sets of target genes, but can also play nonredundant roles depending on the cell type.[7] Nevertheless, both factors ultimately promote oxygen delivery and adaptive processes to hypoxia such as angiogenesis, erythropoiesis, hematopoiesis, and iron supply.[8–14] Their activity is controlled by the HIF-prolyl hydroxylases (PHD1–4). These deoxygenases enable the cell to instantaneously sense and adapt[15] to an inappropriately low oxygen pressure because they need oxygen as a cofactor to initiate the inactivation of HIF (reviewed in Eltzschig and Carmeliet[16] and Laitala and colleagues[17]). PHD2 is considered to be the key oxygen sensor during normoxia and mild hypoxia, and has been associated with a number of physiological and pathological conditions (reviewed in Mamlouk and Wielockx,[18] Singh and colleagues,[19] and Franke and colleagues[20]). Inactivation of PHD2 severely deregulates normal embryonic development in mice, resulting in embryonic lethality, whereas PHD1$^{-/-}$ or PHD3$^{-/-}$ mice develop normally.[21]

Previously, we described a new conditional PHD2 mouse line expressing cre-recombinase under the control of the modified human CD68 promoter (CD68:cre-PHD2$^{f/f}$ mice), commonly defined as a monocyte/macrophage marker. This line is not only deficient for PHD2 in these myeloid cells but in the entire hematopoietic system, in EPO-producing cells in the kidney and the brain, as well as in subsets of epithelial cells (eg, keratinocytes, enterocytes). This resulted in an excessive HIF-2α-induced production of EPO, extreme hematocrit values up to 86%, thrombocytopenia, and splenomegaly.[13] In the current study, we demonstrate that this particular mouse line displays a severe reduction of bone density due to impaired bone formation. This effect is independent of the loss of PHD2 in osteoblasts or the hematopoietic system (including osteoclasts), but directly involves inhibition of HIF-2α degradation, ultimately leading to a consequent hypoxia-independent chronic induction of EPO. Thus, our data propose that the PHD2:HIF-2α:EPO axis is a critical regulator of bone mass.

Materials and Methods

Experimental animals

CD68:cre-PHD2$^{f/f}$ (P2), CD68:cre-PHD2/HIF-1α$^{ff/ff}$ (P2/H1), CD68:cre-PHD2/HIF-2α$^{ff/ff}$ (P2/H2), and EPO transgenic mice (Tg6) as well as their littermate controls were backcrossed at least nine times to C57BL/6 and tested as described.[12,13,22] The Osx:cre[23] and Vav:cre[24] transgenic mouse lines were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and crossed with PHD2$^{f/f}$ mice in our facility. The obtained mouse lines are, respectively: Osx:cre-PHD2$^{f/f}$ and Vav:cre-PHD2$^{f/f}$. Osx:cre-PHD2$^{f/f}$ breeding couples received doxycycline (dox) in their drinking water (10 mg/mL dox in a 3% wt/vol sucrose solution) *ad libitum*. Osx:cre-PHD2$^{f/f}$ offspring received dox-drinking water until 5 weeks after birth. Cre negative PHD2$^{f/f}$ littermate mice were used as controls. In the case of the Osx:cre-PHD2$^{f/f}$, Osx:cre-PHD2$^{+/+}$ mice were also analyzed but their bone density was not significantly different from PHD2$^{f/f}$ mice. CD68:cre-PHD2$^{f/f}$, CD68:cre-PHD2/HIF1$^{ff/ff}$, and EPO-Tg6 mice all display a mild to severe form of erythrocytosis as described.[13,22] All other transgenic lines as well as the WT littermates showed no signs of malformations or sickness. Alzet osmotic pumps (Durect Corporation, Cupertino, CA, USA) filled with recombinant human EPO were transplanted on the back of the mice underneath the skin and were maintained for 30 days. All experiments were performed with male and female mice between the ages of 8 to 12 weeks. Mice were maintained in groups of up to five animals per cage and were kept in a 12-hour light/dark cycle. Water and food was available *ad libitum*. Mice were allocated to groups on availability of the conditional deficient, transgenic mice, and/or their WT littermates. An equal amount of KO versus WT was taken in each experiment when available.

Ethical statement

Mice were anesthetized with a single injection of ketamine (90 mg/kg)/xylazine (10 mg/kg). Before final analysis, mice were euthanized by cervical dislocation. All experiments were conducted at the Medical Theoretical Centre of the Medical Faculty, TU Dresden, Germany. All animal experiments were in accordance with the facility guidelines on animal welfare and were approved by the Landesdirektion Sachsen, Germany (approval numbers: TVV 9/2014 and 24-9168.11-1/2010-47).

Bone structure analysis and histomorphometry

Peripheral quantitative computed tomography (pQCT) and dynamic bone histomorphometry were performed as described.[25,26] Briefly, the distal femur and fourth lumbar vertebra were analyzed using pQCT and a resolution of 70 μm. The cortical bone density was measured at the mid-diaphysis.

For bone histomorphometry, mice received two intraperitoneal injections of calcein (20 mg/kg; Sigma, Germany) on day 10 and day 3 before sacrifice. Bones from the third lumbar vertebra and proximal tibia were fixed in 4% PBS-buffered paraformaldehyde and dehydrated in an ascending ethanol series. Subsequently, bones were embedded in methacrylate and cut into 4-μm sections for staining and 7-μm sections to assess fluorescence labels. The sections were stained with von Kossa and toluidine blue to analyze bone volume/total volume (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), and trabecular thickness (Tb.Th). Unstained sections were analyzed using fluorescence microscopy to determine the mineralized surface/bone surface (MS/BS), the mineral apposition rate (MAR), and the bone formation rate/bone surface (BFR/BS).

Osteoclast parameters were determined on tartrate-resistant acid phosphatase (TRAP)-stained paraffin sections from the third vertebral body and the proximal tibia. Histomorphometric analysis was performed with the Osteomeasure software (OsteoMetrics, Decatur, GA, USA) according to international standards.

Osteoblast cultures and EPO receptor (EPO-R) knockdown

Harvesting of bone marrow stromal cells (BMSCs) from long bones was performed by cutting the bones at both ends and flushing out the bone marrow using DMEM. Cells were centrifuged, resuspended in DMEM + FCS and plated out in flasks or dishes. BMSCs (+ monocytes) specifically adhere to plastic whereas nonadherent cells are discarded after 4 to 6 hours. When cells are semi-confluent (3 to 5 days), osteogenic induction is initiated by adding ascorbate phosphate and β-glycerophosphate (β-GP) to the medium. At different time

points during differentiation, the expression of endothelial (progenitor) (eg, CD31 and VE-Cadherin) or monocyte markers (CD11b, CD45) were tested to exclude the presence of other cell types in the osteoblast (progenitor) population.[27] In addition, cells received different EPO concentrations (Sigma-Aldrich, St. Louis, MO, USA). For knockdown experiments, cells differentiated for 14 days were transfected with 50 nM EPO-R-specific siRNA (ID: s65609) or 50 nM non-targeting siRNA (Life Technologies, Darmstadt, Germany) using Dharmafect I according to the manufacturer's protocol (Fischer Scientific, Frankfurt, Germany). Six hours after transfection, cells were serum-starved overnight and treated with EPO the following day. After 36 hours, RNA was harvested for further analysis. The mineralized matrix was stained with alizarin red S (1%; Sigma-Aldrich), eluted with 100 mM cetylpyridinium chloride, and quantified using a spectrophotometer at 540 nm.

For gene expression analysis, RNA was isolated from day 7 osteoblasts or osteoclast progenitors using the High Pure RNA Isolation kit (Roche, Mannheim, Germany) and reverse transcribed using Superscript II (Invitrogen). The mRNA expression was determined by Applied Biosystems SYBR green-based real-time PCR reactions using a standard protocol (ABI 7500Fast; Applied Biosystems, Darmstadt, Germany). The primer sequences were as follows: β-actin sense (s): ATCTGGCACCACACCTTCT, β-actin, antisense (as): GGGGTGTTGAAGGTCTCAAA; EPO-R s: CCCAAGTTTGAGAGCAAAGC, EPO-R as: TGCAGGCTACATGACT TTCG; runx2 s: CCCAGCCACCTTTACCTACA, runx2 as: TATGG AGTGCTGCTGCTGGTCTG; ALP s: CTACTTGTGTGGCGTGAAGG, ALP as: CTGGTGGCATCTCGTTATCC; OCN s: GCGCTCTGTCTCTCT GACCT, OCN as: ACCTTATTGCCCTCCTGCTT; and PHD2 s: AAG CCCAGTTTGCTGACATT, PHD2 as: CTCGCTCATCTGCATCAAAA. The results were calculated using the $\Delta\Delta C_T$ method, and are presented relative to the control.

Serum measurements

Serum levels of the bone formation markers N-terminal propeptide of type I procollagen (P1NP) and osteocalcin (OCN), and the bone resorption markers type I collagen cross-linked C-telopeptide (CTX) and tartrate-resistant acid phosphatase 5b (TRAP5b) were measured using ELISA according to the manufacturer's protocol (IDS, Frankfurt/Main, Germany).

Statistical analysis

Results are presented as means ± standard deviation (SD). Statistical significance was calculated as two-tailed by the Mann Whitney *U* test unless otherwise stated (GraphPad Prism v5.04) with $p < 0.05$ considered statistically significant. For comparisons with more than two groups, a one-way analysis of variance (ANOVA) was used.

Results

Conditional loss of PHD2 in EPO-producing cells reduces bone density

We recently developed the CD68:cre-PHD2$^{f/f}$ (P2) conditional PHD2-deficient mouse line to study the role of this particular oxygen sensor in physiological and pathological settings.[12,13] In this model, PHD2 is deleted in renal EPO-producing cells, as well as in other cell types including cells of the monocyte/macrophage lineage, neural cells, and astrocytes. Interestingly, we observed that, in addition to high EPO and resulting polycythemia, the long bones of these mutants are far more brittle than of their WT littermates. Therefore, we analyzed the bones of skeletally mature mice and found that P2 mice had a lower total, trabecular, and cortical bone mineral density as

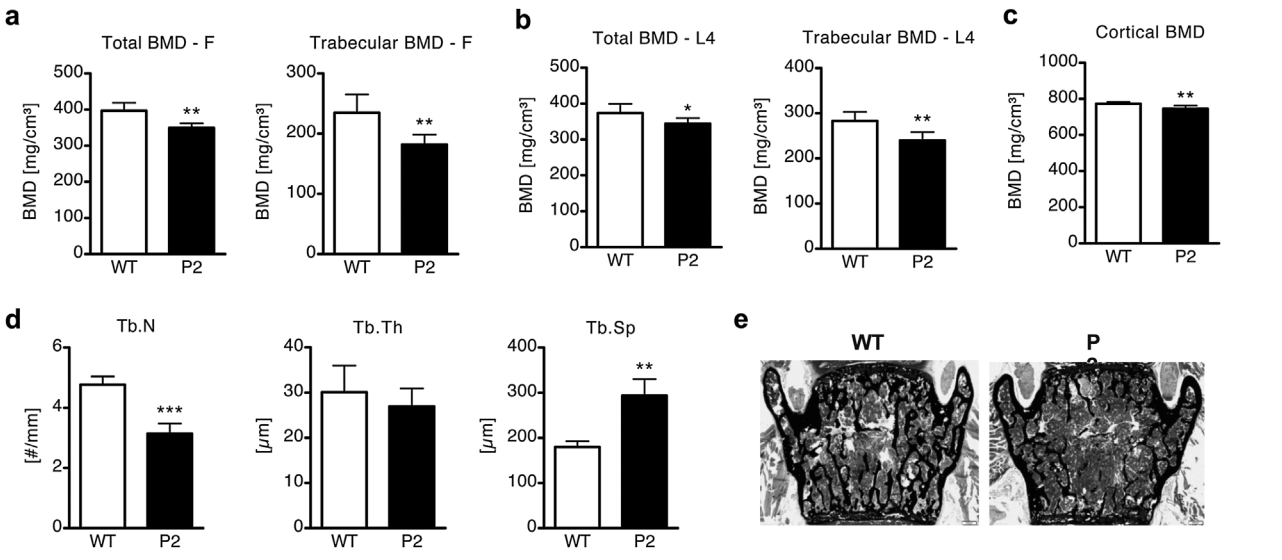

Fig. 1. CD68:Cre-driven PHD2 deletion reduces bone mineral density. Measures of bone density were performed in 8-week-old to 10-week-old P2 mice and their respective littermate controls (WT) using peripheral quantitative computed tomography and histomorphometry. (*A*, *B*) Total and trabecular BMD at the (*A*) distal femur ("F") and (*B*) fourth lumbar vertebra ("L4"). (*C*) Cortical BMD in the femur. (*D*) Tb.N, Tb.Th, and Tb.Sp of the fourth lumbar vertebra. (*E*) Representative von Kossa–stained sections of the fourth lumbar vertebra. Black areas indicate mineralized bone. Scale bars = 200 μm. All data are mean ± standard deviation ($n = 6$ to 10); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. BMD = bone mineral density; Tb.N = trabecular number; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation.

compared to their WT littermates (Fig. 1*A–C*). Low bone density was evident in the axial and appendicular skeleton as well as in the trabecular and cortical bone (Fig. 1*A–C*). Furthermore, although the overall thickness of the trabeculae (Tb.Th) did not differ, the numbers of trabeculae (Tb.N) were dramatically decreased and consequently the trabecular separation (Tb.Sp) was significantly increased (Fig. 1*D*). Representative images of the bone structure are given in Fig. 1*E*. Together, these results show that conditional loss of PHD2 leads to a substantial reduction in bone density in all studied compartments.

Low bone mass in P2 mice is primarily due to reduced osteoblast activity

To obtain more insight into the cellular phenomena driving bone loss in P2 mice, we investigated bone remodeling parameters. Surprisingly, bone resorption was not affected in these mice, because our analyses revealed no difference in the number of osteoclasts or total osteoclast surface (Fig. 2*A*). Furthermore, CTX and TRAP5b, the bone resorption markers in the serum, were not different between the genotypes (Fig. 2*B*, *C*). In contrast, dynamic bone histomorphometry revealed significant reductions in the mineralizing surface, in mineral apposition, and in bone formation rate of P2 mice (Fig. 2*D*). The number of osteoblasts per bone surface was also decreased by 48% (WT: 11.3 ± 4.65 versus P2: 5.92 ± 1.58, $p < 0.05$). In addition, serum levels of the bone formation markers OCN and P1NP were reduced by more than 40% in P2 mice (Fig. 2*E*, *F*), clearly suggesting that P2 mice have reduced osteoblast activity.

Because these mice also display loss of PHD2 mRNA in osteoblasts (WT: 1.0 ± 0.04 versus P2: 0.43 ± 0.13, $p < 0.05$), we first examined the intrinsic effect of PHD2 in osteoblasts. For this purpose, we generated the Osx:cre-PHD2$^{f/f}$ mouse line in which PHD2 is conditionally deleted in all cells of the osteoblastic lineage.[28] As shown by others, these include endosteal osteoblasts, osteoblasts, stromal cells located at the chondro-osseous junction, and a subset of hypertrophic and columnar chondrocytes, but not the hematopoietic lineage (eg, osteoclasts).[29–31] Importantly, these mice displayed no difference in hematocrit value compared to their WT littermates ($49.2\% \pm 0.1\%$ versus $48.4\% \pm 1.4\%$, respectively). In clear contrast to the data obtained in P2 mice, Osx:cre-PHD2$^{f/f}$ mice displayed a significantly higher bone density in both lumbar vertebra and femur (Fig. 3*A*, *B*). Moreover, we found these changes to be directly related to both reduction of the osteoclast number and surface, and to increase in bone formation rate (Fig. 3*C–F*). P2 mice show also reduced PHD2 mRNA in osteoclast (progenitors) (WT: 1.0 ± 0.19 versus P2: 0.42 ± 0.11, $p < 0.05$). Therefore, we also tested mice that target the osteoclasts. However, Vav:cre-PHD2$^{f/f}$ mice deficient for PHD2 in the entire hematopoietic system (WT: 1.0 ± 0.07 versus P2: 0.08 ± 0.05, $p < 0.05$) showed no difference in bone density compared to their WT littermates (Supporting Fig. 1). Together, these data suggest that impaired osteoblast activity

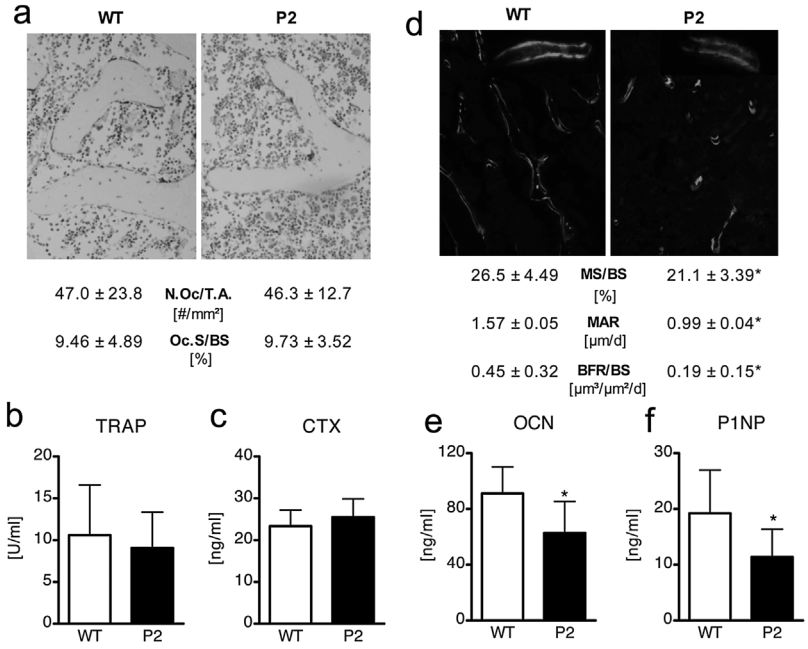

Fig. 2. Bone loss in P2 mice is caused by reduced osteoblast activity. Serum markers of bone remodeling and bone histological data were assessed using ELISAs and dynamic histomorphometry. (*A*) Representative TRAP staining showing osteoclasts in red. Quantification of the N.Oc/T.A, and of Oc.S/BS, are shown below each image and represents the values from 5 to 8 mice per group. Scale bars of the upper panels = 100 μm. (*B*) Serum levels of the bone resorption markers TRAP and (*C*) CTX. (*D*) Representative calcein (green) stained sections of the third lumbar vertebra from each genotype. Values for the MS/BS, the MAR, and BFR/BS, are shown below each image. Scale bars = 100 μm for the larger images, and 20 μm for the top right insets. (*E*) Serum OCN and (*F*) P1NP. ($n = 6$ to 10). All data are mean ± standard deviation. $^{*}p < 0.05$. TRAP = tartrate-resistance acid phosphatase; N.Oc/T.A = osteoclast number/total area; Oc.S/BS = osteoclast surface/bone surface; CTX = C-terminal telopeptides of type I collagen; MS/BS = mineralized surface/bone surface; MAR = mineral apposition rate; BFR/BS = bone formation rate/bone surface; OCN = osteocalcin; P1NP = N-terminal propeptide of type I procollagen.

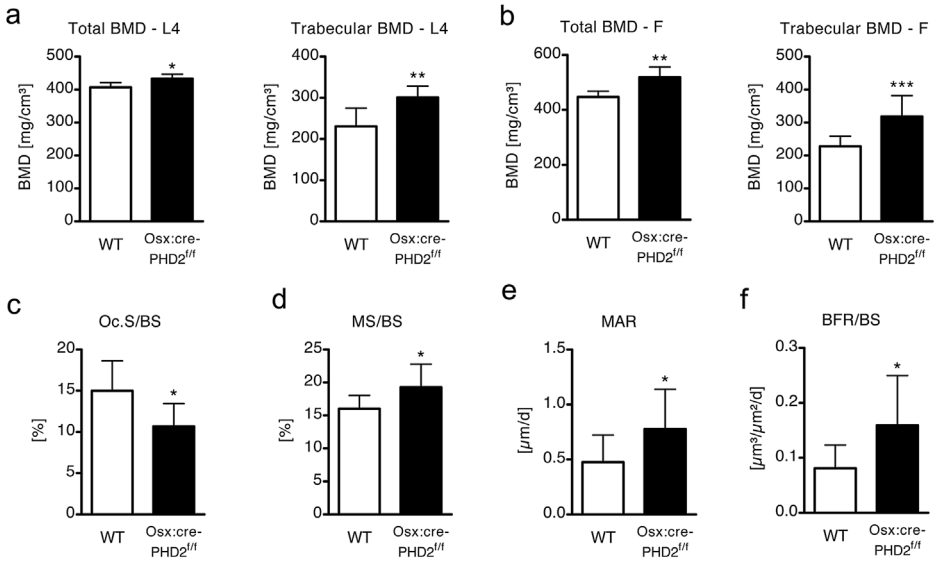

Fig. 3. Conditional loss of PHD2 in osteogenic cells leads to increased bone density. (*A*, *B*) Total and trabecular BMD of 8-week-old to 12-week-old Osx:cre-PHD2$^{f/f}$ mice at the fourth lumbar vertebra (*A*) and femur (*B*) as assessed using peripheral quantitative computed tomography. (*C*) Oc.S/BS, (D) MS/BS, (*E*) MAR, and (*F*) BFR/BS in Osx:cre-PHD2$^{f/f}$-cre mice. ($n = 8$). All data are mean ± standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$. Oc.S/BS = osteoclast surface/bone surface; MS/BS = mineralized surface/bone surface; MAR = mineral apposition rate; BFR/BS = bone formation rate/bone surface.

in P2 mice is independent of any intrinsic PHD2 effect in bone cells. Further, impaired P2 osteoblast activity is not the result of PHD2 deletion within hematopoietic cells.

HIF-2α but not HIF-1α is responsible for the PHD2-induced low bone mass phenotype

Because PHD2 can control the activity of both HIF-1α and HIF-2α, we investigated the role of these HIFα subunits in mediating the observed bone phenotypes. To this end, we used our previously described double deficient mice, CD68:cre-PHD2/HIF-1α$^{ff/ff}$ (P2/H1) and CD68:cre-PHD2/HIF-2α$^{ff/ff}$ (P2/H2), in which CD68:cre drives the deletion of both PHD2 and either the HIF-1α or the HIF-2α alleles, respectively.[13] Our analyses show that the P2 bone phenotype, in which we find loss of total and trabecular bone densities relative to WT littermates, was rescued by the conditional deletion of HIF-2α in the P2/H2 mouse strain. However, not by the conditional deletion of HIF-1α in the P2/H1 mouse model (Fig. 4*A*–*D*). Similarly, loss of cortical bone density and cortical thickness in P2 mice was rescued in P2/H2, but not in P2H1 mice (Supporting Fig. 2A, B).

Again bone histomorphometry measurements were performed in order to analyze the cellular mechanisms underlying the observed phenotypes. In line with the bone density data, bones from P2/H1, but not P2/H2 mice, displayed significant reductions in the mineralizing surface, mineral apposition, and bone formation rate (Fig. 4*E*, *F*), similar to those found in P2 mice. The bone formation serum markers P1NP and OCN were also reduced in P2/H1, but not in P2H2 mice (Fig. 4*G*, *H*). These results suggest that in the presence of a conditional PHD2 deletion, loss of HIF-2α, but not of HIF-1α rescues bone formation. With regard to bone resorption, we found no difference in osteoclast numbers between any of the double deficient lines and their WT littermates (Supporting Fig. 2C–E). Thus, our data support our notion that the impaired bone formation observed in P2 mice is mediated by HIF-2α.

Exogenous EPO administration to mice suppresses bone formation

Our data reveal that correct expression of HIF-2α is essential for the reduced osteoblast activity causing the P2 bone phenotype. Further, our results also show that this is not a cell autonomous effect, because osteoblast-specific PHD2 deletion in mice with normal EPO levels have higher, rather than lower bone density (Fig. 3*A*, *B*). We also showed that reduced osteoblast activity in P2 mice is unlikely to be the result of PHD2 deletion in myeloid or other hematopoietic cells (Supporting Fig. 1A, B). However, we previously found that the CD68:cre also drives PHD2 deletion in renal EPO-producing cells. We therefore examined the role of EPO as it is controlled by the PHD2:HIF-2 axis,[20] particularly because EPO was recently shown to modulate bone metabolism in mice.[32,33] In order to mimic the induced EPO levels that we described in the P2 and P2/H1 mouse models,[13] we administered continuous but moderate amounts of human EPO via osmotic pumps transplanted under the skin of WT mice, delivering 3 U EPO/mouse/day for 30 days. This approach resulted in a small but significant increase in hematocrit (Supporting Fig. 3A). Interestingly, EPO-treated mice displayed reduced bone density in the axial and appendicular skeleton (Fig. 5*A*, *B*; data not shown). Similar to our data obtained in the P2, these mice also revealed a significant decrease in osteoblast activity (Fig. 5*C*–*F*) and numbers (WT: 10.2 ± 0.74 versus 3 U EPO: 8.45 ± 0.62, $p < 0.05$), whereas no difference was detected in osteoclasts (Fig. 5*G*, *H*).

EPO inhibits osteoblast differentiation in a dose-dependent manner

It is known that nonhematopoietic cells, including BMSCs, express EPO receptor (EPO-R).[34,35] To evaluate the expression of EPO-R in mature osteoblasts, we cultured mouse BMSCs for

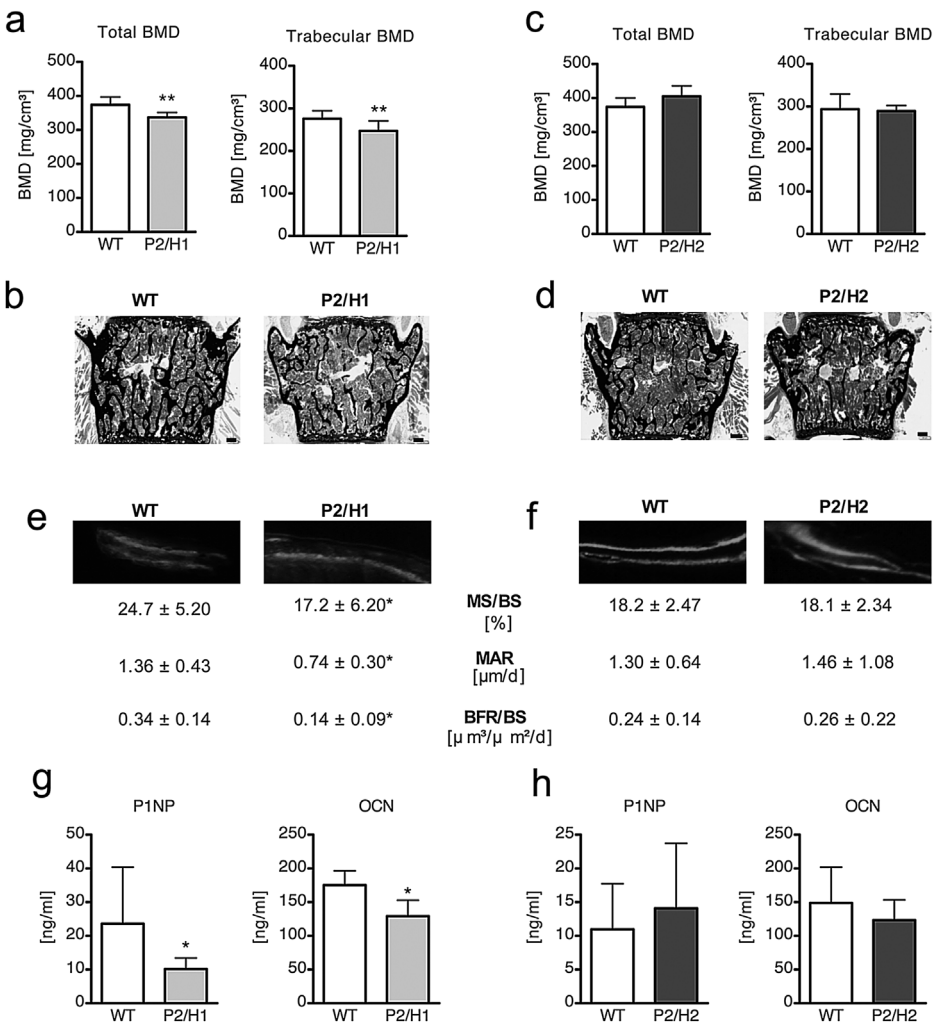

Fig. 4. The bone loss phenotype of P2 mice is rescued by deletion of HIF-2α. Measures of BMD were performed in 8-week-old to 10-week-old conditional P2/H1-deficient and P2/H2-deficient mice and their respective littermate controls (WT) using pQCT. (*A–D*) Total and trabecular BMD at the fourth lumbar vertebra in P2/H1-deficient mice (*A*) and P2/H2-deficient mice (*C*) as well as respective representative von Kossa stainings (*B*, *D*). (*E*, *F*) Representative calcein stained sections of the third lumbar vertebra from each genotype and the values for the MS/BS, the MAR, and BFR/BS. Scale bars = 20 µm. (*G*, *H*) Serum OCN and P1NP of the respective genotypes as measuring using an ELISA. All data are mean ± standard deviation. ($n = 6–8$); $^{*}p < 0.05$, $^{**}p < 0.01$. BMD = bone mineral density; MS/BS = mineralized surface/bone surface; MAR = mineral apposition rate; BFR/BS = bone formation rate/bone surface; OCN = osteocalcin; P1NP = N-terminal propeptide of type I procollagen.

21 days in standard osteogenic conditions that support their differentiation into osteoblasts. As shown in Supporting Fig. 4, we found a fivefold induction of EPO-R mRNA in mature osteoblasts compared to their stromal progenitors. Our in vivo results also show an inhibitory effect of EPO on osteoblasts. Therefore, we assessed the mineralization potential of osteoblasts using a wide range of different EPO concentrations. In a detailed EPO dose-response mineralization assay over 21 days, we found that EPO concentrations between 0.1 and 5 mU/mL significantly inhibited mineralization, whereas doses up to 0.5 U/mL had no effect (Fig. 6*A*). On the contrary, supraphysiological concentrations of EPO were able to enhance mineralization, as was also shown by others.[34]

Furthermore, we differentiated BMSCs for 7 days with EPO using two distinct doses that either inhibited (1 mU/mL) mineralization or did not (25 mU/mL). In line with the latter result, only the low EPO concentration was able to inhibit the expression of the osteoblast specific genes, Runx2, alkaline phosphatase (ALP), and osteocalcin (OCN) (Fig. 6*B*). Moreover, to investigate whether this effect is directly related to EPO:EPO-R signaling, we silenced EPO-R via siRNA (Fig. 6*C*). Interestingly, this completely abrogated the reduction of the osteoblast markers, showing that within a window of low EPO concentrations, the hormone is able to reduce the mineralization capacity of osteoblasts.

High EPO levels induce osteoclastogenesis

Other research groups have reported increased osteoclastogenesis during EPO treatment, leading to bone loss.[32,34] Because previous studies used much higher concentrations of EPO than in our 3-U EPO model, we repeated the latter setup

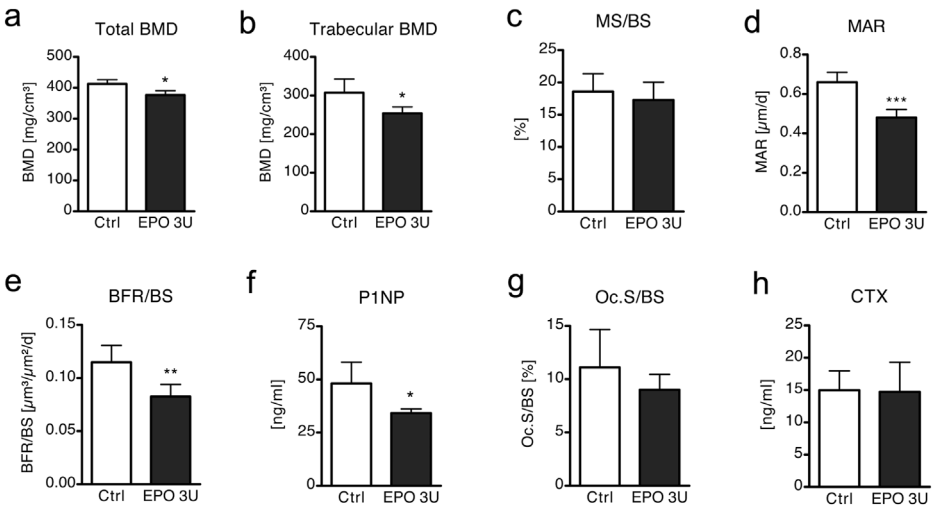

Fig. 5. Mild EPO excess reduced osteoblast activity. (*A*, *B*) Total and trabecular BMD at the fourth lumbar vertebra of 10-week-old mice treated with 3 U/mouse/day EPO or without (Ctrl) in osmotic pumps. (*C–E*) MS/BS, the MAR, and BFR/BS of the third lumbar vertebra from mice treated with or without EPO. (*F*) P1NP concentrations were measured in the serum. (*G*) Number of osteoclasts/bone area was assessed using TRAP staining. (*H*) CTX concentrations were measured in the serum. Significance calculated via an unpaired *t* test ($n = 5$). All data are mean ± standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$. BMD = bone mineral density; EPO = erythropoietin; MS/BS = mineralized surface/bone surface; MAR = mineral apposition rate; BFR/BS = bone formation rate/bone surface; P1NP = N-terminal propeptide of type I procollagen; CTX = C-terminal telopeptides of type I collagen; TRAP = tartrate-resistance acid phosphatase.

using a more than threefold higher dose (10 U EPO/day during 30 days). Our results show, in addition to a significant increase in hematocrit (Supporting Fig. 3B), reduced bone density (Fig. 7*A*, *B*), diminished osteoblast activity (Fig. 7*C*, *D*), as well as increased osteoclast number and osteoclasts activity (Fig. 7*E*, *F*). In line with these findings, we recently showed a similar combined osteoblast/osteoclast phenotype in EPO transgenic mice (Tg6).[33] In addition to the previously documented EPO-induced increase of osteoclast activity, which we also observed in our 10-U EPO model, our results emphasize the effect EPO can exert on osteoblasts (Fig. 7*G–L*). Taken together, our results show that moderate overexpression of EPO reduces bone formation in vivo. When EPO reaches higher concentrations it additionally induces osteoclastogenesis.

Discussion

In the current study we used our erythrocytotic CD68:cre-PHD2$^{f/f}$ (P2) mouse line[12] to investigate the effect of this oxygen sensor and two of its substrates, HIF-1α and HIF-2α, on bone metabolism. Here we show that the P2 mice display reduced bone mass due to diminished osteoblast activity. Using a combined set of in vivo and in vitro approaches, we provide evidence indicating a detrimental role for HIF-2α and its downstream mediator EPO on bone formation. Furthermore, we have also been able to show that specific loss of PHD2 in the osteoblastic lineage has a completely opposite effect, because these mice display higher bone volume. This clearly identifies this oxygen sensor in osteoblasts as a negative regulator of bone mass.

We recently carried out detailed analysis of our P2 mice with regard to erythropoiesis, hematopoiesis, and tumor development.[12,13,36] During the course of these studies we noticed a high degree of fragility of the bones. Our research results now show that the bone density of the P2 mouse femur and lumbar vertebra are significantly reduced compared to WT littermates. Our data also reveal that this phenotype is completely restored when PHD2 and HIF-2α are simultaneously knocked-out. By contrast, the bone phenotype of mice in which both PHD2 and HIF-1α are conditionally ablated did not differ from the bone phenotype of the P2 mice.

In search of the mechanism for the reduced bone density in the P2 mouse, we first investigated the potential role of PHD2 in osteoclasts and osteoblasts. Because our P2 mice display loss of the enzyme in the hematopoietic system, including osteoclasts,[12] as well as in osteoblast (progenitors) it could be envisaged that PHD2 exerts direct effects on bone cells. However, cell-specific knockout mice for both cell types showed no comparable bone phenotype to that of the P2 mice. On the contrary, mice lacking PHD2 in the osteoblasts (Osx:cre-PHD2$^{f/f}$) had a significantly higher bone density compared to their WT littermates. This finding is in contrast to a recent report showing that mice deficient for PHD2 in Col1α2-expressing cells (Col1α2:cre-PHD2$^{f/f}$) have a reduced bone density.[37] However, apart from osteoblasts, the Col1α2:cre-line also targets many other cells, including glomeruli and proximal tubules of adult kidneys,[38] possibly explaining why the Col1α2:cre-PHD2$^{f/f}$ mice displayed a significant increase in circulating EPO levels accompanied by erythrocytosis and bone loss.[37] Recently, we showed in our P2 mice that loss of PHD2 in a subset of renal cells is responsible for the overproduction of EPO and consequent increase in hematocrit.[13] Importantly, our Osx:cre-PHD2$^{f/f}$ mice revealed none of these features. Therefore, it is possible that the reduced bone density described by Cheng and colleagues[37] in the Col1α2:cre-PHD2$^{f/f}$ model has a similar underlying mechanism than the one we describe here for the P2 mice, because both mouse lines show similar EPO/erythrocytotic phenotypes. More recently, Wu and colleagues[39] also showed enhanced bone formation in osteoblast-specific (Osx:cre)

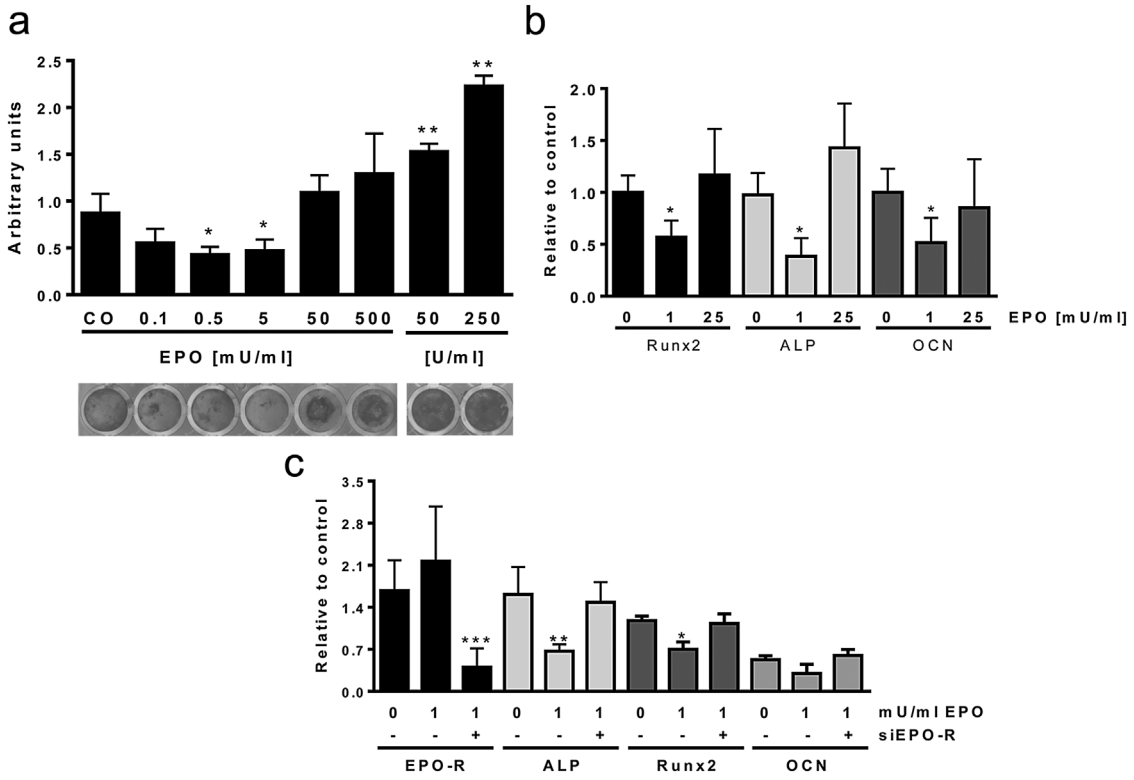

Fig. 6. Low doses of EPO inhibit osteoblast activity in vitro. (*A*) Osteoblasts were differentiated in the presence of a range of EPO concentrations as shown, for 21 days. Mineralized matrix was stained with Alizarin red S and quantified by eluting the dye and measuring its absorbance ($n = 6$). (*B*) Osteoblasts were differentiated from BMSC using osteogenic medium without or with (1 or 25 mU/mL) EPO. On day 7, the mRNA levels of osteoblast markers (runx2, ALP, OCN) were determined using qPCR. Expression was normalized to β-actin ($n = 4$). (*C*) Osteoblasts were differentiated for 14 days and treated with 50 nM scrambled siRNA (–) or siEPO-R and subsequently treated with 1 mU/mL EPO for 36 hours. mRNA levels were measured using qPCR ($n = 3$). All data are mean ± standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$ versus control (0 mU/mL EPO). ALP = alkaline phosphatase; OCN = osteocalcin; siEPO-R = siRNA against EPO-R.

PHD2-deficient mice, but only in combination with loss of at least one other PHD (PHD1 and/or PHD3). Furthermore, their phenotype was osteoprotegerin (OPG)-dependent, whereas we were not able to show changes in OPG expression in only PHD2-silenced osteoblasts (our unpublished data). At this point it is unclear why loss of PHD2 in osteoblasts in our mice is sufficient to induce the observed bone phenotype. However, a possible explanation could be found in the difference in the genetic background of the mice.[39] These data are also supported by earlier work from the Clemens' group in which they showed very dense and highly vascularized long bones in mice deficient for VHL in osteoblasts; an effect that was at least in part dependent on HIF1α.[40,41]

Our results as well as those of others show that EPO plays a critical role in the modulation of bone metabolism.[32–34] However, in contrast to our results with low endogenous overexpression or moderate exogenous doses of EPO, Shiozawa and colleagues'[34] report showed that very high doses of EPO result in increased bone formation in newborns and 4-week-old to 6-week-old young mice. Furthermore, their data suggested an effect of EPO on osteoblasts and osteoclasts.[34] Our previous study using similarly high doses in adult mice (10 to 12 weeks), however, could not confirm these results because these also showed reduced bone density.[33] Singbrant and colleagues,[32] on the other hand, found a decrease in bone volume when lower doses of EPO were given for 10 days. Singbrant and colleagues suggested an increase in the number of osteoclasts and osteoblasts, even though the bone formation rate was unchanged.[32] Furthermore, using cell lineage tracing, they have suggested that mesenchymal and osteoblastic-enriched populations do not express the EPO-R. Although our in vitro differentiation assays clearly advocate for the presence and a functional role of the EPO-R in osteoblastic cells, future experiments in mice deficient for EPO-R in this lineage will be essential to undoubtedly prove the in vivo role of the EPO/EPO-R axis during bone homeostasis.

Our erythrocytotic P2 mice, as was also suggested by Cheng and colleagues[37] in their mice, show reduced bone volume dependent on an impairment of bone formation but no increased bone resorption. Although our P2 mice have significantly higher levels of circulating EPO compared to WT littermates (~6.5-fold),[13] they are significantly lower than the transgenic mouse line overexpressing human EPO (Tg6) (~29-fold versus WT littermates).[33] In line with this, we found a similar reduction in bone density in Tg6 mice, which was dependent on a diminished osteoblast activity and enhanced osteoclastogenesis, supporting our conclusion that at low doses EPO only suppresses bone formation whereas at higher doses it also stimulates bone resorption.[33]

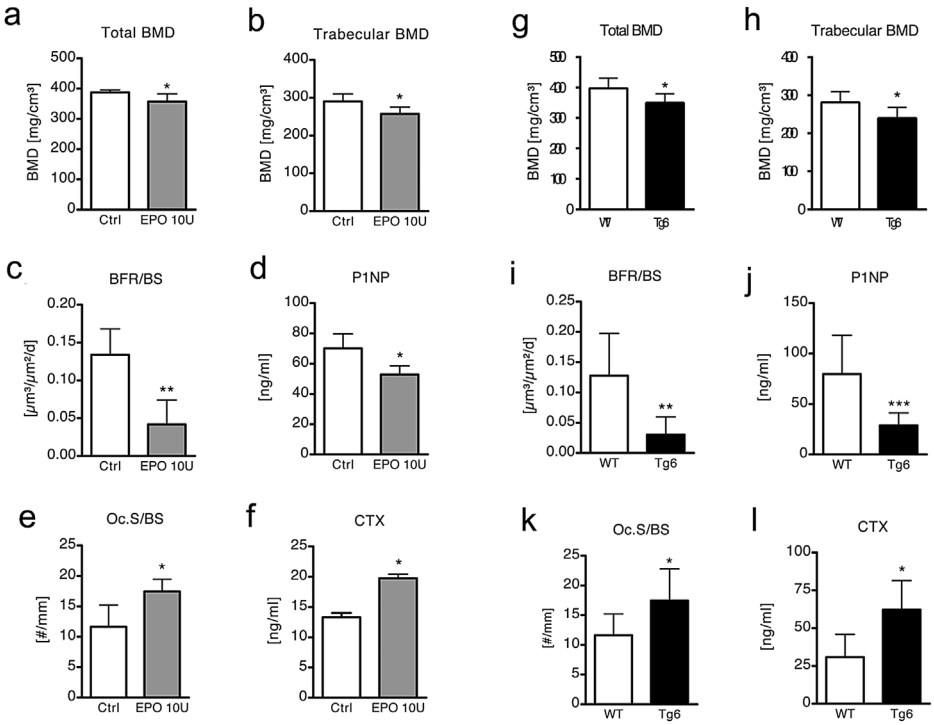

Fig. 7. High EPO concentrations reduced bone formation and concomitantly stimulated bone resorption. (*A*, *B*) Total and trabecular BMD at the fourth lumbar vertebra of mice treated with 10 U/mouse/day EPO or without (Ctrl) in osmotic pumps. (*C*, *D*) BFR/BS of the third lumbar vertebra as well as serum P1NP from mice treated with or without EPO. (*E*, *F*) N.Oc/B.Pm and serum CTX concentrations in mice treated with or without EPO. (*G*, *H*) Total and trabecular BMD at the fourth lumbar vertebra of Tg6 mice. (*I*, *J*) BFR/BS of the third lumbar vertebra as well as serum P1NP from Tg6 mice. (*K*, *L*) N.Oc/B.Pm and serum CTX concentrations in Tg6 mice. Significance calculated via an unpaired *t* test ($n = 6$ to 8). All data are mean ± standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. BMD = bone mineral density; EPO = erythropoietin; BFR/BS = bone formation rate/bone surface; P1NP = N-terminal propeptide of type I procollagen; N.Oc/B.Pm = number of osteoclasts/bone perimeter; CTX = C-terminal telopeptides of type I collagen; Tg6 = EPO transgenic mice.

To mimic the P2 bone phenotype in WT mice, we used continuous administration of low EPO concentrations, 3 U EPO/day for 4 weeks. This setup resulted in about fourfold higher circulating EPO levels (human + mouse) and enhanced hematocrit by roughly 15%. Interestingly, this resulted in an osteoblast-driven bone phenotype comparable with the effect seen in CD68-Cre:PHD2$^{f/f}$ mice. These observations are consistent with literature reports of impaired bone formation in polycythemia vera patients more than four decades ago.[42] These patients are extremely sensitive to EPO because of the constitutive activation of the EPO-signaling pathway at the EPO-R level.[20] Importantly, when we increased the EPO concentration in the osmotic pump model (10 U EPO/day), we also found induced bone resorption in our mice, which is very comparable to the phenotype observed in the Tg6 mice (Fig. 7*G–L*; and Hiram-Bab and colleagues[33]).

Although it has been shown that under certain hypoxic conditions osteoblasts can produce EPO,[29] it is less clear and even controversial to what extent EPO can directly stimulate osteoblasts (progenitors).[32] Our comprehensive in vitro results reveal an intriguing dose-dependent effect: supraphysiological EPO concentrations induced mineralization, as was shown by others,[34] whereas concentrations between 5 and 0.5 mU/mL inhibited osteoblast mineralization; an effect that we revealed to be directly EPO-R–dependent. Although the outcome resembles the observations we made in vivo, it still needs to be shown to what extent this concentration effect influences the osteoblastic niche in our mice.

Taken together, our data show that loss of PHD2 in EPO-producing cells (eg, kidney) results in reduced osteoblast function and diminished bone formation, leading to low bone mass. Using a genetic approach we show that this phenotype is directly HIF-2α–dependent, and at least in part induced by its downstream effector EPO. Our work highlights the need for further research to unravel the precise nature of EPO signaling in cells of the bone tissue. This will help to better understand the role of EPO in osteohematology and identify potential limitations of the clinical use of PHD inhibitors, or EPO in the treatment of anemia in end-stage kidney disease or myelodysplastic syndromes.

Disclosures

All authors declare no conflict of interest.

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Authors' roles: MR and KF designed and performed the experiments, analyzed the data, and wrote the manuscript; MM and RPS designed and performed experiments; SHB and UP provided tools; DN, YG, MG, MS, TC, and LCH provided tools, contributed to the discussions and to writing the manuscript. BW designed the study, supervised the overall project, analyzed the data, and wrote the manuscript.

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