Hepatic hypoxia-inducible factor-2 down-regulates hepcidin expression in mice through an erythropoietin-mediated increase in erythropoiesis

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ABSTRACT

Background
Iron metabolism, regulated by the iron hormone hepcidin, and oxygen homeostasis, dependent on hypoxia-inducible factors, are strongly interconnected. We previously reported that in mice in which both liver hypoxia-inducible factors-1 and -2 are stabilized (the hepatocyte von Hippel-Lindau knockout mouse model), hepcidin expression was strongly repressed and we hypothesized that hypoxia-inducible factor-2 could be the major regulatory component contributing to the hepcidin down-regulation.

Design and Methods
We generated and analyzed hepatocyte-specific knockout mice harboring either hypoxia-inducible factor-2α deficiency (*Hif2a* knockout) or constitutive hypoxia-inducible factor-2α stabilization (*Vhlh/Hif1a* knockout) and *ex vivo* systems (primary hepatocyte cultures). *Hif2a* knockout mice were fed an iron-deficient diet for 2 months and *Vhlh/Hif1a* knockout mice were treated with neutralizing erythropoietin antibody.

Results
We demonstrated that hypoxia-inducible factor-2 is dispensable in hepcidin gene regulation in the context of an adaptive response to iron-deficiency anemia. However, its overexpression in the double *Vhlh/Hif1a* hepatocyte-specific knockout mice indirectly down-regulates hepcidin expression through increased erythropoiesis and erythropoietin production. Experiments in primary hepatocytes confirmed the non-autonomous role of hypoxia-inducible factor-2 in hepcidin regulation.

Conclusions
While our results indicate that hypoxia-inducible factor-2 is not directly involved in hepcidin repression, they highlight the contribution of hepatic hypoxia-inducible factor-2 to the repression of hepcidin through erythropoietin-mediated increased erythropoiesis, a result of potential clinical interest.

Key words: hepatic HIF-2, hepcidin expression, erythropoiesis.

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The online version of this article has a Supplementary Appendix.

Introduction

An adequate supply of oxygen in tissues is necessary for survival and normal organ function. The number of circulating red blood cells is the major determinant of tissue oxygenation. As a consequence, lack of oxygen (hypoxia) triggers induction of erythropoietin to increase the production of new red blood cells. The liver is the primary source of erythropoietin during embryogenesis, however in adults, the site of erythropoietin production switches from the fetal liver to the kidney.[1]

Hypoxia-inducible factor-1 (HIF-1) was initially identified in 1992 as a transcriptional factor able to regulate erythropoietin production.[2] A number of laboratories have since demonstrated that HIF-1 is implicated in most aspects of hypoxia-induced gene expression, and operates not only in kidneys but also in a wide range of organs and cell types. HIF is a heterodimeric transcription factor stabilized by low oxygen concentrations. HIF consists of two helix-loop-helix proteins: an α-regulatory subunit, which is the oxygen and iron-responsive component, and the β-subunit, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT), which is constitutively expressed. Three regulatory HIF subunits have been cloned and named HIF-1α, HIF-2α and HIF-3α. In the presence of oxygen, the regulatory subunit is hydroxylated on two proline residues by the oxygen and iron-dependent prolyl-hydroxylases (PHD); the α subunit is then targeted for degradation to the proteasome by interacting with the von Hippel-Lindau (VHL) E3 ubiquitin ligase tumor suppressor protein. Under hypoxia, the activity of PHD is inhibited, therefore permitting HIF-α to escape degradation and translocate into the nucleus, where it binds to HIF-β/ARNT. The HIF heterodimer binds to hypoxic response elements (HRE) of target gene regulatory sequences, and recruits transcriptional cofactors such as core binding protein (CBP) and p300 to induce the expression of genes involved in the control of metabolism and angiogenesis, as well as apoptosis, cellular stress and other critical processes. More than a decade after the discovery of HIF which led in turn to the discovery of VHL and various PHD, mutations in genes encoding all three of these essential components of the oxygen-sensing axis have been identified in humans with familial erythrocytosis.[3]

To perform their duty as oxygen carriers, erythrocytes require iron and failure to incorporate adequate iron into heme results in impaired erythrocyte maturation, leading to microcytic, hypochromic anemia. The most important systemic factor that influences iron availability is hepcidin, a circulating peptide that maintains iron homeostasis. Hepcidin is an hyposideremic hormone made predominantly by hepatocytes; it acts to down-regulate iron absorption by the duodenal enterocytes and iron release by the macrophages.[4]

Hepcidin expression is increased by iron loading, thus avoiding an excess of free toxic iron in the body, and decreased in response to hypoxia, iron deficiency and increased erythropoiesis allowing iron supply to match the erythropoietic demand.[4]

Recently, the bone morphogenetic protein 6 (BMP6)/hemojuvelin (HJV) signaling cascade has emerged as the principal pathway in the regulation of hepcidin gene expression.[4] BMP6 signals through a BMP-receptor complex that requires HJV as a co-receptor. In addition, type II transmembrane serine proteinase (TMPRSS6) encoding liver matriptase 2 has recently been identified as a repressor of hepcidin gene expression[5] able to antagonize hepcidin induction by BMP6 by cleaving HJV from the cell membrane.[6] Finally, HJV was shown to be cleaved by the furin proconvertase, which releases a soluble form of HJV that suppresses BMP signaling and hepcidin expression by acting as a decoy that competes with membrane HJV for BMP ligands.[7]

We previously reported that HIF control iron homeostasis by repressing hepcidin synthesis in the liver[8] and the VHLR200W mutation has been shown to be associated with down-regulation of hepcidin expression in patients with Chuvash polycythemia.[9] A marked down-regulation of hepcidin was observed in conditional knockout of *Vhlh* in the liver, in which both HIF-1 and HIF-2 were stabilized.[8] However, deletion of *Hif1a* alone in the liver of adult mice accounted for only a small fraction of hepcidin repression in response to an iron-deficient diet, thus possibly suggesting that HIF-2 may be a putative candidate contributing to the observed hepcidin down-regulation.

To resolve this issue and to understand the molecular mechanisms, direct or indirect, of HIF in the regulation of hepcidin gene expression better, we developed and analyzed hepcidin expression by a combination of *in vivo* (hepatocyte-specific knockout mice harboring either HIF-2α deficiency or constitutive HIF-2α stabilization) and *ex vivo* systems (primary hepatocyte cultures). In this study, we demonstrated that HIF-2 is not involved in the repression of hepcidin in the setting of iron deficiency. However, we showed that its overexpression in the double *Vhlh/Hif1a* hepatocyte-specific knockout mice, indirectly down-regulates hepcidin expression through increased erythropoiesis and erythropoietin production, and not through transcriptional activation of TMPRSS6, the negative regulator of the BMP/HJV pathway, as recently suggested by an *in vitro* study.[10]

Design and Methods

Animals

All mice used in the experiments were cared for according to criteria outlined by the European Convention for the Protection of Laboratory Animals. Animal studies described here were reviewed and approved (Agreement n. P2.CP.151.10.) by the *Président du Comité d'Ethique pour l'Expérimentation Animale Paris Descartes*. Mice with hepatocyte-specific inactivation of *Hif2a* (referred to as *Hif2a* KO) were generated by cross-breeding Albumin-*Cre* transgenic mice with *Hif2a*$^{lox/lox}$ mice (provided by Celeste Simon, University of Pennsylvania, USA) and compared to wild-type littermates (*Hif2a* WT). Mice with hepatocyte-specific inactivation of both *Hif1a and Vhlh* (*Vhlh*$^{lox/lox}$/*Hif1a*$^{lox/lox}$/Albumin*Cre*, referred to as *Vhlh/Hif1a* KO) were generated by breeding *Vhlh*$^{lox/lox}$/*Hif1a*$^{lox/lox}$ and *Vhlh*$^{lox/lox}$/*Hif1a*$^{lox/lox}$ Albumin*Cre* mice. Three- to 4-week old *Vhlh/ Hif1a* KO males and females were used and compared to littermates of all other genotypes. All mouse strains were reared on a C57BL/6 background. When indicated, 4-week old male mice were fed an iron-deficient diet for 2 months (3 ppm iron; Scientific Animal Food & Engineering).

Treatment with anti-erythropoietin blocking serum

Three-week old mice were injected with anti-erythropoietin rabbit serum or 0.5M NaCl (placebo). Injections were performed for 5 consecutive days and mice were sacrificed 18 h after the last

injection. Since the neutralizing capacity of the anti-erythropoietin serum is 50 ng of recombinant erythropoietin (5 Epo units) for 100 μL of serum antibody and the estimated amount of erythropoietin in a normal 3-week old mice is 0.25 ng, we injected 300 μL of a 1:60 NaCl dilution of the anti-erythropoietin serum/day, i.e. a dose able to neutralize a 10-fold excess of circulating erythropoietin. Assessing that circulating erythropoietin levels were at least 60-fold increased in the *Vhlh/Hif1a* KO mice, these mice received 300 μL of the original anti-erythropoietin serum/day.

Reticulocytes and red blood cell counts

Hematologic parameters were measured using a Coulter MAXM automatic analyzer (Beckman Coulter) as previously described.[11] Reticulocytes counts were determined according to Lee *et al.*[12] Briefly, 1 μL of total blood was incubated with 1 mL of thiazole orange dye solution (100 ng/mL) in phosphate-buffered saline for 15 min at room temperature prior to analysis. An unstained blood sample was used as a negative control. All samples were analyzed on a FACSCanto II (BD Biosciences).

Erythropoietin enzyme-linked immunosorbent assay

Erythropoietin protein levels in plasma were determined using a Quantikine mouse erythropoietin enzyme-linked immunosorbent assay kit (R&D Systems).

Isolation and culture of primary hepatocytes

Hepatocytes were isolated from 2- to 4-month old *Hif2a* KO and *Hif2a* WT mice of matched sex. Hepatocytes were seeded in 6-well plates at a density 300,000 cells/well and cultured in standard conditions (5% CO_2, 37°C) in M199 medium containing 2% Ultroser G, for 4 h (adapted from[13]). After cell attachment, the medium was replaced by fresh M199 medium supplemented with 10% calf serum (Invitrogen).

Generation of the hypoxia-inducible factor-2α adenovirus construct

Human HIF-2α adenovirus constructs used to transfect primary hepatocytes were generated by subcloning the 2.6 kb human EPAS1 ORF into the pAd-Track-CMV vector, followed by recombination with the pAdEasy-1 vector and transfection into the HEK293-AV packaging cell line as outlined by He *et al.*[14]

Reverse transcription and real-time quantitative polymerase chain reaction

Total RNA was extracted from whole liver or primary hepatocytes and homogenized in 1 mL of TRIZOL reagent (Invitrogen). Reverse transcription was done with 2-3 μg of total RNA. Quantitative polymerase chain reaction was performed with 2 μL of a 1:10 dilution of reverse-transcribed total RNA and 10 μM of each primer diluted in 1× LightCycler DNA Master SYBR Green I mix using a LightCycler apparatus (Roche Applied Science). All samples were normalized to the threshold cycle value for 18S or cyclophilin-A. The following primer sequences were used : *Hepcidin1* forward 5'-CCTATCTCCATCAACAGAT-3'; *Hepcidin1* reverse 5'-TGCAACAGATACCACACTG-3'; *EPO* forward 5'-CACAACCCATCGTGACATTTTC-3'; *EPO* reverse 5'-CATCTGCGACAGTCGAGTTCTG-3'; *Furin* forward 5'-CAGCCTCGGTACACACAGAT-3'; *Furin* reverse 5'-AGCTACACCTACGCCACAGA-3'; *Tmprss6* forward 5'-CCTGGTGAGTTCCTCTGCTC-3'; *Tmprss6* reverse 5'-CTTGGCACTGTTCTTCGTCA-3'; *Hif1a* forward 5'-TGAGCTTGCTCATCAGTTGC-3'; *Hif1a* reverse 5'-CCATCTGTGCCTTCATCTCA-3'; *Hif2a* forward 5'-TGAGTTGGCTCATGAGTTGC-3'; *Hif2a* reverse 5'-TTGCTGATGTTTTCCGACAG-3'; *BMP6* forward 5'-GTTCCGCGTCTACAAGGACT-3'; *BMP6* reverse 5'-CAGCCAACCTTCTTCTGAGG-3'; *Hjv* forward 5'-TCTGACCTGAGTGAGACTGC-3'; *Hjv* reverse 5'-GATGATGAGCCTCCTACCTA-3'.

Statistical analysis

All values in the figures are the mean ± SEM. Statistical analysis was performed using GraphPad Prism 4.0 and an unpaired (2-tailed) Student's t-test or one-way ANOVA followed by a Bonferroni's post-test when more than two groups were compared respectively. Statistical significance is indicated by * symbols (*P< 0.05, ** P<0.01, ***P< 0.001).

Results

Hypoxia-inducible factor-2α is not involved in the regulation of hepcidin gene expression in the setting of iron-deficiency anemia

To investigate the role of HIF-2α in hepcidin regulation, we have generated mice deficient for *Hif2a* specifically in hepatocytes by breeding *Hif2a*$^{lox/lox}$ mice with a transgenic strain expressing Cre recombinase under the control of the murine albumin promoter. The deletion efficiency of *Hif2a* in the liver was approximately 66% as determined by quantitative polymerase chain reaction on genomic DNA from the liver. However, HIF-2α mRNA levels were decreased by 95% in primary hepatocytes derived from *Hif2a* KO *versus* *Hif2a* WT littermates (*Online Supplementary Figure S1*). Hepcidin mRNA levels were similar in the livers of *Hif2a* KO and WT littermates on a standard diet. After 8 weeks of an iron-deficient diet, livers from WT and *Hif2a* KO mice showed similar repression of hepcidin mRNA (Figure 1A). BMP6 mRNA levels were decreased in both WT and *Hif2a* KO mice under iron deficiency, compared to controls (*Online Supplementary Figure S2*). Interestingly, plasma erythropoietin was also found to be similarly up-regulated in both *Hif2a* KO and WT mice (Figure 1A). While erythropoietin levels were undetectable in the liver (*data not shown*), renal erythropoietin was strongly induced in both WT and *Hif2a* KO mice under iron deficiency indicating that the increase of systemic erythropoietin in iron deficiency is not dependent on hepatic HIF-2α (Figure 1A). Hematologic parameters, such as red blood cell content, hematocrit and hemoglobin levels were also similarly decreased in the *Hif2a* KO and WT littermates fed on the iron-deficient diet compared to mice on a standard diet (Figure 1B). No significant differences in plasma or liver iron were found between WT and *Hif2a* KO mice (Figure 1C). Our findings suggest that, while HIF-1α is a weak repressor of hepcidin,[8] HIF-2 is not involved in hepcidin gene regulation in the context of an adaptive response to iron-deficiency anemia.

Constitutive hypoxia-inducible factor-2 activation in hepatocyte-specific Vhlh/Hif1a null mice represses hepcidin

To examine the effect of HIF-2 stabilization on hepcidin expression, we generated, on a pure C57BL/6 background, the *Vhlh/Hif1a* KO murine model, which lacks both VHL and HIF-1α in hepatocytes. These mice, similarly to the *Vhlh* KO mice - which constitutively express both HIF-1α and HIF-2α[8,15] - died between 3 and 5 weeks of age, presented with alopecia, weight loss, severe hepatomegaly and splenomegaly as well as iron deficiency (Figure 2A). We found that, as compared to control littermates, hepatic hepcidin mRNA levels were repressed in the *Vhlh/Hif1a* KO

(Figure 2B), as strongly as in the *Vhlh* KO model previously described[8] (*Online Supplementary Figure S3*). Whether hepcidin is regulated directly or through alternative pathways by HIF has been the subject of intense research *in vitro*.[10,16-18] Our previous *in vitro* studies suggested a direct regulation of hepcidin by HIF.[8] However, our *in vivo* data could not exclude indirect alternative pathways. Furin gene expression was demonstrated to be increased by hypoxia via HIF-1.[7,19] It was, therefore, proposed that this up-regulation of furin could lead to the increase of soluble HJV production and, in turn, hepcidin gene repression. However, we found that in the liver of *Vhlh/Hif1a* KO mice, furin mRNA levels were decreased rather than increased compared with levels in control littermates (Figure 2B). More recently, it was shown, *in vitro*, that *TMPRSS6* expression was up-regulated by both HIF-1α and HIF-2α in hepatoma cell lines, leading to an increase in membrane HJV shedding and a decrease in hepcidin promoter responsiveness.[10] *In vivo*, we found that TMPRSS6 mRNA levels were decreased in *Vhlh/Hif1a* KO mice (Figure 2B) as compared to WT littermates suggesting that hepcidin repression by HIF-2 is not a consequence of an increase in TMPRSS6 signaling.

The double *Vhlh/Hif1a* KO mutant mice showed a strong increase in erythropoiesis and up-regulation of erythropoietin mRNA in the liver and in the serum as compared to the amount in WT mice (Figure 2C), correlated with high levels of hemoglobin and reticulocyte counts (Figure 2D). Interestingly, the increase of erythropoietin mRNA in the kidney seen in the 3-week old control mice was blunted in the double *Vhlh/Hif1a* KO mutant mice as if the forced dramatic increase in liver erythropoietin levels had altered the regulation of erythropoietin mRNA in the kidney (Figure 2C). Collectively, these results confirmed that HIF-2 but not HIF-1 is the main regulator of hepatic erythropoietin production[20] and hepcidin repression in the *Vhlh*-deficient background.

Hepcidin repression by hypoxia-inducible factor-2 is dependent on erythropoietin-mediated increased erythropoiesis

Erythropoietin and increased erythropoiesis have been reported to decrease hepcidin levels in mice[21-23] and in humans.[24] Erythropoietin has been shown to be a HIF-2 target gene in both liver[20] and kidney.[25] We, therefore, exam-

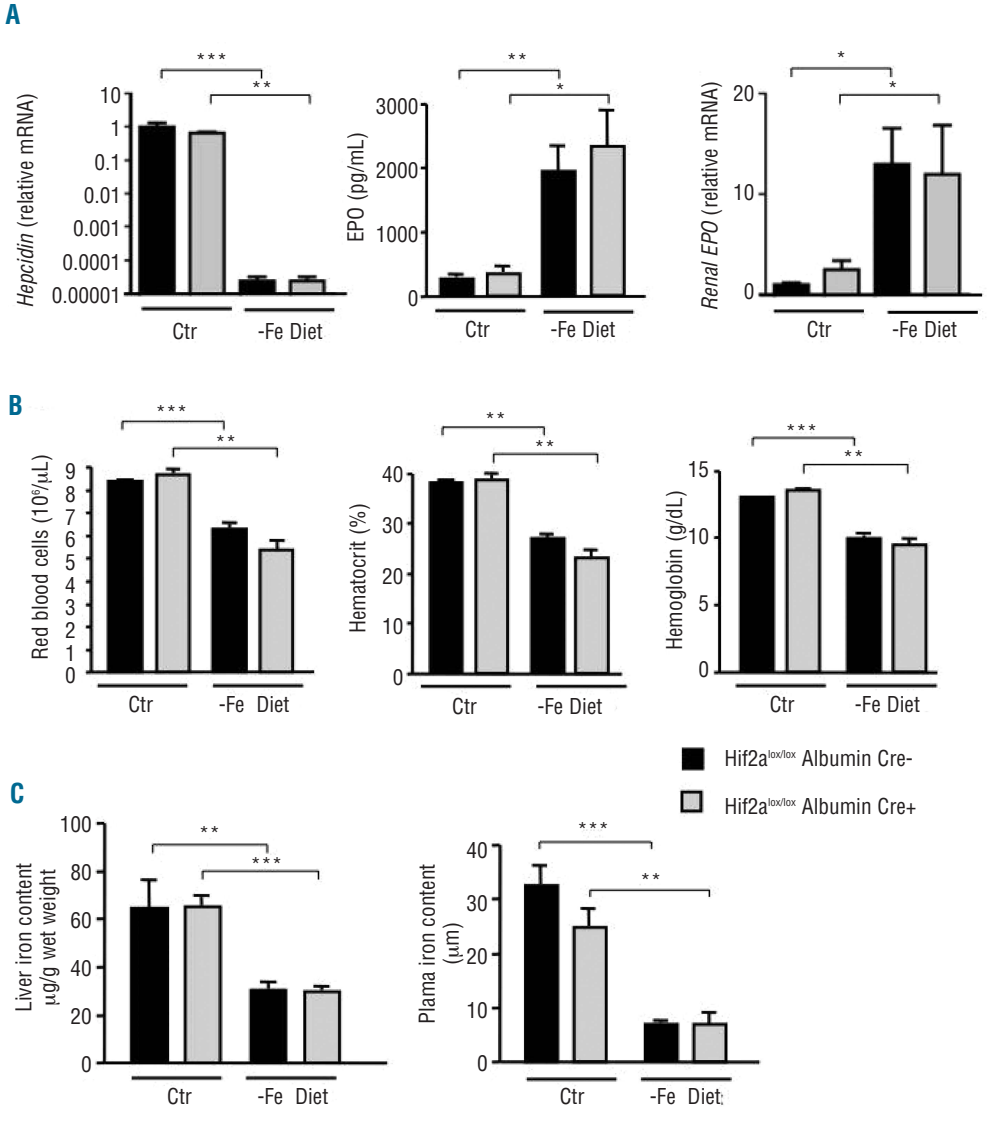

Figure 1. Mice with HIF2-α-deficient liver respond normally to diet-induced iron deficiency. *Hif2a*$^{lox/lox}$Albumin Cre- and *Hif2a*$^{lox/lox}$Albumin Cre+ littermates were fed an iron deficient (-Fe diet) or control diet (Ctr) for 2 months after weaning. (A) Hepatic hepcidin and renal erythropoietin (EPO) relative mRNA expression normalized to cyclophilin-A mRNA. Results are expressed as a fold change compared to the *Hif2a*$^{lox/lox}$Albumin Cre- mice on a control diet. EPO in plasma of WT and *Hif2a*$^{lox/lox}$Albumin Cre+ mice fed an iron deficient or control diet. (B) Hematologic parameters. (C) Liver and plasma iron contents. n ≥ 4 for each group.

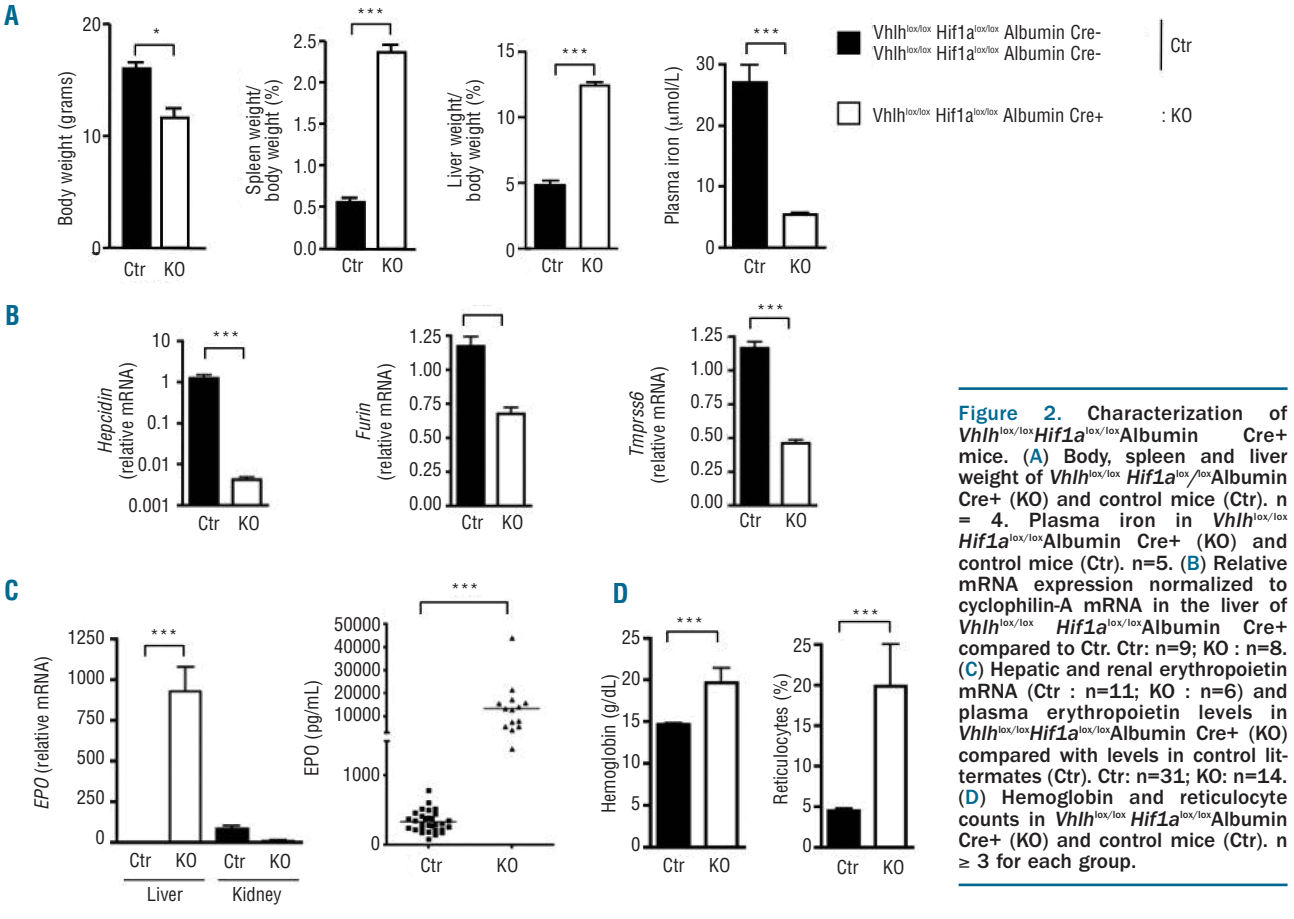

Figure 2. Characterization of *Vhlh*$^{lox/lox}$*Hif1a*$^{lox/lox}$Albumin Cre+ mice. (A) Body, spleen and liver weight of *Vhlh*$^{lox/lox}$ *Hif1a*$^{lox/lox}$Albumin Cre+ (KO) and control mice (Ctr). n = 4. Plasma iron in *Vhlh*$^{lox/lox}$ *Hif1a*$^{lox/lox}$Albumin Cre+ (KO) and control mice (Ctr). n=5. (B) Relative mRNA expression normalized to cyclophilin-A mRNA in the liver of *Vhlh*$^{lox/lox}$ *Hif1a*$^{lox/lox}$Albumin Cre+ compared to Ctr. Ctr: n=9; KO : n=8. (C) Hepatic and renal erythropoietin mRNA (Ctr : n=11; KO : n=6) and plasma erythropoietin levels in *Vhlh*$^{lox/lox}$*Hif1a*$^{lox/lox}$Albumin Cre+ (KO) compared with levels in control littermates (Ctr). Ctr: n=31; KO: n=14. (D) Hemoglobin and reticulocyte counts in *Vhlh*$^{lox/lox}$ *Hif1a*$^{lox/lox}$Albumin Cre+ (KO) and control mice (Ctr). n ≥ 3 for each group.

ined whether increased erythropoiesis as a consequence of HIF-2 stabilization in the liver was the underlying cause of the hepcidin reduction. To this aim, inbred *Vhlh/Hif1a* KO mice and control littermates were injected with neutralizing erythropoietin antiserum for 5 consecutive days.

Upon anti-erythropoietin injection, erythropoiesis was dramatically reduced in both control and *Vhlh/Hif1a* KO mice, as measured by reticulocyte counts (Figure 3A). As expected, the blood parameters (red blood cells, hemoglobin, hematocrit) were consecutively decreased in both control and *Vhlh/Hif1a* KO mice after the neutralizing erythropoietin antibody injection (Figure 3A).

The decrease of erythropoiesis increased hepcidin mRNA levels in control mice as previously reported[23] (Figure 3B). Interestingly, hepcidin levels in *Vhlh/Hif1a* KO mice injected with neutralizing erythropoietin serum returned to values similar to those of control mice injected with NaCl (Figure 3B). It is worth noting that in conditions of abolished erythropoiesis, hepcidin mRNA values in *Vhlh/Hif1a* KO mice were still slightly lower than those in control mice.

Altogether, these results suggest that stabilized HIF-2α in the liver does not repress hepcidin directly but through an erythropoietic drive due to erythropoietin overexpression.

Cell-autonomous regulation of hepcidin expression is independent of hypoxia-inducible factor-2

To further ensure the absence of a direct effect of HIF-2 on hepcidin gene expression, we sought to analyze hepcidin gene expression in primary hepatocytes, independently of the systemic effect of erythropoiesis. We first validated our model of primary hepatocytes by showing conditions (BMP2 treatment or infection by an adenovirus expressing TMPRSS6) in which hepcidin can be either up- or down-regulated (*Online Supplementary Figure S4*). For the purpose of our study, we generated HIF-2α–overexpressing adenovirus (AV-HIF-2). Primary hepatocytes were infected with either a GFP-overexpressing adenovirus as a control or AV-HIF-2 at a multiplicity of infection (MOI) of 10. While AV-HIF-2 promotes a 160-fold induction of erythropoietin levels compared to AV-GFP infection, hepcidin levels were not affected by HIF-2 overexpression (Figure 4). Using our model of primary hepatocytes, we also confirmed the absence of regulation of TMPRSS6 and furin by HIF-2. Collectively, these data suggest that *in vitro* stabilization of HIF-2α alone in primary mouse hepatocytes is not sufficient to repress hepcidin expression.

Discussion

The current study addresses the role of hepatic HIF-2 in the repression of hepcidin expression by the use of complementary *ex vivo* and *in vivo* experimental models. Models of dietary iron deficiency and of constitutive HIF-2 activation (in hepatocyte-specific VHL HIF-1α KO mice and in primary hepatocytes) were used.

Our previous work, using the iron chelator deferoxamine as a HIF stabilizer, suggested a direct repression of hepcidin by HIF-1 *in vitro*.[8] However, other studies in HepG2 cells showed that overexpression or knockdown of HIF-1 did not affect hepcidin expression[18] and have suggested indirect pathways including 2-oxoglutarate-[16] or ROS-dependent[17]

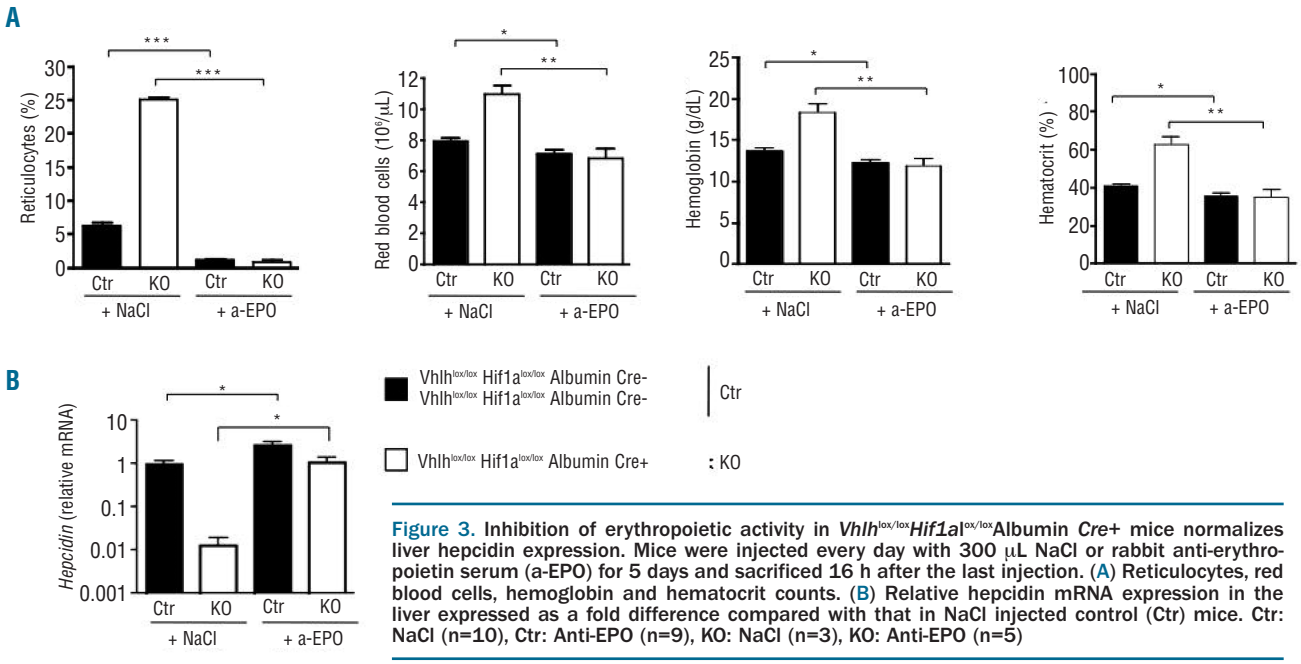

Figure 3. Inhibition of erythropoietic activity in *Vhlh*$^{lox/lox}$*Hif1a*$^{lox/lox}$Albumin *Cre+* mice normalizes liver hepcidin expression. Mice were injected every day with 300 μL NaCl or rabbit anti-erythropoietin serum (a-EPO) for 5 days and sacrificed 16 h after the last injection. (A) Reticulocytes, red blood cells, hemoglobin and hematocrit counts. (B) Relative hepcidin mRNA expression in the liver expressed as a fold difference compared with that in NaCl injected control (Ctr) mice. Ctr: NaCl (n=10), Ctr: Anti-EPO (n=9), KO: NaCl (n=3), KO: Anti-EPO (n=5)

pathways. Moreover, HIF-2 but not HIF-1 seems to play a major role in iron metabolism and erythropoiesis.[26] HIF-2 regulates erythropoietin and key iron-related genes in the liver[25] and we recently showed that HIF-2 but not HIF-1 regulates iron absorption in enterocytes.[11]

In a model of constitutive HIF-2 activation in hepatocytes (*Vhlh/Hif1a* KO), we found hepcidin to be strongly repressed. We aimed to determine by which pathway HIF-2 regulates hepcidin *in vivo* in this model. HIF-2 may down-regulate hepcidin expression by affecting the BMP/HJV pathway. BMP-6, recently shown to be an important hepcidin regulator, is increased in models with hepatic iron overload, and decreased in response to iron deficiency.[27-29] However, BMP6 mRNA levels were found to be increased, rather than decreased, in the *Vhlh/Hif1a* KO model and cannot account for the decrease in hepcidin (*Online Supplementary Figure S3*). HJV mRNA levels were decreased in livers from *Vhlh/Hif1a* KO mice as compared to in WT littermates (*Online Supplementary Figure S3*). Furin, which cleaves HJV and produces soluble HJV, has been identified as a HIF-1 target gene and could also have affected hepcidin expression by decreasing the BMP/HJV pathway.[7,19] However, furin mRNA levels were not increased in the liver of mice overexpressing HIF-2 (Figure 2) or in *Vhlh* KO mice overexpressing both HIF isoforms (*data not shown*). Lakhal and colleagues recently demonstrated *in vitro* that TMPRSS6 is a new HIF-1 and HIF-2 target gene. In hepatoma cells, both HIF stabilization by hypoxia and chemical inducers induce an increase in TMPRSS6 mRNA, therefore decreasing transcriptional activity of the hepcidin promoter by impairing BMP/HJV signaling.[10] However, we found a decrease rather than an increase in TMPRSS6 mRNA levels in our HIF-2 overexpression model *in vivo*. The decrease in HJV, furin and TMPRSS6 mRNA levels observed in the liver of *Vhlh/Hif1a* KO mice may be due to an *in vivo* compensatory mechanism as the levels of these genes were unchanged in primary hepatocytes upon AV-HIF-2 infection. The expression of these genes was not changed in either the *Hif2a* KO mice or WT littermates fed on the iron-deficient diet compared to the expression in mice on a standard diet (*Online Supplementary Figure S2*). Accordingly, Krijt *et al.* recently reported that tissue hypoxia, resulting from repeated phlebotomies, does not transcriptionally regulate furin or TMPRSS6.[30]

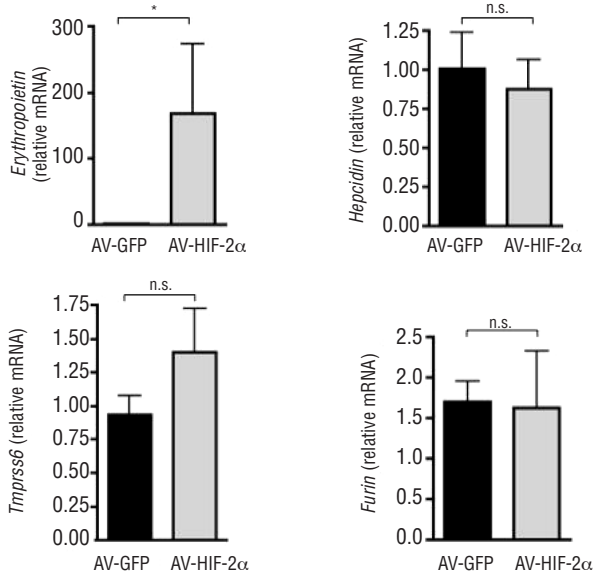

Figure 4. HIF-2α overexpression does not repress hepcidin expression in primary hepatocytes. Infection of wild-type primary hepatocytes with a control GFP-adenovirus (AV-GFP) (black bar) or with a HIF2-α-overexpressing adenovirus (AV-HIF-2α) (gray bar) at a multiplicity of infection (MOI) of 10. Relative mRNA expression normalized to cyclophilin-A. Representation of three independent experiments performed in triplicate.

We next addressed the putative role of erythropoietin in

hepcidin repression in our model since erythropoietin administration is known to result in a strong down-regulation of hepcidin expression.[21] Irradiation of the bone marrow or inhibition of erythropoiesis by chemical inhibitors in mice abolished the effect of erythropoietin treatment or phlebotomy on hepcidin expression,[22,23] suggesting that the action of erythropoietin in repressing hepcidin was not direct but relied on the erythropoietic activity. However, Pinto *et al.* suggested a direct effect of erythropoietin on hepcidin expression through erythropoietin-receptor-mediated regulation of the transcription factor C/EBPα.[31] Nevertheless, we did not detect erythropoietin-receptor expression in liver cells using highly sensitive detection methods.[32] Since erythropoietin is preferentially regulated by HIF-2 but not by HIF-1 in the liver,[20] we examined whether HIF-2 contributes to hepcidin repression through the transcription of its target gene erythropoietin and the subsequent increase of erythropoiesis. Our *in vivo* data clearly demonstrated that hepcidin repression by HIF-2 was due to the increase in erythropoiesis. However, while the neutralizing erythropoietin antibody completely reversed the increased erythropoiesis in *Vhlh/Hif1a* KO mice, hepcidin levels remained slightly lower in the knockout mice than in the control mice. This difference may be due to a dose or timing effect of the neutralizing erythropoietin serum.

Regulation of hepcidin by erythropoietic activity is of particular importance in iron loading anemias such as β-thalessemias. Patients with thalassemia intermedia have very low hepcidin levels, despite high serum iron, and develop fatal tissue iron overload.[33] However, the underlying mechanism by which erythropoiesis decreases hepcidin levels has not yet been demonstrated. One hypothesis that has been put forward is that a plasma circulating erythroid factor could be responsible for this regulation. GDF15 and TWSG1 have been proposed as potential candidates.[34,35] However, administration of erythropoietin to healthy volunteers has been shown to suppress circulating hepcidin, independently of GDF15.[24]

The development of the *Hif2a* KO mice has allowed us to demonstrate that HIF-2 is not involved in hepcidin repression triggered by iron-deficiency anemia. Several HIF-2-independent mechanisms could contribute to this process. As previously mentioned, TMPRSS6 is required in the liver to sense the iron deficiency and decrease hepcidin expression.[36] TMPRSS6 could, therefore, contribute to the hepcidin down-regulation observed in our models of iron deprivation. A decrease in BMP signaling could also be considered, as previously suggested.[27]

Interestingly, it was recently proposed that hypoxia inhibits hepcidin expression in hepatoma cells via a marked decrease in SMAD4 mRNA expression,[37] indicating that the involvement of the BMP/SMAD signaling in hypoxia deserves further investigations. *In vivo*, hypoxia has been shown to decrease hepcidin expression in both acute and chronic experimental models[21,38,39] as well as in humans.[40] Hypoxia is also known to induce the expression of renal erythropoietin, thereby promoting increased erythropoiesis to compensate for the decrease in ambient oxygen. The delayed hepcidin decrease in response to hypoxia *in vivo* could, therefore, be explained by the time needed to mount an increase in erythropoietic activity. In support of this, hepcidin was less repressed in experimental models of short-term hypoxia *in vivo* than in response to erythropoietin injection or experimental models of increased erythropoiesis induced by phlebotomy.[38]

It has recently been reported that hepatocyte-derived HIF-2 can substitute as the main regulator of systemic erythropoietin homeostasis when erythropoietin production in the kidney is impaired.[25] Interestingly, we showed, in the *Vhlh/Hif1a* KO mouse model, that liver erythropoietin contributes to plasma erythropoietin. The lack of renal erythropoietin production could arise through several mechanisms and awaits further investigations.

Importantly, our study showing the ability of hepatic HIF-2 to repress hepcidin through increased erythropoiesis in order to increase iron absorption may be clinically interesting in the treatment of patients with chronic kidney disease, resulting in renal anemia.

Authorship and Disclosures

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