REGULAR ARTICLE

blood advances

Osteocyte-derived erythroferrone regulates liver hepcidin during stress erythropoiesis

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Key Points

- Osteocytes produce ERFE, a hormone that regulates red cell production.
- ERFE produced by osteocytes in vivo contributes to suppressing liver hepcidin production.

Our knowledge of which bone marrow (BM) cells affect red cell production is still incomplete. To explore the role of osteocytes in the process, we performed bulk RNA sequencing of osteocytes isolated from control and phlebotomized mice. The second top upregulated gene after phlebotomy was erythroferrone (*Erfe*). *Erfe* expression in osteocytes was also upregulated after erythropoietin (EPO) treatment and hypoxia in vitro. To explore whether osteocytes contribute to systemic ERFE levels, we generated 2 mouse models. We first transplanted wild-type BM in $Erfe^{-/-}$ mice, creating a model where ERFE is produced in the BM but not by osteocytes. After phlebotomy, liver hepcidin suppression was significantly lower in mice where the osteocytes could not produce ERFE. To confirm that osteocytes are responsible for this difference, we generated mice lacking EPO receptors in osteocytes by crossing $Epor^{flox/flox}$ and *Dmp1-Cre* mice. After phlebotomy, these mice demonstrated reduced hepcidin suppression in the liver and higher circulating serum hepcidin levels compared with controls. Our work identified a novel function of osteocytes in suppressing systemic hepcidin levels during stress erythropoiesis.

Introduction

Erythropoiesis results from a fine-tuned differentiation of hematopoietic stem cells to mature red blood cells (RBCs). Erythropoietin (EPO), made by interstitial fibroblasts in the kidney,[1] is a master regulator of erythropoiesis. RBC production requires a large amount of circulating iron. Hepcidin, a hormone produced by the liver, regulates systemic iron levels. Downregulation of Hamp is required to increase the bioavailability of iron for RBC formation. Erythroferrone (ERFE) suppresses the production of Hamp by the liver. ERFE is produced in the bone marrow (BM) by erythroblasts in response to erythropoietic stimuli, such as blood loss, hemolysis, hypoxia, or an increase in EPO levels.[2,3] The process of RBC development occurs along the central macrophages in erythroblastic islands within the BM,[4] first described by Bessis in 1958.[5]

In addition to the central macrophages, other cells such as BM stromal cells,[6] osteoblasts (bone-forming cells),[7] and osteoclasts (bone-resorbing cells)[8,9] also support erythropoiesis either directly or indirectly.[10] Osteoblasts were found to produce sufficient EPO in the BM microenvironment to protect mice from stress-induced anemia.[7] Osteoclasts are required for the EPO-induced erythropoietic response; inhibition of osteoclast activity using bisphosphonate blunts this response.[8] However, it is not known whether osteocytes, the most abundant bone cell (95%) that live for years, unlike

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post-mitotic osteoblasts and osteoclasts,[11] have a role in erythropoiesis. Osteocytes are terminally differentiated osteoblasts that reside in the bone matrix.[12,13] Osteocytes play a vital role in skeletal homeostasis, hematopoietic stem cell/progenitor regulation,[14] myelopoiesis,[15] and neutrophil development.[16] They secrete endocrine signals and regulate systemic phosphate homeostasis,[17] adipogenesis,[18] and development of B cells and stromal cells in the thymus.[19]

In this work, we explored the role of osteocytes in erythropoiesis. We reveal that osteocytes secrete ERFE in response to erythropoietic stimuli, such as phlebotomy, EPO stimulus or treatment, and hypoxia. Mice with specific deletion of EPO receptors (*Epor*) in their osteocytes had significantly blunted *Hamp* expression in their liver and higher circulating HAMP levels after phlebotomy. Our findings suggest that osteocytes significantly contribute to the circulating levels of ERFE to suppress hepcidin during stress erythropoiesis.

Materials and methods

Animal studies

Four-month-old male C57BL/6 mice were used for bulk RNA sequencing (RNAseq). Transgenic (Tg) mice with an osteocyte-specific deletion of *Epor* Tg were generated by crossing *Dmp1-Cre* mice (The Jackson Laboratory, Bar Harbor, ME) with $Epor^{flox/flox}$ mice[20] (generous gift from Hong Wu, University of California, Los Angeles, CA). Mice that express only Cre recombinase (Cre) were used as littermate controls. $Erfe^{-/-}$ mice[21] were a generous gift from Tomas Ganz, University of California, Los Angeles. All $Erfe^{-/-}$ and wild-type (WT) control mice used in this study were generated from heterozygous ($Erfe^{+/-}$) breeding pairs, ensuring that experimental animals were true littermates. All mice were maintained on a C57BL/6 background. When feasible, WT and $Erfe^{-/-}$ littermates were co-housed to further reduce environmental variation.

For erythropoietic stimulus, mice were phlebotomized by retro-orbital puncture (500 μL) or treated with a single dose of 200 U of human EPO (NDC55513-126-10, Epogen; Amgen). Analysis was done after 16 hours. RNA was prepared from the livers as described subsequently, and serum Hamp levels were measured using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Novus Ms hepcidin ELISA, catalog no. NBP2-82129). Recovery from anemia was monitored every 3 days after phlebotomy for 9 days. Blood (50 μL) was collected by retro-orbital puncture at each time point. Complete blood cell counts were obtained using the IDEXX ProCyte Dx hematology analyzer. Animal housing and maintenance were in full compliance with the criteria of the National Institutes of Health for the care and use of laboratory animals. The Animal Care and Use Committee of the National Institute of Dental and Craniofacial Research, National Institutes of Health, approved all procedures under Animal Study Protocol no. 23-1117.

OEBT isolation and culture

Osteocyte-enriched bone tubes (OEBTs) were generated from the tibia and femur. The BM was isolated by centrifugation as previously described.[22] Osteocytes were isolated by a modified protocol of Stern and Bonewald.[23] The bones were subjected to sequential digestion performed in a 37°C water bath using collagenase buffer (alpha-minimum essential medium [αMEM], HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [25mM], bovine serum albumin [0.1%], penicillin-streptomycin [1%], collagenase type IV [2 mg/mL], DNAse [40 μg/mL]) and EDTA buffer (phosphate-buffered saline, bovine serum albumin [0.1%], EDTA [5mM]) as follows: collagenase, 20 minutes; EDTA, 20 minutes; collagenase, 1 hour; collagenase, 1 hour; EDTA, 45 minutes. Between the steps, prewarmed phosphate-buffered saline was used for washing. After the final digestion, the tubes were washed and used for RNA extraction or cell culture. For RNA expression analysis, the tubes were cultured in αMEM medium with 0.2% polyvinyl alcohol and penicillin-streptomycin (1%) and stimulated with EPO (10 U/mL). For ERFE culture medium analysis, total BM cells were cultured in Iscove's modified Dulbecco's medium with 10% fetal bovine serum. OEBTs were cultured in αMEM medium with 10% fetal bovine serum for 3 days, and ERFE was measured by sandwich ELISA (Biomatik, catalog no. EKF58568).

For STAT5 signal inhibition, the OEBTs were pretreated for 2 hours with the following 2 different STAT5 inhibitors: pimozide (PIMO) (10μM Sigma; catalog no. P1793) or 2-[(4-oxo-4H-1-benzopyran-3-yl)methylene]hydrazide-3-pyridine carboxylic acid] (200μM, Cayman Chemical; catalog no. 15784). Next, the pretreated OEBTs were stimulated with 10 U/mL EPO, and after 16 hours, the tubes were collected to study RNA expression as described subsequently.

Isolation of BM RBCs

Total BM cells (1×10^7) were stained with anti-mouse biotin-Ter119 antibody (Thermo Fisher Scientific, catalog no. 13-0114-82), and MagCellect streptavidin ferrofluid magnetic beads (R&D Systems; MAG999B) were used for selection using a MagCellect magnet. Both selected $Ter119^+$ and $Ter119^-$ (from the flow-through) cells underwent RNA analysis.

BM transplantation

For BM transplantation, 1×10^6 BM cells isolated from CD45.1-positive WT mice were administered intravenously to rescue lethally irradiated (10 Gy) CD45.2 $Erfe^{+/+}$ and $Erfe^{-/-}$ recipient mice at 6 to 8 weeks of age. Recipient mice were given sulfamethoxazole- and trimethoprim-containing water for 3 weeks, followed by regular water. BM engraftment was confirmed by flow cytometric analysis of CD45.2-positive donor-derived cells in the peripheral blood of recipient mice. After 3 months following BM transplantation, the mice were subjected to phlebotomy. After 16 hours, the livers of mice were collected and analyzed for Hamp expression.

Bulk RNAseq and analysis

RNA was converted into complementary DNA using the SMART-seq UltraLow version 4 kit (Clontech, Mountain View, CA [now Takara]; catalog no. 634890). Illumina complementary DNA-sequencing libraries were prepared using the Nextera XT kit (Illumina, San Diego, CA). Libraries were sequenced on an Illumina HiSeq 1500 sequencer, in the 126 base pair paired-end mode. Raw sequences underwent initial quality control analysis and were then aligned to the GRCm38 version of the mouse genome with STAR v.2.5.2a. Raw gene read counts produced by STAR were filtered to remove low-expressing genes and further processed in

R (R Development Core Team, 2011) using the edgeR package[24] and the START application.[25] Enrichr (maayanlab.cloud/Enrichr) was used to generate bar charts from Molecular Signatures Database Hallmark 2020 of significantly upregulated or downregulated genes by phlebotomy in OEBTs. Predicted protein–protein interactions of upregulated or downregulated genes were mapped using the STRING database[26] and clustered by functional interconnection using the MCL (Markov Cluster Algorithm), applying an inflation parameter of 1.8.

Statistical analysis

Statistical analyses were performed with Prism 8 (GraphPad Software, Inc) using unpaired *t* test. Significance is indicated by $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.

Results

Osteocytes produce ERFE in stress erythropoiesis

To learn about the osteocyte response to erythroid stress, 4-month-old male C57BL/6 mice were phlebotomized. After 16 hours, OEBTs (referred to as "osteocytes") from control and phlebotomized mice were collected for RNAseq. A total of 247 differentially expressed genes were identified using START, revealing upregulation of genes activated by interferon response and downregulation of genes involved in mesenchymal cell proliferation and the canonical WNT pathway (supplemental Figure 1). A list of strongly regulated genes was created using a $\log^2$ change of 1 or more as a cutoff, with an adjusted *P* value of $< .05$. A total of 28 strongly differentially expressed genes were identified in osteocytes from control vs phlebotomized mice (Figure 1A and Gene Expression Omnibus data set GSE241575). The second most significantly upregulated gene in osteocytes after phlebotomy was *Erfe* (Figure 1A-B). Because ERFE production is said to be induced by EPO,[21] we looked at the expression of the *Epor* in osteocytes and found no difference between control and phlebotomized animals (Figure 1C). We validated the *Erfe* expression levels using quantitative polymerase chain reaction of the same samples submitted for RNAseq. *Erfe* was upregulated 16-fold in osteocytes from phlebotomized vs control mice (Figure 1D). Osteocyte marker genes were dentin matrix acidic phosphoprotein (*Dmp1*), fibroblast growth factor 23 (*Fgf23*), matrix extracellular pohosphoglycoprotein (*Mepe*), neuropeptide Y (*Npy*), podoplanin (*Pdpn*), phosphate regulating endopeptidase homolog X-linked (*Phex*), and sclerostin (*Sost*); they were not significantly different between the groups (supplemental Figure 2).

EPO has been reported to induce *Erfe* expression only in the BM and spleen.[21] We compared the *Erfe* expression 16 hours after EPO injection in total BM cells, $Ter119^{+}$, $Ter119^{-}$ cells, and osteocytes. In the control group, *Erfe* expression was similar in total BM, $Ter119^{+}$ cells, and osteocytes. $Ter119^{-}$ cells had a sixfold lower expression of *Erfe* than the other cell populations. Following EPO treatment, *Erfe* expression was significantly upregulated in all 4-cell populations, with highest expression in $Ter119^{+}$ cells (6.5-fold), followed by osteocytes (4.8-fold) (Figure 2A).

We next asked whether osteocytes secrete ERFE in response to EPO stimulation. We collected the total BM cells and OEBTs from WT mice, placed them in culture medium, and treated them with EPO (10 U/mL) for 3 days. We then measured ERFE levels in the medium using ELISA. ERFE levels were significantly higher in both EPO-treated BM cells and bone tubes than in controls to which no EPO had been added (Figure 2B). Thus, both BM cells and osteocytes appear to secrete ERFE in response to EPO stimulation. To confirm this, we also measured RBC parameters in Tg mice with Epor–negative osteocytes.[27] When these animals were injected with EPO, their RBC indices were significantly reduced compared with the age-matched WT controls 10 days post-injection (Figure 2C). This illustrates the importance of osteocytes during stress-induced erythropoiesis. Hypoxia promotes erythropoiesis in the BM. This effect is mediated by oxygen sensors regulating the induction of EPO in interstitial fibroblasts in the kidney through hypoxia-inducible factor.[28,29] Dimethyloxalylglycine (DMOG), an inhibitor of the oxygen-sensing prolyl hydroxylases and a hypoxia-mimetic agent, did not induce *Erfe* expression in the BM cells.[21] To see whether hypoxia can regulate *Erfe* expression in osteocytes, they were cultured in a hypoxic incubator (1.2% oxygen) alongside BM cells for 16 to 18 hours. Hypoxia significantly upregulated *Erfe* expression in both total BM (Figure 2D) and osteocytes (Figure 2E). As a positive control for hypoxia, *Vegfa* expression was analyzed and found upregulated as expected in BM cells (supplemental Figure 3). To determine whether osteocyte *Erfe* expression is regulated through STAT5 signaling, as it is in erythroblasts, we treated osteocytes with 2 unrelated STAT5 inhibitors (pimozide- and nicotinohydrazide-labeled PIMO and nicotine-hydrazide in Figure 2F) for 2 hours before incubation with EPO for 16 hours. Inhibition of the JAK2-STAT5 signaling did not affect EPO-induced *Erfe* expression in the osteocytes (Figure 2F). Overall, these results reveal that *Erfe* in the osteocytes is regulated by both EPO and hypoxia, and *Erfe* expression in the osteocytes does not seem to be regulated by the STAT5 signaling pathway.

Osteocytes contribute to systemic ERFE levels

To determine whether loss of *Erfe* affects specific osteocyte marker genes, we analyzed their expression in $Erfe^{-/-}$ and $Erfe^{+/+}$ mice in steady state and after phlebotomy. In steady state, *Dmp1* expression was significantly downregulated in $Erfe^{-/-}$mice. However, *Fgf23* expression was significantly upregulated, and no significant changes were observed in the expression of *Mepe*, *Sost*, *Phex*, *E11*, and *Npy* in $Erfe^{-/-}$ compared with $Erfe^{+/+}$ mice. After phlebotomy, expression of *Dmp1*, *Mepe*, and *Phex* was significantly downregulated, whereas expression of *Sost*, *Fgf23*, *E11*, and *Npy* was similar in $Erfe^{-/-}$ and $Erfe^{+/+}$ mice (supplemental Figure 4). These results suggest that *Erfe* might also play a role in skeletal mineralization; this function needs further investigation.

To determine whether osteocytes contribute to circulating levels of ERFE in stress erythropoiesis, we generated 2 mouse models. In 1 model, we performed BM transplantation of WT CD45.1 donor mice to CD45.2-recipient $Erfe^{-/-}$ and $Erfe^{+/+}$ mice. The transplanted BM can produce ERFE in both $Erfe^{-/-}$ and $Erfe^{+/+}$ mice, but the osteocytes in the $Erfe^{-/-}$ mice cannot. If the osteocyte-derived ERFE contributes to the suppression of liver Hamp, we would find an increase (reduced suppression) of liver Hamp in $Erfe^{-/-}$ mice. After 3 months from transplantation, the mice were subjected to phlebotomy, and liver Hamp expression was analyzed. Hamp suppression was significantly inhibited in $Erfe^{-/-}$ mice transplanted with WT BM (Figure 3A) suggesting that osteocytes secrete significant levels of ERFE to suppress Hamp during stress erythropoiesis. For the second model, we generated

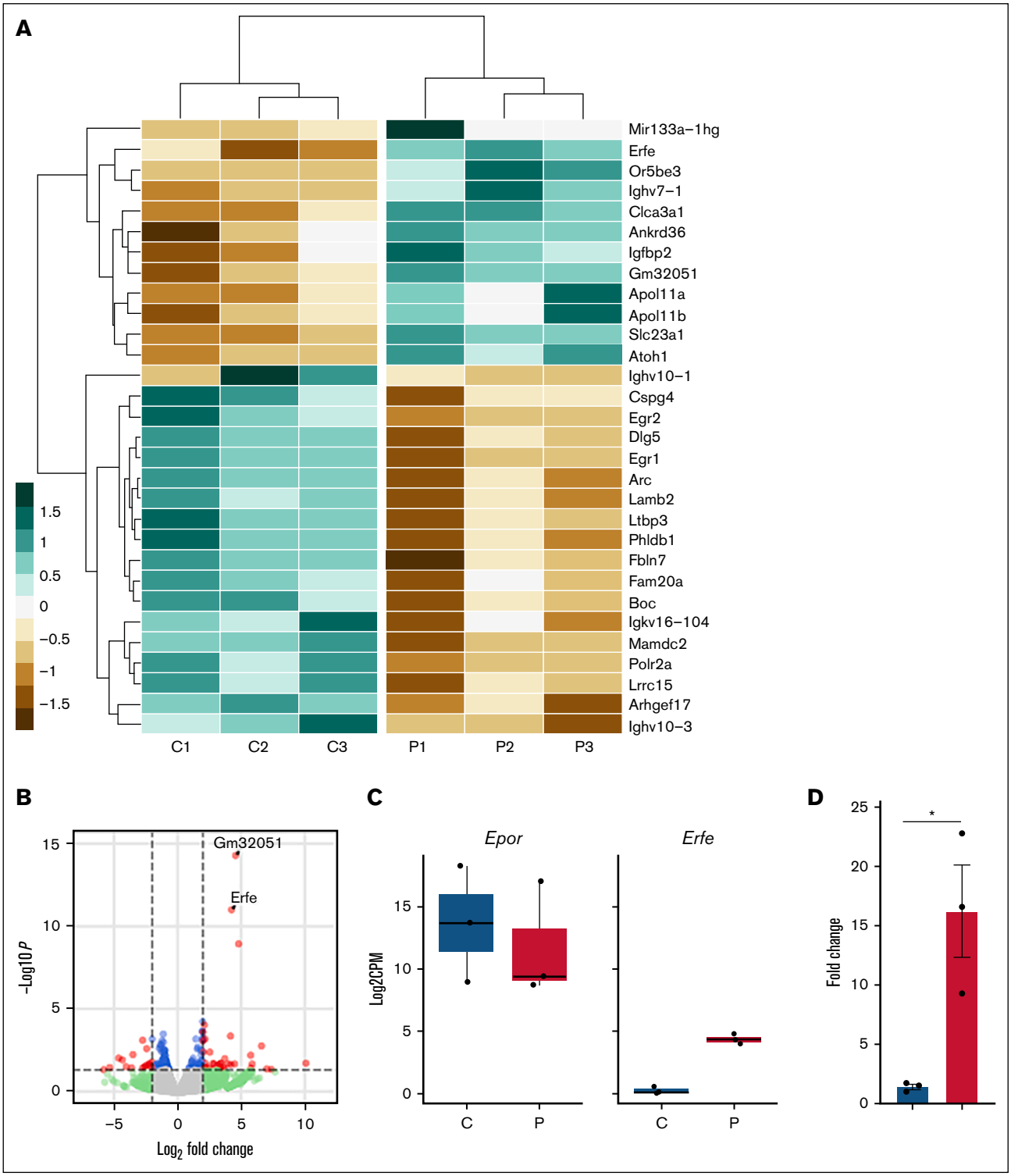

Figure 1. *Erfe* is the top upregulated gene in osteocytes after phlebotomy. (A) Heat map revealing the top 30 differentially expressed genes between control and phlebotomized mice of osteocytes. (B) The volcano plot illustrates that the most significant and highly expressed gene is *Erfe* after phlebotomy. (C) Box plots of *Epor* expression in the osteocytes are unchanged after phlebotomy, whereas the expression of *Erfe* is significantly increased. (D) quantitative polymerase chain reaction confirmation of *Erfe* expression of the RNAseq samples (n = 3, per group). C, control; P, phlebotomized.

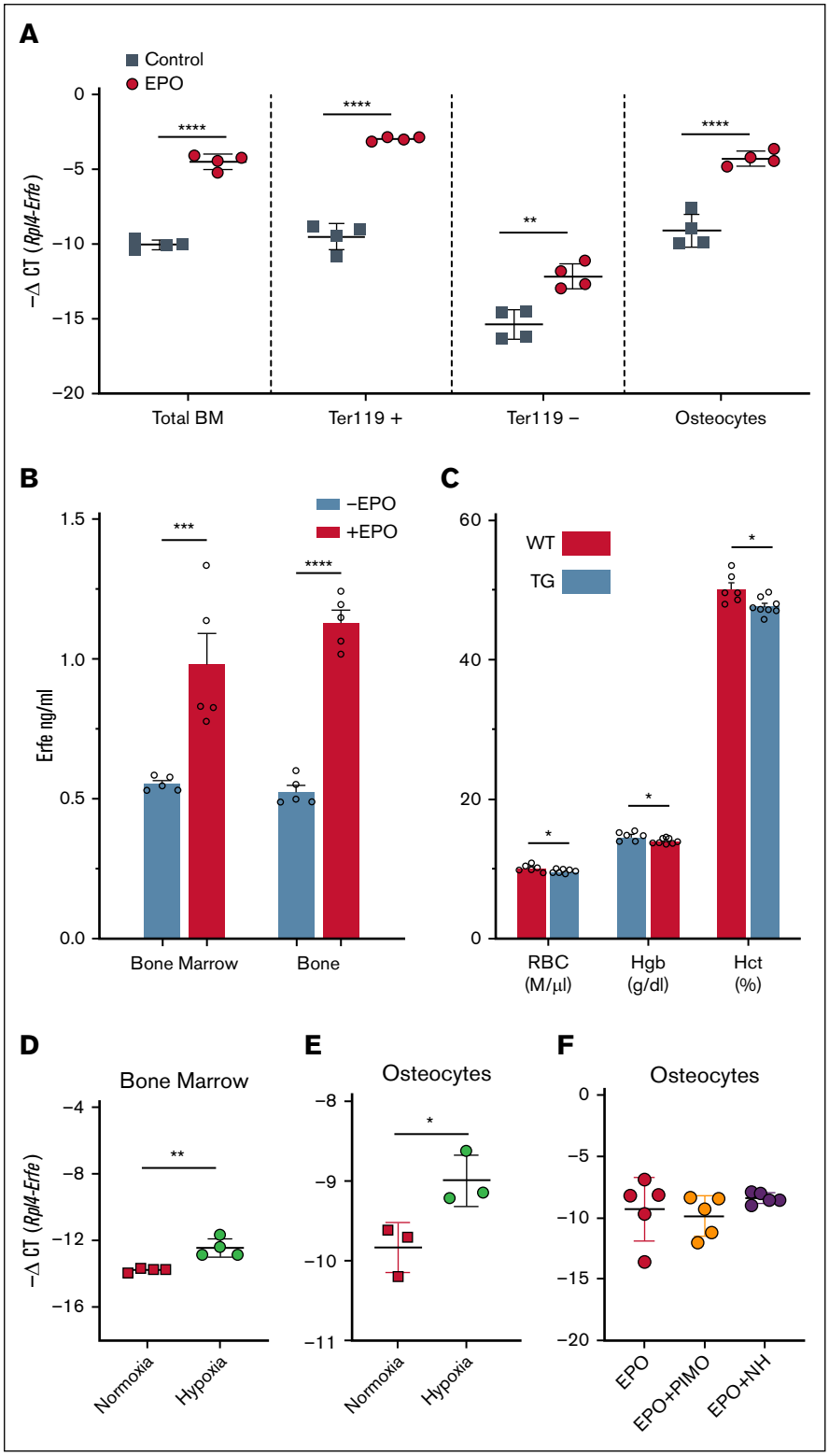

Figure 2. *Erfe* expression is increased in osteocytes by EPO and hypoxia. (A) Expression of *Erfe* in total BM cells, Ter119^{+} and Ter119^{-} cells, and osteocytes, 16 hours after EPO injection (n = 4, per group). (B) ERFE levels in medium after BM or osteocytes were cultured with and without EPO (10 U/mL) (n = 5, per group). (C) Red blood indices were measured using a procyte IDEXX hematology analyzer in Tg mice and age-matched WT mice after EPO injections (200 U) on day 10. (D-E) *Erfe* expression in total BM and osteocytes cultured for 16 to 18 hours in normoxia or hypoxia (n = 3). (F) *Erfe* expression in osteocytes after EPO stimulation and using STAT5 inhibitors PIMO and *N*′-((4-oxo-4H-chromen-3-yl) methylene)) nicotine-hydrazide (NH) using in vitro culture (n = 5, per group). Data are presented as mean ± SE. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001. Hgb, hemoglobin; Hct, hematocrit.

mice in which the *Epor* were conditionally ablated in the osteocytes using the osteocyte-specific *Dmp1-Cre* mice (hereon called Tg). We confirmed that *Epor* expression in osteocytes was significantly decreased in Tg compared with control mice, but not in other tissues such as the spleen, liver, and BM (supplemental Figure 5). Control and Tg mice were subjected to phlebotomy, and Hamp levels in the liver and serum were analyzed. Suppression of Hamp expression in the liver was significantly inhibited in

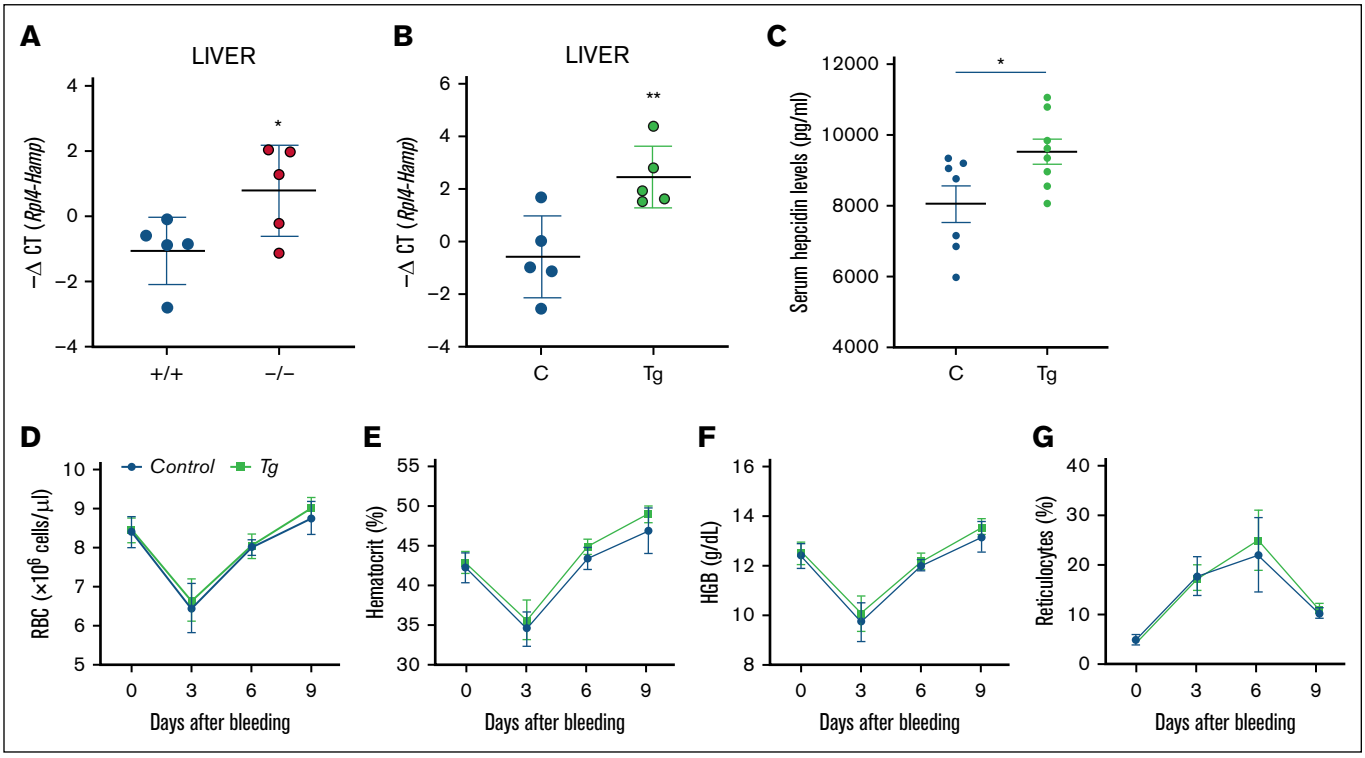

Figure 3. ERFE secreted by osteocytes inhibits liver Hamp expression after phlebotomy. (A) *Hamp* expression in the liver after phlebotomy in *Erfe*$^{-/-}$ and *Erfe*$^{+/+}$ mice (CD45.2) transplanted with WT (CD45.1) BM; n = 5, per group. (B) Expression of *Hamp* in the liver after phlebotomy of control and osteocyte *Epor*-deficient mice (Tg); n = 5 per group. (C) Serum Hamp levels after phlebotomy of control and osteocyte *Epor*-deficient mice (Tg); n = 7 per group. (D-G) Control and Tg were subjected to phlebotomy and hematological parameters were monitored for 9 days (every 3 days); (D) RBC, (E) hematocrit, (F) hemoglobin, and (G) reticulocytes. Data are presented as mean ± standard deviation *$P < .05$, **$P < .01$. The first data points are from the first sample of the blood at the start of the experiment from previously nonphlebotomized mice and the later time points reflect the effects of continuous blood draws.

the Tg group compared with the control group (Figure 3B). Similarly, serum Hamp levels are higher in the Tg group compared with the control group (Figure 3C). To determine whether the loss of EPO signaling in osteocytes affects recovery after hemorrhage, control and Tg mice were subjected to phlebotomy and monitored for 9 days. Throughout this period, there were no significant changes in RBC (Figure 3D), hematocrit (Figure 3E), hemoglobin (Figure 3F), and reticulocytes (Figure 3G). In addition, total serum iron levels in males and females were not significantly different between Tg and control animals in steady state (supplemental Figure 6). To confirm that there are no contaminating erythroid cells in our osteocyte preparations, we used quantitative polymerase chain reaction in the osteocytes of bone tubes and in the BM cells that were magnetically separated into Ter119^{+} and Ter119^{-} populations. Two specific markers of erythroid cells (*Gypa* and *Klf1*) were detectable in the BM but were under detection limit in the bone tubes (supplemental Figure 7). Furthermore, osteocyte-producing ERFE was also supported by immunostaining in mouse and human bone (supplemental Figures 8 and 9).

Overall, these results suggest that during stress erythropoiesis, ERFE produced by osteocytes contributes to the suppression of Hamp in the liver and that Epor in osteocytes might not have a role in the recovery after hemorrhage.

Discussion

In this work, we explored the role of osteocytes in erythropoiesis. We report a novel function of osteocytes in regulating systemic Hamp levels through ERFE secretion during stress erythropoiesis. Kautz et al[21] reported that *Erfe* is expressed in different tissues. However, *Erfe* expression was affected only in the BM and spleen after EPO stimulation. Erythroblasts in the BM are the source of *Erfe* during stress erythropoiesis. In their study, the authors did not analyze *Erfe* expression in the bones.[21] Our work reveals that *Erfe* expression in osteocytes was also stimulated by EPO and phlebotomy. This suggests that osteocytes respond to erythropoietic stimuli similarly to erythroblasts. Interestingly, *Erfe* was also expressed by other bone cells such as osteoblasts and osteoclasts.[10] However, stimulation with exogenous EPO did not upregulate the expression of *Erfe* in these cells. This observation suggests that osteocytes are the only bone cells that could regulate systemic iron levels through ERFE secretion to suppress liver Hamp and help mobilize iron.

To study osteocytes, we first generated OEBTs (osteocytes) from mouse tibia and femur. In this culture model, the osteocytes are in their native bone matrix and retain their extensive canalicular network, which is important to maintain the phenotype and function of osteocytes ex vivo. The role of systemic interferon signaling to decrease iron availability through Hamp stimulation in viral

infections is well known.[3] In our model, differential gene expression analysis after phlebotomy revealed upregulation of interferon signaling, whereas genes related to skeletal homeostasis (in WNT [wingless/integrated]/β-catenin or BMP2 [bone morphogenetic protein 2] pathways) were downregulated. This response, in addition to *Erfe* being the second highest expressed gene in osteocytes after phlebotomy, suggests a novel mechanism when during acute anemia osteocytes choose to prioritize iron availability for erythropoiesis over bone remodeling. This increased expression of *Erfe* seemed similar to that of erythroblasts reported by Kautz et al.[21] To confirm this, we compared the expression of *Erfe* in erythroblasts and nonerythroblasts (including osteocytes) after EPO stimulation. *Erfe* expression was increased 6.5-fold in erythroblasts and 4.8-fold in osteocytes vs controls. Interestingly, nonerythroblasts in the BM (Ter119^{-} cells) also produce ERFE in response to EPO but much less than erythroblasts.

In addition to EPO, hypoxia also stimulates erythropoiesis. Hypoxia stimulates Epor expression and regulates iron metabolism.[28] Kautz et al[21] reported that treatment of BM cells ex vivo using DMOG, a hypoxia-mimetic agent, did not affect *Erfe* expression even though it strongly induced *Vegfα* mRNA expression. Unlike Kautz et al, we found that hypoxia did induce *Erfe* expression in BM and osteocytes.[21] The discrepancy might be due to using a hypoxia-mimetic agent by Kautz et al[21] rather than culturing cells in a hypoxic incubator as we did. In fact, in a recent study, Chen et al[30] compared DMOG application with culturing cells at low oxygen, using a pheochromocytoma cell line (PC12), and found that a reduced oxygen environment is not accurately mimicked by DMOG. Our results also reveal that hypoxia is less potent in inducing *Erfe* expression than EPO. Although EPO regulates *Erfe* expression through the JAK2-STAT5 signaling pathway in erythroblasts,[21] in osteocytes this does not seem to be the case. Regulation of *Erfe* expression in osteocytes and erythroblasts should be evaluated in more detail. Overall, our work reveals that ERFE is secreted not only by erythroblasts but also by other BM cells and that osteocytes participate in suppressing liver Hamp production in response to acute erythropoietic stimuli. One should keep in mind that deleting Epor from osteocytes will undoubtedly affect other EPO-responsive agents that osteocytes make. We thought that serum total iron levels would be different between Tg and control mice in steady state but found no statistically significant differences (supplemental Figure 6). This might be due to other osteocyte-produced hormones that respond to EPO, such as FGF-23, a hormone regulating phosphate and vitamin D, first described ~20 years ago.[31] FGF-23 is a negative regulator of erythropoiesis.[32] Clinkenbeard et al[33] revealed that EPO directly affects FGF-23 production in the cortical bone in vivo in mice.

Our findings also correlate well with other available data on osteocytes and FGF-23. Data from patients with intact kidney function recently pointed at a direct role of the hypoxia-inducible factor/EPO pathways in regulating FGF-23 transcription and translation.[34] Specific genes (*Egln1/Phd2*) involved in oxygen sensing by osteocytes have also been recently described by Noonan et al[35] who also concluded that in addition to sensing oxygen, osteocytes also respond to the changes in concentration of bioavailable iron. Osteocytes are known to be the main source of FGF-23,[36] and their EPOR, that we characterized in this work, is likely to be responsible for the increased production of FGF-23 by osteocytes in hypoxia.

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Authorship

Contribution: V.D.M. designed the study, performed the experiments, performed data analysis, wrote the draft, and prepared the draft figures; A.P. performed the in vitro experiments, helped analyze data, and participated in finalizing the text and figures; L.V.-C. performed the osteocyte-enriched bone tube quantitative polymerase chain reaction experiment required for the revision; I.S. performed irradiation and BM transplants, was responsible for breeding and genotyping mice, and performed all ELISA experiments; L.F.de.C. performed additional analysis, contributed to the discussion, and prepared a supplementary figure; E.M. oversaw the design, analyzed the data, performed more detailed RNA sequencing analysis (with Daniel Martin), prepared the final figures, and wrote the final version of the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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