Brief report

Deletion of HIF-2α in the enterocytes decreases the severity of tissue iron loading in hepcidin knockout mice

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Hereditary hemochromatosis (HH) is a highly prevalent genetic disorder characterized by excessive parenchymal iron accumulation leading to liver cirrhosis, diabetes, and in some cases hepatocellular carcinoma. HH is caused by mutations in the genes encoding upstream regulators of hepcidin or more rarely in the hepcidin gene itself. A deficit in hepcidin results in intestinal iron hyperabsorption; however, the local effectors mediating the up-regulation of iron absorption genes are unknown. We hypothesized that HIF-2 could mediate high iron absorption rates in HH. We generated *Hepc*$^{-/-}$ mice (a murine model of hemochromatosis) lacking HIF-2 in the intestine and showed that duodenal HIF-2 was essential for the up-regulation of genes involved in intestinal iron import and the consequent iron accumulation in the liver and pancreas. This study highlights a role of HIF-2 in the dysregulation of iron absorption and chronic iron accumulation, as observed in patients with hemochromatosis. (*Blood*. 2012;119(2): 587-590)

Introduction

Hereditary hemochromatosis (HH) is a heterogeneous genetic disease characterized by excessive iron accumulation in the liver and parenchyma. Clinical manifestations include liver cirrhosis, diabetes, cardiomyopathy, arthropathy, hypermelanotic skin pigmentation, and hepatocellular carcinoma. HH is typically caused by mutations in genes encoding either upstream signaling molecules involved in the induction of hepcidin expression (HFE, transferrin receptor 2, and hemojuvelin) or more rarely in the hepcidin gene itself.[1,2] Iron absorption in the duodenum is the only way to control iron entry in the body and is finely regulated in response to systemic iron requirements.[3] At the apical brush border of duodenal enterocytes, duodenal cytochrome b (DCYTB) facilitates non-heme iron uptake by divalent metal transporter 1 (DMT1), whereas ferroportin (FPN) exports iron across the basolateral membrane.[4]

Hepcidin is the central regulatory molecule of systemic iron homeostasis[5] and regulates cellular iron efflux by binding to FPN and inducing its internalization and subsequent degradation in the lysosome.[6] Although hepcidin is known to act at a systemic level to regulate the rate of iron absorption by controlling the amount of iron exported across the basolateral membrane by FPN, the local effectors mediating the up-regulation of apical iron absorption genes in hemochromatosis are unknown. We and others have previously demonstrated that the hypoxia-inducible factor-2α (HIF-2α) transcription factor, and not HIF-1α, regulates DMT1, DCYTB, and FPN expression in the duodenum at basal level, iron deficiency, and in conditions of increased erythropoiesis.[7-9] HIF-1 and HIF-2 are heterodimeric transcriptional factors and central mediators of cellular and systemic adaptation to hypoxia. In the presence of oxygen, the HIF-α subunit is hydroxylated by oxygen- and iron-dependent prolyl hydroxylases and targeted to the proteasome after the binding to the von Hippel-Lindau protein. On hypoxia (or iron deficiency), HIF-α is stabilized and binds to the HIF-β constitutive subunit to induce the transcription of target genes.[10]

We hypothesized that HIF-2 could be a mediator of high iron absorption rates in HH and addressed this question by breeding the hepcidin knockout mice (*Hepc*$^{-/-}$), a model of severe iron overload, with mice lacking HIF-2 in the intestinal epithelium.

Methods

Animals

Animal studies described here were reviewed and approved (Agreement P2.CP.151.10.) by the Président du Comité d'Ethique pour l'Expérimentation Animale Paris Descartes. We intercrossed mice homozygous for germline knockout of hepcidin[11] and mice with loss of HIF-2α specifically in the intestinal epithelium Hif-2α$^{lox/lox}$Villin-*Cre*$^{+}$[7], both in a C57BL/6J genetic background, to produce the *Hepc*$^{-/-}$/*Hif*-2α$^{lox/lox}$/Villin-Cre^{+} mouse strain (referred as *Hepc*$^{-/-}$*Hif*-2α$^{\Delta int}$). Finally, we interbred Hepc$^{-/-}$HIF-2α$^{\Delta int}$ and *Hepc*$^{-/-}$/*Hif*-2α$^{lox/lox}$/VillinCre^{-} mice (here referred as Hepc$^{-/-}$). Male mice were analyzed at the age of 5 months and compared with control genotypes, including *Hepc*$^{+/+}$ *Hif*-2α$^{lox/lox}$/VillinCre^{-} and *Hepc*$^{+/-}$ *Hif*-2α$^{lox/lox}$/VillinCre^{-} (referred as controls [CTR]).

Reverse transcription and real-time quantitative PCR

RNA extraction, reverse transcription, quantitative PCR, and sequences of the primers used have been previously described.[7] All samples were normalized to the threshold cycle value for cyclophilin.

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Western blot

Frozen whole duodenal tissue was homogenized using a pestle and smash, and extraction of membrane proteins was performed as previously described.[7] The following antibodies were used: DMT1 antibody recognizing both DMT1-IRE and non-IRE isoforms[12] (kind gift of François Cannone-Hergaux), DCYTB antibody (Alpha Diagnostic DCYTB11-A), and FPN antibody (Alpha Diagnostic, MTP11-A).

Iron measurements and immunostaining

Plasma and tissue iron were quantified colorimetrically by a previously described method.[7] For histology, tissues were fixed in 4% formaldehyde and embedded in paraffin and stained with Perls Prussian blue and nuclear fast red counterstain.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 4.0, and the significance of experimental differences was evaluated by 1-way ANOVA followed by a Bonferroni posttest. Values in the figures are expressed as mean ± SEM.

Results and discussion

To test whether HIF-2 can mediate the up-regulation of iron absorption genes in HH, we generated *Hepc*$^{-/-}$ mice deleted for HIF-2α in the duodenum (*Hepc*$^{-/-}$*Hif-2α*$^{\Delta int}$ mice). These mice do not exhibit any overt phenotypic abnormalities. We previously reported that DMT1, DCYTB, and FPN protein levels were increased in the duodenum of hepcidin-deficient mice (the Usf2$^{-/-}$ mouse model[13]). We confirmed this result (Figure 1B) and further demonstrated that *Hepc*$^{-/-}$ mice presented high levels of DMT1, DCYTB, and FPN mRNA (although to a lesser extent) compared with control mice (Figure 1A), suggesting that a transcriptional control of these genes takes place in the duodenum

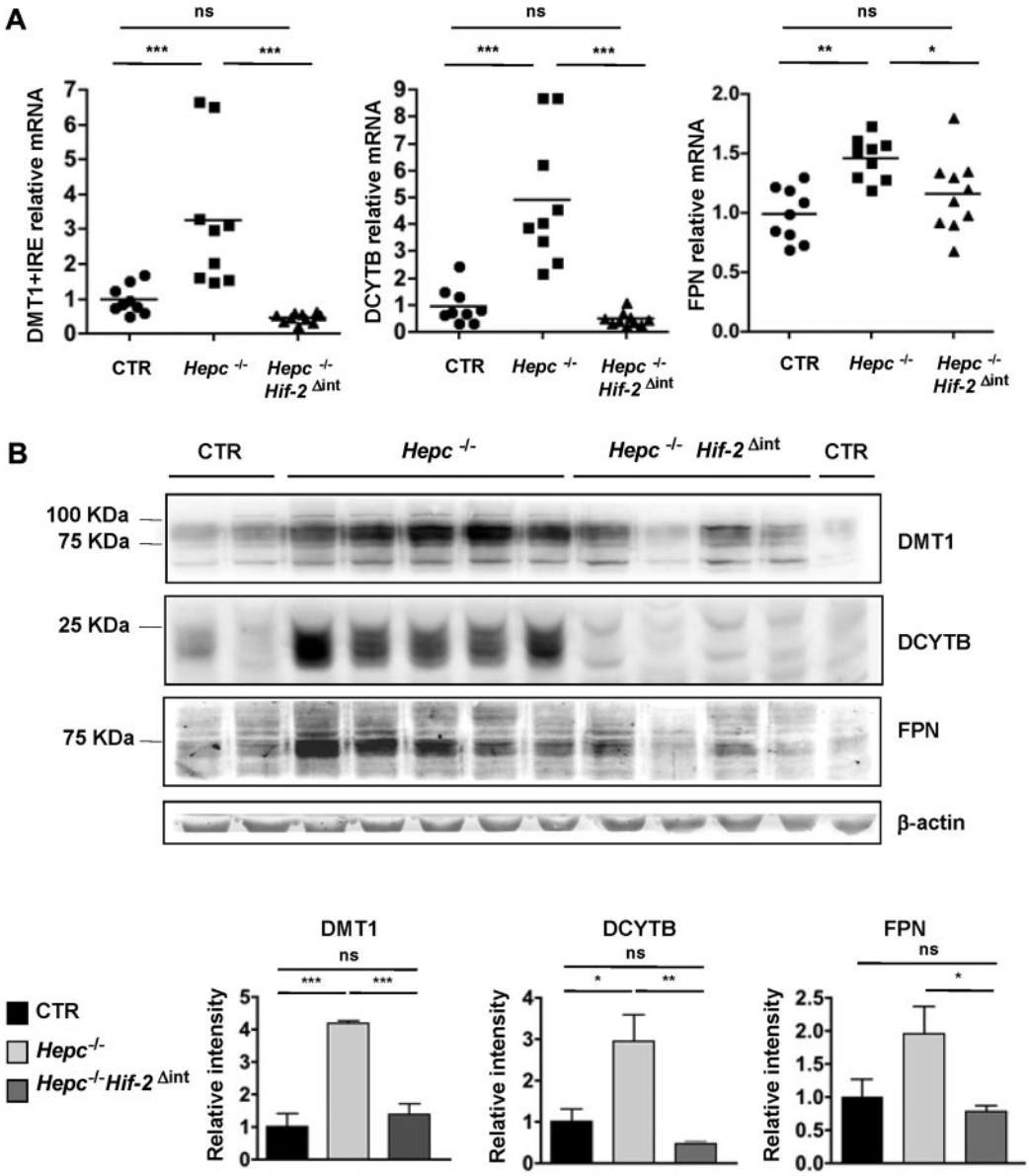

Figure 1. Iron absorption genes are decreased in *Hepc*$^{-/-}$*Hif-2α*$^{\Delta int}$ compared with *Hepc*$^{-/-}$ mice. (A) Relative mRNA expression of *DMT1 + IRE*, *DCYTB*, and *FPN* normalized to *Cyclophilin* in the duodenum of *Hepc*$^{-/-}$*Hif-2α*$^{\Delta int}$ (●; n = 9) versus Hepc$^{-/-}$ (■; n = 9) and CTR (▲; n = 9) mice. (B) Western blot of FPN, DMT1, and DCYTB on membrane extracts of whole duodenum from *Hepc*$^{-/-}$*Hif-2*$^{\Delta int}$ and *Hepc*$^{-/-}$ mice versus CTR littermates. Expression was normalized to β-actin. Results were quantified using ImageJ Version 1.43r software (http://rsb.info.nih.gov/ij/). All genotypes used contain the Hif-2α$^{lox/lox}$ allele. *$P < .05$. **$P < .01$. ***$P < .001$. ns indicates not significant.

of these mice. The levels of DMT1, DCYTB, and FPN transcript and protein were fully attenuated in *Hepc*$^{-/-}$*Hif-2α*$^{\Delta int}$ (Figure 1A-B) compared with *Hepc*$^{-/-}$ mice with levels not statistically different from wild-type mice. The duodenal deletion of HIF-2α decreased significantly FPN protein levels, despite the lack of hepcidin, which should prevent FPN degradation by systemic regulation.

We next asked whether the decrease of genes involved in iron absorption at the apical (DMT1 and DCYTB) and the basolateral membrane (FPN) was sufficient to prevent the hyperabsorption characteristic of the *Hepc*$^{-/-}$ mice. Interestingly, the double knockout presented a significantly decreased accumulation of nonheme iron in the liver and pancreas compared with *Hepc*$^{-/-}$ littermates. This was assessed both quantitatively (Figure 2A) and

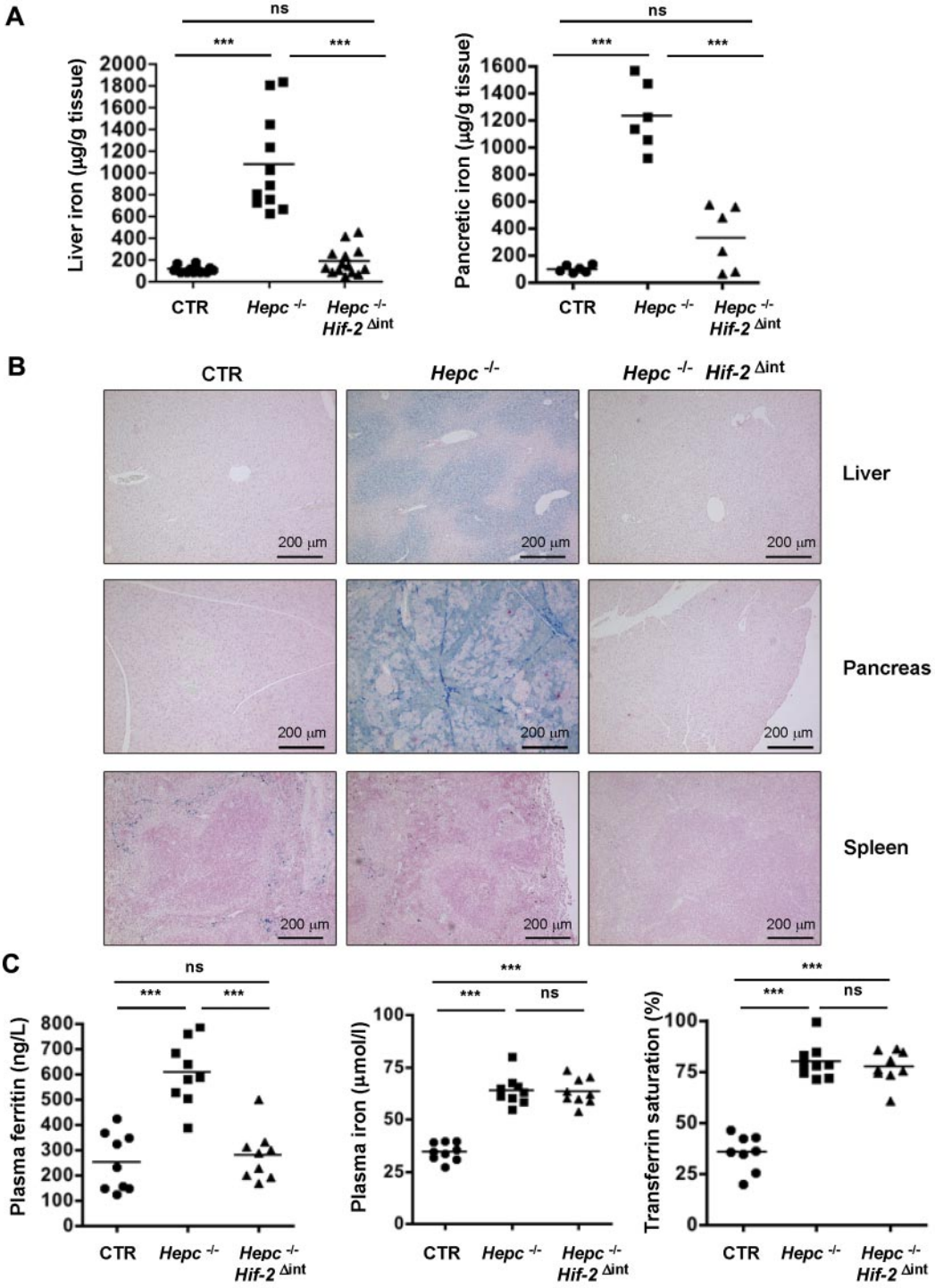

Figure 2. Iron parameters are decreased in the *Hepc*$^{-/-}$*Hif-2α*$^{\Delta int}$ mice compared with Hepc$^{-/-}$ mice. (A) Quantification of liver (n = 12 per group) and pancreas (n = 6 per group) iron levels in *Hepc*$^{-/-}$*Hif-2α*$^{\Delta int}$ (●) and Hepc$^{-/-}$ (■) versus CTR (▲) mice. (B) Perls' blue staining of the liver, pancreas, and spleen of CTR, Hepc$^{-/-}$, and *Hepc*$^{-/-}$*Hif-2α*$^{\Delta int}$ mice. One representative picture of each genotype is shown. Bars represent 200 µm. (10×/0.45, Nikon E800 microscope, CDD QICAM cooled camera [QImaging, QCapture Version 2.98.2 software [Qualitative Imaging Corporation]). (C) Plasma ferritin, plasma iron, and transferrin saturation in *Hepc*$^{-/-}$*Hif-2α*$^{\Delta int}$ (▲; n = 9) versus *Hepc*$^{-/-}$ (■; n = 9) and CTR (● n = 9) mice. ***$P < .001$. ns indicates not significant.

qualitatively by Perls' blue staining (Figure 2B). Plasma ferritin levels, reflecting parenchymal iron storage, were significantly diminished in *Hepc*$^{-/-}$ mice lacking duodenal HIF-2, compared with *Hepc*$^{-/-}$ mice (Figure 2C). However, plasma iron concentrations or transferrin saturation (Figure 2C) did not differ between the *Hepc*$^{-/-}$*Hif-2α*$^{\Delta int}$ and *Hepc*$^{-/-}$ littermates, suggesting a contribution of the iron recycled from the spleen, an organ that is not affected by the deletion of Hif-2α.[14] Indeed, most circulating iron is provided by macrophage iron recycling, and this process seems not affected in the *Hepc*$^{-/-}$*Hif-2α*$^{\Delta int}$ mice compared with the *Hepc*$^{-/-}$ mice, as shown by the lack of detectable iron in the macrophages of the spleen in both models (Figure 2B). Interestingly, hematologic parameters (hemoglobin, hematocrit, mean corpuscular volume) were decreased in the *Hepc*$^{-/-}$HIF-2α$^{\Delta int}$ mice compared with the *Hepc*$^{-/-}$ mice and not statistically different from wild-type mice (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

In conclusion, our data suggest that HIF-2 contributes to the intestinal iron hyperabsorption in a mouse model of HH but may not overcome all of the negative consequences of the abnormal iron metabolism. Associations between single nucleotide polymorphisms at Hif-2α locus and blood-related phenotypes have been recently demonstrated.[15,16] It would be of interest to determine whether HIF-2α polymorphisms could be found associated with iron burden in hemochromatosis. Current treatments for iron overload disorders are limited to phlebotomy or, in case of severe anemia, cardiac failure, or poor tolerance, to chelation therapies.[2] Here, we propose that therapeutic intervention on intestinal HIF-2α activity might be beneficial to reduce the rates of iron absorption and parenchymal iron overload.

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Authorship

Contribution: M.M., P.M., S.D., and J.-C.D. performed experiments; M.M., S.V., and C.P. wrote the manuscript; and all authors conceived, analyzed, and interpreted the experiments.

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