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Letter
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# Hfe Is Highly Expressed in Liver Sinusoidal Endothelial Cells But Is Not Needed to Maintain Systemic Iron Homeostasis In Vivo

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Hereditary hemochromatosis (HH) type 1 is an iron overload disorder caused by a homozygous p.C282Y mutation in the *Hfe* (High Fe) gene. In the absence of treatments, severe clinical consequences arise such as liver cirrhosis, cardiac dysfunction, and diabetes.[1] HH is caused by an inappropriately low expression of the hepatic peptide hormone hepcidin that controls the rate of iron efflux into the bloodstream by inducing the degradation of the iron exporter ferroportin.[2] *Hepcidin* transcription is controlled by the bone morphogenetic proteins (BMP)2 and BMP6 released from liver sinusoidal endothelial cells (LSECs) and that bind the cognate BMP receptor on hepatocytes.[3,4]

Our previous work established that cell-type-specific deletion of *Hfe* in mouse hepatocytes in vivo largely explains the anomalies of systemic iron metabolism observed in constitutive *Hfe* knock-out mice, resembling those of patients suffering from HH.[5] However, these results could not exclude the possibility that HFE needs to be co-expressed in other hepatic cell types to exploit its hepcidin regulatory function. This hypothesis has been investigated in mice with specific deletion of *Hfe* in myeloid-derived cells, including liver resident macrophages (named Kupffer cells). The lack of HFE in Kupffer cells does not generate an hemochromatosis-like phenotype but rather a mild iron deficiency at a very advanced age.[6] This demonstrates that there is no combinatorial effect of hepatocyte and macrophage HFE on hepcidin regulation.

LSECs play a critical role in the communication with hepatocytes to control Hepcidin expression. Therefore, we investigated whether LSECs express Hfe and whether it participates in the regulation of the son of mother against decapentaplegic (SMAD) signaling pathway that controls Hepcidin expression. By analyzing publicly available RNA sequencing repositories, we show that LSECs express ≈7-fold higher levels of Hfe messenger RNA (mRNA) compared with hepatocytes (Figure 1A). We validated this result in quantitative real time PCR assays on hepatocytes and LSECs purified through CD146 immunoselection demonstrating a similar difference in *Hfe* mRNA expression (Figure 1B). In contrast to the data here reported, it was previously shown that rat endothelial cells express a lower amount of HFE in comparison to hepatocytes,[7,8] suggesting a species-specific *Hfe* expression pattern. However, the analysis of *Hfe* mRNA expression in single-cell RNA sequencing of human liver cells (from the human protein atlas repository) overlaps with mouse data (data not shown). This indicates that the understanding of the role of HFE in murine liver endothelial cells may have a great translational relevance. Therefore, to investigate the potential biological function of HFE in LSECs, we generated LSEC-specific *Hfe* knock-out mice ($Hfe^{LSEC\text{-}KO}$) by crossing $Hfe^{fl/fl}$ ($Hfe^{flx}$)[9] with endothelial-specific *Stab2*-Cre ($Stab2^{Cre+}$) mice[10] (Figure 1C). CRE-mediated *Hfe* recombination occurred with high specificity and efficiency in LSECs but not hepatocytes (Figure 1D), as also shown by *Hfe* mRNA expression in isolated liver cells (Figure 2G). Importantly, we show that serum iron (Figure S1A, http://links.lww.com/HS/A213), transferrin saturation (Figure S1B, http://links.lww.com/HS/A213), spleen and liver iron content (Figure S1C and D, http://links.lww.com/HS/A213), hepatic Hepcidin, Bmp2 and Bmp6 mRNA expression (Figure S1E–G, http://links.lww.com/HS/A213) remained unaltered in $Stab2^{Cre+}$ mice, thus excluding toxicity induced by expression of the Cre recombinase.

We next focused our analysis on $Hfe^{LSEC\text{-}KO}$ mice. At birth, $Hfe^{LSEC\text{-}KO}$ male and female mice are born at Mendelian ratio, are phenotypically indistinguishable from $Hfe^{flx}$ littermates and reach adulthood without overt abnormalities (not shown). At 20 weeks of age, $Hfe^{LSEC\text{-}KO}$ animals showed normal body weight (Figure 1E), exhibited an overall healthy phenotype, were fertile and displayed normal posture and behavior (not shown).

Unlike what is observed in patients with HH type 1 and constitutive *Hfe* knock-out mice, HFE-deficiency specifically in LSECs did not alter erythroid hematological parameters, as indicated by the measurement of red blood cell count, hemoglobin, mean corpuscular volume, and hematocrit (Figure 1F–I). Furthermore, systemic iron indices in $Hfe^{LSEC\text{-}KO}$ mice are comparable to controls, as shown by normal serum iron and transferrin saturation levels (Figure 1J and K and Figure S2A and B, http://links.lww.com/HS/A213), splenic (Figure 1L and Figure S2C, http://links.lww.com/HS/A213) and hepatic

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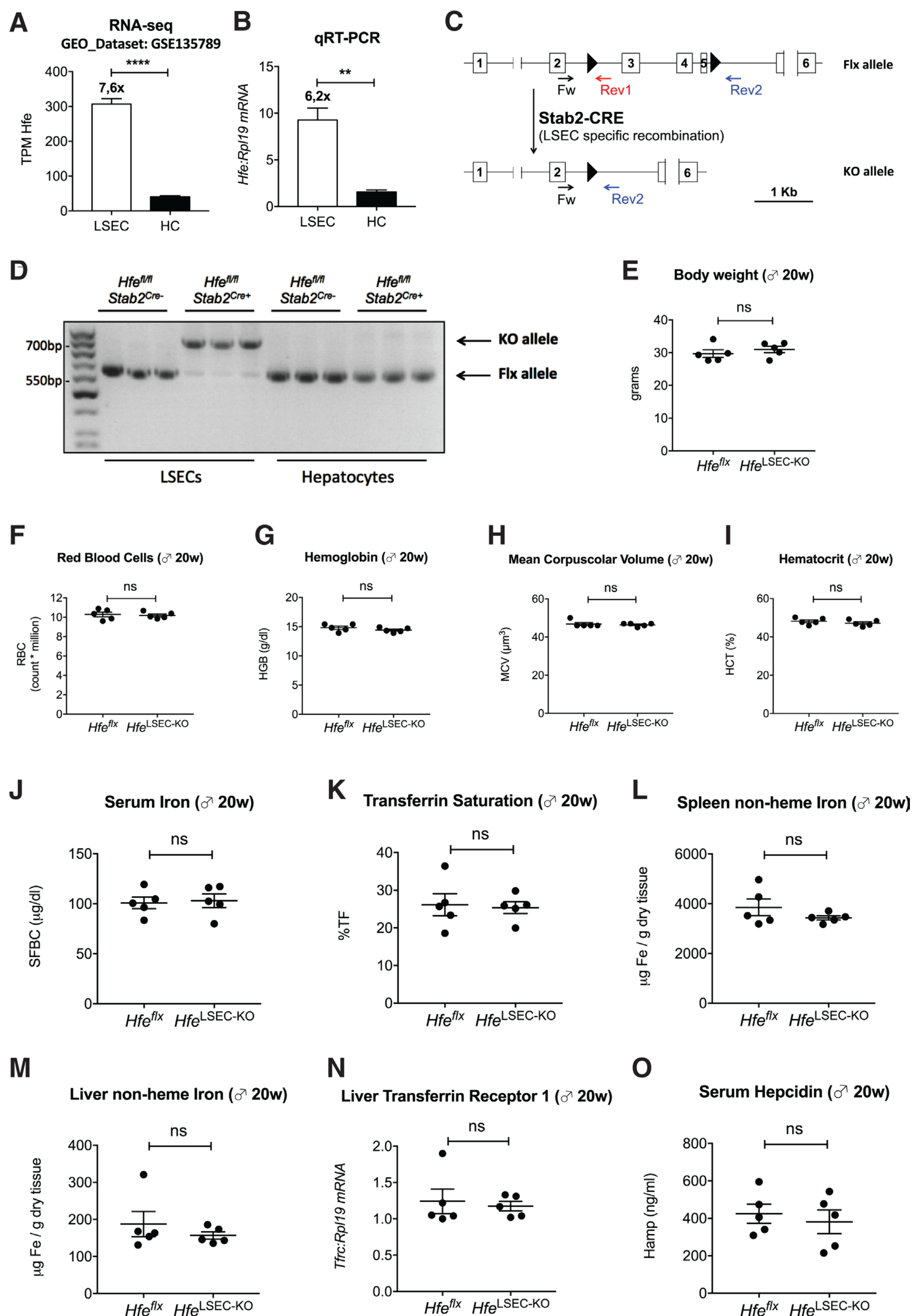


**Figure 1. Iron parameters are not affected in *Hfe*$^{LSEC\text{-}KO}$ male mice.** (A and B), *Hfe* mRNA expression calculated from (A) RNA sequencing analysis (GSE135790) or (B) qRT-PCR analysis of LSECs and HCs isolated from wild-type male mice (n = 4), as indicated. (C), Schematic representation of the *Hfe* gene before and after CRE-mediated recombination. (D), *Hfe* genotyping of DNA extracted from isolated LSEC and HCs of *Hfe*$^{fl/fl}$; Stab2$^{Cre-}$ (*Hfe*$^{flx}$) and *Hfe*$^{fl/fl}$; Stab2$^{Cre+}$ (*Hfe*$^{LSEC\text{-}KO}$) mice. (E–O), Characterization of iron parameters in 20-wk-old male *Hfe*$^{flx}$ and *Hfe*$^{LSEC\text{-}KO}$ mice (n = 5 per group): (E) body weight, (F–I) hematological parameters, (J) serum iron, (K) transferrin saturation, (L and M) splenic and hepatic nonheme iron content, (N) hepatic *Tfr1* mRNA levels, and (O) serum hepcidin analysis. qRT-PCR data were normalized to the housekeeping gene *Rpl19*. Data are reported as mean ± SEM. Student *t* test *P* value: ns, $^{**}P < 0.01$ and $^{****}P < 0.0001$. CRE = causes recombination; Flx = floxed; Fw, Rev1, Rev2 = genotyping primer location; GEO = gene expression omnibus; Hamp = hepcidin; HC = hepatocyte; HCT = hematocrit; HFE = High Fe; HGB = hemoglobin; LSEC = liver sinusoidal endothelial cell; MCV = mean corpuscular volume; mRNA = messenger RNA; ns = nonsignificant; qRT-PCR = quantitative real time PCR; SFBC = serum iron bound concentration; %TF = transferrin saturation; Tfrc = transferrin receptor; TPM = transcripts per kilobase million.

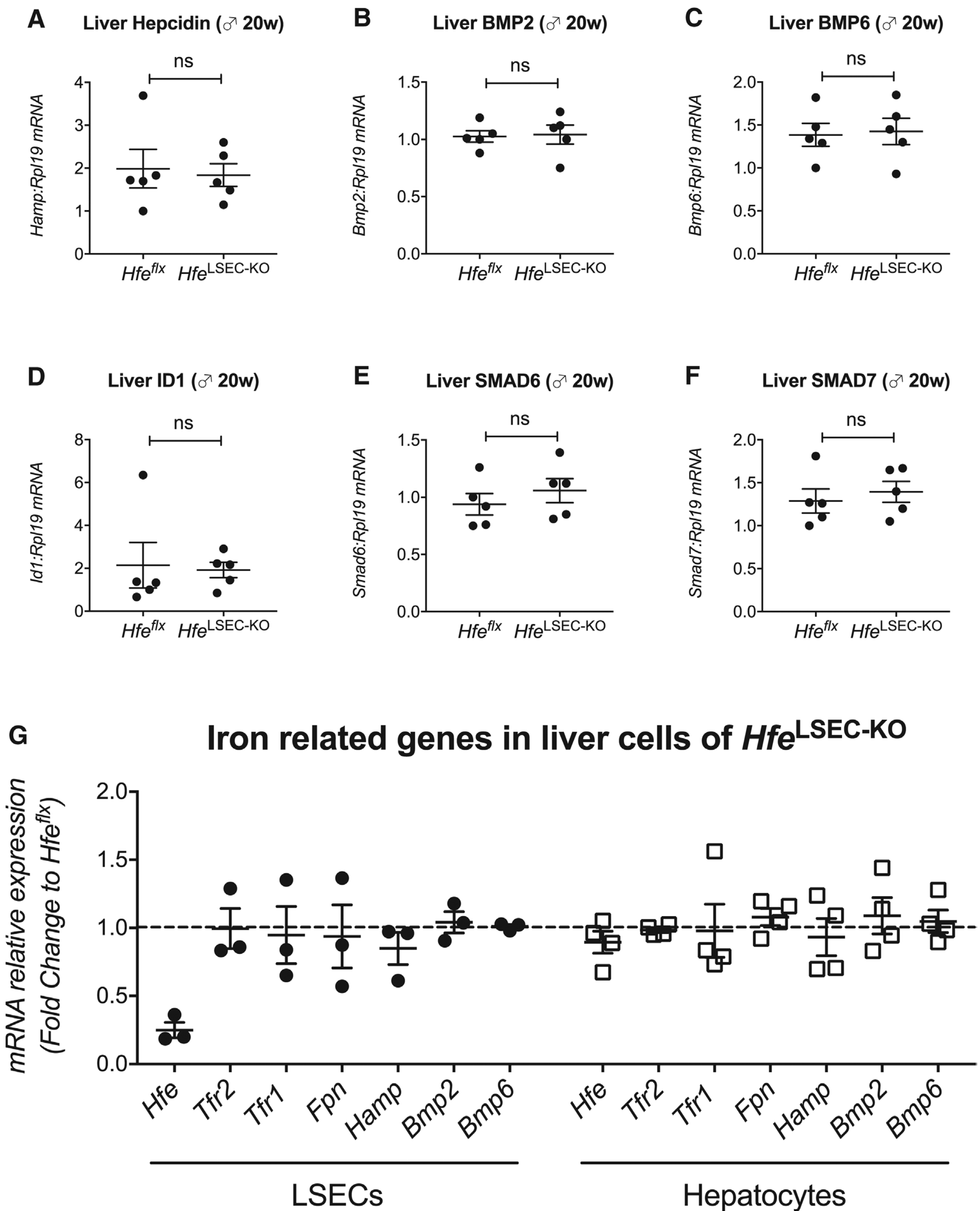


**Figure 2. The BMP/SMAD signaling pathway is not affected in the liver of male *Hfe*$^{LSEC-KO}$ mice.** Livers from 20-wk-old *Hfe*$^{flx}$ and *Hfe*$^{LSEC-KO}$ male mice (n = 5 per group) were analyzed for the mRNA expression of (A) *Hepcidin* (*Hamp*), (B) *Bmp2*, (C) *Bmp6*, (D) *Id1*, (E) *Smad6*, and (F) *Smad7*. (G), mRNA expression of *Hfe*, *Tfr2*, *Tfr1*, *Fpn*, *Hamp*, *Bmp2*, and *Bmp6* of LSECs and hepatocytes isolated from 15-wk-old *Hfe*$^{flx}$ and *Hfe*$^{LSEC-KO}$ male mice (n = 3, 4 per group) were analyzed. (G), Data are reported as mRNA expression of *Hfe*$^{LSEC-KO}$ isolated cells relative to *Hfe*$^{flx}$ (dashed line). qRT-PCR data were normalized to the housekeeping gene *Rpl19*. Data are reported as mean ± SEM. BMP = bone morphogenetic protein; Fpn = ferroportin; Hamp = hepcidin; HFE = High Fe; Id1 = inhibitor of DNA binding; LSEC = liver sinusoidal endothelial cell; mRNA = messenger RNA; ns = nonsignificant; qRT-PCR = quantitative real time PCR; SMAD = son of mother against decapentaplegic; Tfr = transferrin receptor.

nonheme iron levels (Figure 1M and Figure S2D, http://links.lww.com/HS/A213), and unaltered *Transferrin receptor 1* (*Tfrc*) mRNA expression (Figure 1N). Moreover, serum hepcidin is unchanged (Figure 1O and Figure S2E, http://links.lww.com/HS/A213) and mirrors hepatic *Hepcidin* mRNA levels (Figure 2A and Figure S2F, http://links.lww.com/HS/A213). Likewise, *Bmp2* and *Bmp6* levels are normal (Figure 2B and C) as well as BMP target genes, such as *Id1*, *Smad6*, and *Smad7* (Figure 2D–F). These results demonstrate that HFE-deficiency in LSECs of 20-week-old male (Figures 1 and 2) and

female (Figure S2, http://links.lww.com/HS/A213) mice does not affect systemic iron metabolism. Furthermore, we did not detect any cell-type-specific change in LSECs and hepatocytes isolated from *Hfe*$^{LSEC\text{-}KO}$ of the iron-related genes *Tfr2*, *Tfr1*, *Ferroportin*, *Hepcidin*, *Bmp2*, and *Bmp6* (Figure 2G), indicating unaltered cellular iron status. Whereas, *Hfe* is reduced exclusively in LSECs, as expected (Figure 2G).

The phenotypic expression of HFE-hemochromatosis increases with age. We therefore explored iron-related parameters in older mice with LSEC-specific HFE-deficiency. However, systemic iron metabolism remained unaltered also in 31-week-old mice (Figure S3, http://links.lww.com/HS/A213).

Taken together, these results clearly demonstrate that, despite the high-level expression of HFE in LSECs, endothelial-specific deletion of the hemochromatosis gene *Hfe* does not alter systemic iron homeostasis under steady state conditions in adult male and female mice. Future studies will have to explore whether HFE in LSECs may influence circulating or liver iron sensing in response to iron challenges or play a role in molecular processes not involved in iron metabolism.

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## AUTHOR CONTRIBUTIONS

SC, KM, and SA performed the experiments. SC and SA analyzed the results. SC, SA, and MUM wrote the manuscript. MUM and SA supervised the project.

## DISCLOSURES

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