Hepatic expression of hemochromatosis genes in two mouse strains after phlebotomy and iron overload

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Background and Objectives. Iron homeostasis is tightly regulated in mammals according to the needs of erythropoiesis and the iron stores present. This regulation is disrupted in hereditary hemochromatosis (HH), a genetic disorder characterized by increased intestinal iron absorption, leading to iron overload. The genes coding for HFE, transferrin receptor 2 (TFR2), ferroportin (SLC40A1 or FPN1), hepcidin (HEPC) and hemojuvelin (HJV or RGMC) are responsible for different types of genetic iron overload. All these genes are highly expressed in the liver and their protein products are likely components of a single hepcidin-related pathway. In order to gain insights into the molecular relationship among the HH proteins we evaluated the hepatic expression of HH genes in conditions of iron restriction or overload.

Design and Methods. Data were obtained after phlebotomy, to activate the *erythroid regulators* and following parenteral iron dextran loading, to activate the *store* regulators, in two mice strains (C57BL/6 and DBA/2). HH genes and proteins expression were analyzed by quantitative real time polymerase chain reaction and by Western blotting, respectively.

Results. *Hepc* RNA was reduced after phlebotomy and increased in iron overload. A statistically significant reduction of hepatic *Fpn1* RNA expression was observed after phlebotomy; this effect was more evident in the DBA/2 strain. *Fpn1* increased in C57BL/6 mice, but not in the DBA/2 ones in parenteral iron loading. *Fpn1* protein did not change substantially in either condition. *Hfe*, *Rgmc* and *Tfr2* expression was not influenced by phlebotomy. In parenteral iron overload, *Tfr2* gene and protein expression decreased concomitant to the increase in *Hepc*, while *Hfe* RNA remained constant.

Interpretations and Conclusions. Our results indicate that regulation of hepatic *Fpn1* differs from that reported for duodenal *Fpn1*. Furthermore, taken the differences in gene expression in dietary overload (increased *Hfe* but not *Tfr2*), distinct roles are suggested for *Hfe* and *Tfr2* in *Hepc* activation.

Key words: iron, hemochromatosis, mouse, hepcidin, ferroportin.
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Iron is an essential element for growth and functionality of all mammalian cells, thus its balance is maintained through a tight regulation of intestinal absorption, in order to avoid excessive uptake and iron-mediated cell toxicity. This regulation is titrated on the basis of iron needs for erythropoiesis and on the consistency of iron stores present, according to the proposed model of *erythroid* and *store* regulators.[1,2] Advances in understanding iron homeostasis derive from molecular genetic studies of hereditary hemochromatosis (HH), a genetic disorder characterized by inappropriately high intestinal iron absorption. Identification of the genes involved in HH has revealed new proteins regulating iron homeostasis. Disruption of HFE produces classic, adult-onset HH[3], whereas mutations of hepcidin (HEPC)[4] and of hemojuvelin (HJV or RGMC)[5] lead to the juvenile form, characterized by early onset of severe iron loading. Mutations of transferrin receptor 2 (TFR2) cause type 3 HH, with an early presentation but an intermediate clinical phenotype.[6] The inactivation of each of these four genes results in the same pattern of hepatocyte iron accumulation with reticuloendothelial sparing,[7] although preferential iron deposition in the heart and pituitary gland is observed in the juvenile disease.[8] SLC40A1 gene, encoding ferroportin1 (Fpn1)/IREG1/MTP1, is mutated in type 4 HH,[9,10] which is also known as *ferroportin disease*, and has a dominant inheritance and preferential iron storage in macrophages in most patients.[11]

Among the HH proteins, hepcidin has a key control role and is considered the final effector of the pathway that regulates iron homeostasis. In mice models, its overexpression causes severe iron deficiency,[12] whereas its ablation results in iron overload.[13] The central position of the liver in iron homeostasis is inferred by the observation that all HH genes are highly expressed in hepatocytes.[5,13-15] However, the interrelationships among the different proteins and their relationship with the

storage and *erythroid* regulators remain poorly understood. The evidence is that HFE modulates hepcidin, since the levels of hepcidin are inappropriate to the degree of iron loading in both *Hfe*-deficient animals [16,17] and HFE C282Y homozygous patients.[18] Furthermore, hepcidin is able to correct the *Hfe* defect in transgenic mice.[19] Hepcidin is absent or present at only low levels in the urine of juvenile patients with HJV mutations,[5] suggesting that HJV is hepcidin-related. Low hepcidin levels are also found in patients with TFR2 mutations,[20] implying that TFR2 is another modulator of hepcidin. Furthermore, hepcidin is regulated by hypoxia/anemia both in human hepatoma cells and in animal models[21,22] and is strikingly increased in inflammation.[23]

Recent data have shown that in cultured cells hepcidin interacts directly with ferroportin 1, causing its internalization and lysosomal degradation.[24] This is expected to occur physiologically in duodenal cells to impair iron absorption and in macrophages to restrict iron release in the presence of high hepcidin levels. Indeed in *Hepc*-deficient mice a high level of expression of *Fpn1* was observed in duodenum, spleen and liver macrophages.[25]

The purpose of this study was to evaluate the variations of hepatic expression of the HH genes and especially the *Hepc/Fpn1* relationships, after phlebotomy and parenteral iron overload in two mouse strains characterized by different *Hepc* expression.

Design and Methods

Animal care

Mice purchased from Harlan S.r.l, were housed in the barrier facility at the Department of Clinical and Biological Sciences, University of Turin, Italy and maintained on a standard diet.

All procedures in the mice were carried out in compliance with the guidelines of Institutional Animal Care and Use Committee at our University. All experiments were performed on animals of ten weeks of age. Because of significant differences in iron loading between sexes, only females were used.[26] The studies were carried out in C57BL/6 and in DBA/2 mice, since these strains are characterized by a distinct response to iron variation, the former being more resistant and the latter more susceptible to iron loading.[27]

Treatment protocols

Induction of anemia through phlebotomy

To induce acute anemia, 0.5 mL of blood were extracted by a single retro-orbital puncture from the previously anesthetized mice (Avertin, 2,2,2,-tribromoethanol; Sigma-Aldrich, St Louis, MO, USA). Animals were sacrificed at 6, 9, 12, 15, 48, 72 hours and 7 days after treatment and tissues were collected for RNA and protein extraction. Five animals were used for each time point and nine served as controls.

Induction of parenteral iron overload

Secondary iron overload was induced by two intraperitoneal administrations of 20 mg (1 g/kg body weight) iron dextran (Sigma Chemicals, St Louis, MO, USA) separated by 15 days. Animals were sacrificed two weeks after the second injection. Each animal's liver was dissected and snap-frozen immediately for RNA and protein analysis. Five animals were treated and five served as controls.

Measurements of hematologic parameters

Blood obtained by phlebotomy was collected into tubes containing EDTA (Sarstedt s-monovette, Aktiengesellschaft & Co., Numbrecht, Germany). Blood cell counts and erythrocyte parameters were determined using an EPICS Coulter Profile II (Coulter Electronics, Hialeah, FL, USA) automatic analyzer.

Histology and Perls' staining

For histological studies and iron staining, tissues were fixed in aqueous formaldehyde solution (buffered 4% vol/vol) and embedded in paraffin. For histological assessment of non-heme iron deposition, slides of liver sections were stained with Perls' Prussian blue. Hematoxylin-eosin (H&E) counterstaining was performed to mark the nucleus and cytoplasm by standard procedures.

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

For reverse transcription, starting from 1 μg of total RNA, random hexamers at a concentration of 25 μM and 100 U of the reverse transcriptase (Applera, Milan, Italy) were added to the reaction mixture.

Levels of gene expression were measured by quantitative real time PCR (qRT-PCR). The PCR reactions and fluorescence measurements were performed using an iCycler (Bio-Rad Laboratories, Hercules, CA, USA). The amount of HH gene transcripts was evaluated using a qRT-PCR assay based on specific sets of primers, designed to be complementary to the different exon sequences to avoid co-amplification of genomic DNA, and probes (Assays-on-Demand, Gene Expression Products) supplied by Applied Biosystems (Foster City, CA, USA). Hepc primers used for qRT-PCR amplify both *Hepc 1* and *Hepc 2*. For the PCR reaction, 5 μL of cDNA were added to 15 μL of PCR reaction mix containing 10 μL of TaqMan Universal PCR Master Mix (Applera), 1 μL of assay in a final volume of 20 μL. The PCR procedure was started with a step of 2 min at 50°C to activate the UNG enzyme, followed by 10 min at 95°C to inactivate the UNG

enzyme and to provide a *hot start* activating the AmpliTaq polymerase. Next, 50 cycles of denaturation (95°C for 15 sec), followed by annealing and extension (60°C for 60 sec) were performed. All analyses were carried out in triplicate; results showing a discrepancy greater than one cycle threshold in one of the wells were excluded.

Northern blot

RNA preparation and Northern-blot analysis were performed with standard methods. Serum amyloid A3 probe was kindly provided by Fiorella Altruda, University of Turin, Italy. The blot was rehybridized with a GAPDH probe in order to normalize the amounts of RNA.

Data analysis

The values obtained were normalized using β-glucuronidase (GUS) as a control gene. The results were analyzed using the ΔΔCt method, as the efficiencies of amplification of both the target and reference genes were determined and found to be approximately equal. Briefly, the threshold cycle (C_T) indicates the cycle number at which the amount of the amplicon reaches the fixed threshold (usually 50%). ΔC_T is the difference between threshold cycle for the gene of interest and the reference gene ($C_{Ttarget} - C_{Treference}$). $\Delta\Delta C_T$ is the difference between ΔC_T of the sample and ΔC_T of a RNA calibrator. A pool of normal mice liver RNA was used as calibrator. The final result is expressed as $2^{-(\Delta\Delta CT)}$.

The statistical significance of the differences of mRNA expression between controls and treated mice was evaluated using one way ANOVA analysis followed by Dunnet's post-hoc test for phlebotomy experiments and Student's t test (unpaired, two tailed) for the iron overload experiments.

Western blot

Western blotting was perfomed with anti-human Tfr2 antibody (14E8),[28] which has demonstrated cross-reactivity with mouse proteins. Rabbit anti-mouse polyclonal anti-Fpn1 antibodies were kindly provided by David Haile, University of Texas, USA.[29] Hepatic tissue was collected, homogenized and lysed. Ten micrograms of proteins were run in 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted according to standard protocols. All Western blot analyses were performed on both C57BL/6 and DBA/2 mice tissues.

Results

Hepc expression in basal conditions differed in the two strains of animals studied and also in individual animals. As previously reported,[26] *Hepc* levels, as assessed by RT PCR, were higher in DBA/2 mice than in the C57BL/6 strain, and were predominantly accounted for by *Hepc2,* whereas C57BL/6 expressed more *Hepc1* (*data not shown*). The assay used for qRT-PCR experiments cannot discriminate *Hepc1* and *Hepc2*. However, since it has been demonstrated that *Hepc2* has no effect on iron metabolism,[30] we assumed that the differences observed are due to inter-strain variations in *Hepc1*.

Phlebotomy protocol

Induction of anemia was demonstrated at 6 hours after phlebotomy by measuring hemoglobin levels (Figure 1). Hemoglobin concentration then remained stable and returned to normal levels 7 days after treatment in both strains. *Hepc* expression was up-regulated at 6 hours, decreased at 12-15 hours and recovered at 48-72 hours in both strains. *Hepc* expression was significantly lower ($p<0.05$) within 12 hours after phlebotomy (9 hours in DBA/2) in the animals with induced anemia than in the non-treated animals in both strains (Figure 1A). Northern blot of the same samples showed that serum amyloid A3, a marker of inflammation,[31] increased at 6-15 hours (*data not shown*).

Fpn1 transcripts decreased significantly 6 hours after phlebotomy ($p<0.05$), before the *Hepc* decrease in both strains (Figure 1B). *Fpn1* levels returned to normal 9 hours after phlebotomy in C57BL/6 mice while they paralleled *Hepc* levels in DBA/2 animals. At the protein level, *Fpn1* was slightly decreased in C57BL/6 mice at 6-12 hours after phlebotomy, then remained stable; DBA/2 *Fpn1* did not show significant variations (Figure 1C).

Hfe, *Rgmc* and *Tfr2* transcript levels did not change significantly after phlebotomy in either strain (*data not shown*).

Parenteral iron loading

The protocol of parenteral iron dextran administration we used dramatically increased reticuloendothelial and also parenchymal iron. We did not measure liver iron concentration but, as shown by Perls' staining (Figure 2), iron deposition in C57BL/6 and DBA/2 liver was massive. *Hepc* levels were significantly up-regulated in iron-loaded mice. *Hepc* expression increased by about 7-fold relative to basal levels in C57BL/6 mice ($p<0.001$) and 3-fold in DBA/2 mice ($p<0.001$) (Figure 3A).

Tfr2 transcript levels were downregulated in iron-loaded animals. The reduction was approximately 2-fold in both the C57BL/6 and DBA/2 strains with a statistically significant difference as compared to controls ($p<0.001$ and $p<0.01$, respectively) (Figure 3B). Tfr2 protein levels were reduced accordingly in C57BL/6 mice (Figure 3D upper panel). *Fpn1* tran-

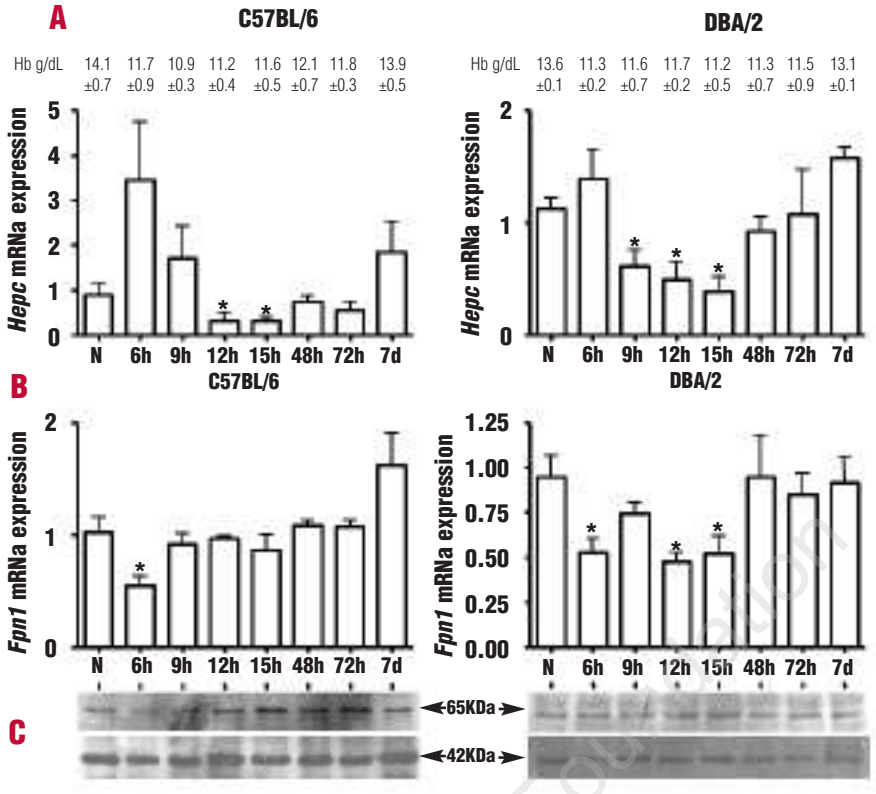

Figure 1. *Hepc* and hepatic *Fpn1* variations in phlebotomized mice. A. Hepatic expression of *Hepc in* C57BL/6 (left) and DBA/2 (right) phlebotomized mice. Hemoglobin values (Hb) are reported. The time course of the experiment (h/d= hours/days after phlebotomy) is illustrated. Five animals were used for each point and nine served as controls (N). The statistical analysis was performed using the ANOVA test, as described in the *Design and Methods*. *Hepc* expression data were normalized to *Gus* cDNA. The asterisk indicates a statistically significant difference ($p<0.05$) vs controls. Results are expressed as mean ± SEM (standard error mean). B. Results of real time PCR (qRT-PCR) of hepatic *Fpn1* mRNA. h/d= hours/days after phlebotomy. N = control mice. Results are expressed as mean + SEM. Statistical analysis was performed as above. *Fpn1* expression data were normalized to Gus (β-glucuronidase) cDNA. The asterisk indicates a statistically significant difference ($p<0.05$) vs controls. C. Western blot of liver Fpn1 (upper lanes) compared with β-actin (lower lanes) of phlebotomized C57BL/6 and DBA/2 mice. The time course of the experiment is reported above the lanes. Molecular sizes in kilodalton (kDa) are shown. N: normal mice.

script levels increased significantly in C57BL/6 mice ($p<0.001$), but did not change after iron loading in DBA/2 animals (Figure 3C). The levels of *Fpn1* protein remained rather stable in both strains (Figure 3D lower panel and *data not shown*). No significant variations were observed for *Hfe* and *Rgmc* transcripts (*data not shown*).

Discussion

The liver is central to the regulation of iron homeostasis in mammals, as demonstrated by the high hepatic expression of HH genes, especially *Hepc*. The evidence is that both *erythroid* and *storage* needs are mediated by *Hepc*,[24] but whether and how the other genes respond to the regulators remains unknown. We have studied hepatic expression of murine HH genes in conditions that modulate iron requirements and *Hepc* production, activating either the *storage* (*Hepc* increase) or the erythroid (*Hepc* decrease) regulators.

A dramatic decrease of *Hepc* in parallel with anemia had been previously observed after multiple blood withdrawal (total 1.5 mL in 24 hours) in C57BL/6 mice.[21] In this study after a single withdrawal of 0.5 mL of blood we observed *Hepc* up-regulation at 6 hours, but thereafter a striking reduction occurred with normalization within 72 hours. The increase at 6 hours is inflammation-related as demonstrated by the simultaneous increase of the inflammatory serum amyloid A3 protein RNA.[31]

The reduction in *Hepc* occurred before any possible change of marrow erythroblast activity and is likely hypoxia-related, consistent with hypoxia being a major determinant of *Hepc* suppression.[21] As shown in Figure 1, *Hepc* levels returned to normal before correction of anemia, suggesting that hypoxia was rapidly compensated. We did not observe significant variation of expression of any of the other genes

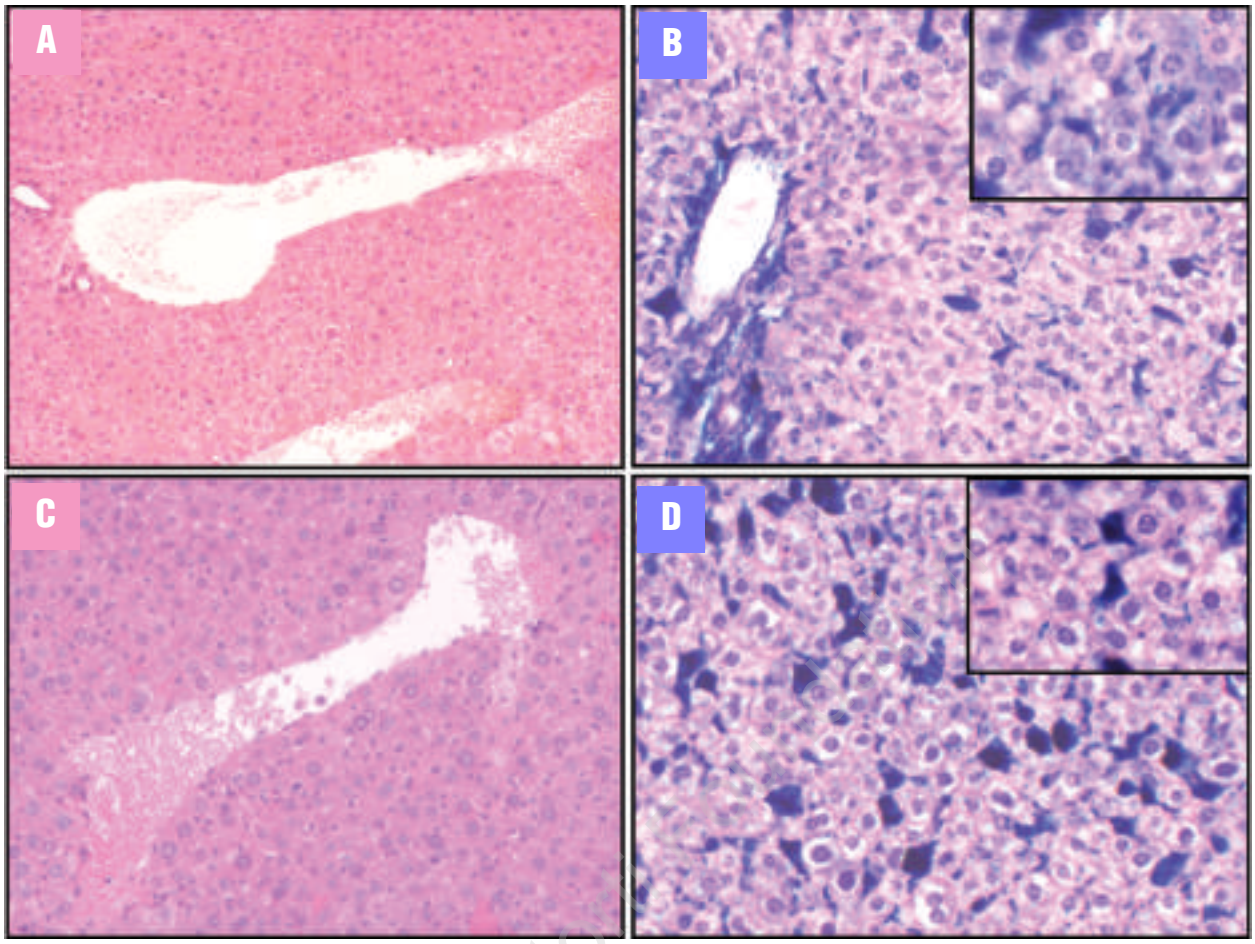

Figure 2. Histology and Perls' staining of mice liver in basal conditions and after iron overload. Panels A and C show the histology of C57BL/6 and DBA/2 control mouse liver respectively (×100), with normal lobular architecture. Panels B and D show heavy iron deposition in Küppfer cells and hepatocytes (Perls' staining, ×100) of the iron dextran treated C57BL/6 and DBA/2 mice, respectively. The inset is a higher-power view (×200).

(*Tfr2, Hfe, Rgmc*) in parallel with the decrease in *Hepc*, indicating that these genes are not transcriptionally co-regulated with *Hepc*. The only exception concerned *Fpn1*. After phlebotomy *Fpn1* RNA was significantly but transiently reduced in both strains, likely as a result of inflammation, as reported in rat liver in response to lipopolysaccharide.[32] From 9 hours liver *Fpn1* RNA parallels *Hepc* RNA variations in DBA/2, while its level overlaps that of controls in C57BL/6. Fpn1 protein levels remained stable, except for a slight reduction in C57BL/6 mice in the first hours after phlebotomy. As expected, a remarkable increase in *Hepc* was observed in mice treated with intraperitoneal iron injections, with this increase being more striking in C57BL/6 mice than in the DBA/2 animals. Interestingly, increased *Fpn1* RNA expression was recorded only in the C57BL/6 strain, but Fpn1 protein levels, although with individual variations, did not change significantly.

Decreased/stable hepatic *Fpn1* after phlebotomy and stable levels in iron overload suggest a distinct regulation of liver *Fpn1*, as compared with duodenal *Fpn1*, whose levels are inversely related to *Hepc* levels.[33] *Fpn1* RNA has a functional 5' UTR IRE motif[29,34] and may undergo post-transcriptional regulation. *In situ* hybridization studies have demonstrated that *Fpn1* mRNA in rat liver shows a decreasing gradient of intensity from periportal to central hepatocytes, indicating a correlation of *Fpn1* expression with iron deposition.[14] The discrepancy between hepatic and duodenal *Fpn1* expression might also be related to different rates of protein degradation. Iron-driven post-transcriptional regulation could operate in the liver and be overtaken by the *Hepc* effect in duodenum and macrophages. A distinct *Fpn1* regulation could reflect a different iron storage function of liver and macrophages. Unfortunately, in our study we cannot separate the contribution of Küppfer cells and that of hepatocytes. Preserved Fpn1 activity would permit iron export from hepatic stores when intestinal iron absorption and macrophage release are inhibited by high *Hepc* levels. After phlebotomy when

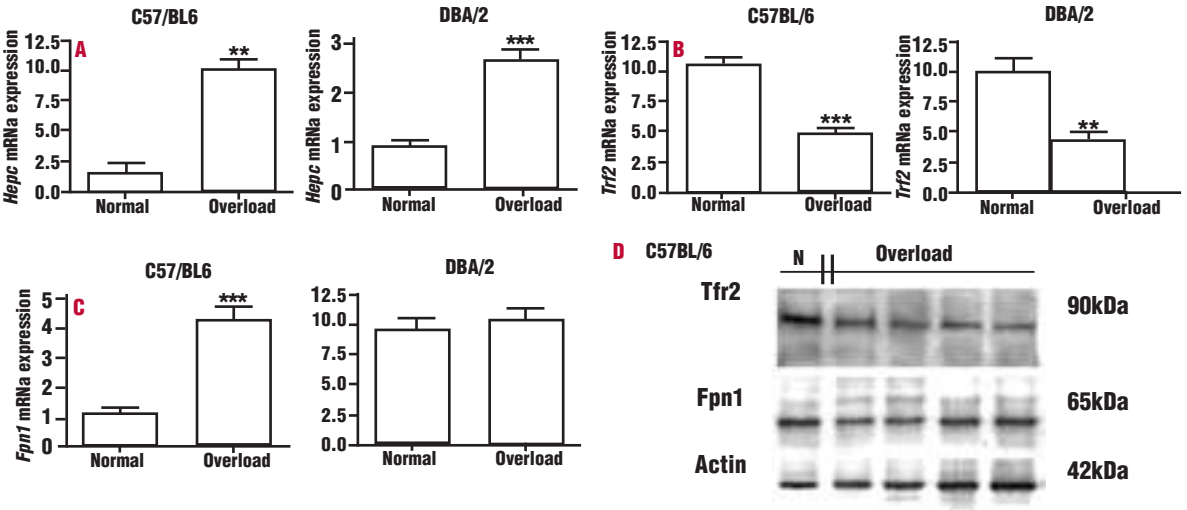

Figure 3. Expression of HH genes and proteins in iron-loaded animals. A-C. Results of qRT-PCR experiments of hepatic mRNA of C57BL/6 (left panels) and DBA/2 (right panels) mice after treatment with iron dextran. Five animals were treated and five served as controls. Statistical evaluation was performed using the Student's t test. The asterisks indicate statistically significant differences vs controls (**p<0.01; ***p<0.001). D. Western blot of liver proteins (Tfr2, Fpn1 compared with beta actin) of C57BL/6 iron-overloaded mice. Band sizes in kilodalton (kDa) are shown on the right. N: normal mice.

Hepc is low, lack of increase of Fpn1 protein could preserve hepatocyte iron, if iron storage were limited. The finding that in *Hepc*-deficient, *Usf2* -/- mice, which are characterized by high liver iron, Fpn1 protein is increased but prevalently in Küppfer cells might be in keeping with our hypothesis.[25] In parenteral iron overload a consistent reduction of *Tfr2* transcript and protein was observed in both strains, irrespective of the degree of *Hepc* activation, whereas *Hfe* expression did not change. A modest reduction of *Tfr2* expression after secondary iron overload in normal mice was observed by analyzing liver RNA on an iron chip.[17] In dietary iron overload *Tfr2* expression is unchanged, whereas that of *Hfe* is increased[35,36] (*and data not shown*), suggesting that transcription of the two genes responds to different signals. Since Tfr2 protein is stabilized *in vitro* by exposure to diferric transferrin, TFR2 has been proposed to be a sensor of transferrin saturation.[37,38] TFR2 is an hepcidin activator *in vivo*, since patients with TFR2 mutations, have low or undetectable urinary levels of hepcidin,[19] but its function is likely distinct from that of HFE. TFR2 could activate hepcidin production, to decrease iron absorption following increase of transferrin saturation. The downregulation of *Tfr2* we observed in chronic iron overload, may suggest that other mechanisms maintain the persistent *Hepc* activation.

All authors meet the criteria for being contributing authors. AR and CC were responsible for the study design, AB, PV, PP and AR did the experimental procedures on animals, FD, EGr, SC and EGo performed the molecular analyses of the samples. All the authors participated in the data interpretation, the drafting of the paper and its final version revision with their suggestions and criticisms. The authors declare that they have no potential conflict of interest.

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