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SHORT COMMUNICATION

A High Serum Iron Level Causes Mouse Retinal Iron Accumulation Despite an Intact Blood-Retinal Barrier

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The retina can be shielded by the blood-retinal barrier. Because photoreceptors are damaged by excess iron, it is important to understand whether the blood-retinal barrier protects against high serum iron levels. Bone morphogenic protein 6 (Bmp6) knockout mice have serum iron overload. Herein, we tested whether the previously documented retinal iron accumulation in Bmp6 knockout mice might result from the high serum iron levels or, alternatively, low levels of retinal hepcidin, an iron regulatory hormone whose transcription can be up-regulated by Bmp6. Furthermore, to determine whether increases in serum iron can elevate retinal iron levels, we i.v. injected iron into wild-type mice. Retinas were analyzed by real-time quantitative PCR and immunofluorescence to assess the levels of iron-regulated genes/proteins and oxidative stress. Retinal hepcidin mRNA levels in Bmp6 knockout retinas were the same as, or greater than, those in age-matched wild-type retinas, indicating that Bmp6 knockout does not cause retinal hepcidin deficiency. Changes in mRNA levels of L ferritin and transferrin receptor indicated increased retinal iron levels in i.v. iron-injected wild-type mice. Oxidative stress markers were elevated in photoreceptors of mice receiving i.v. iron. These findings suggest that elevated serum iron levels can overwhelm local retinal iron regulatory mechanisms. *(Am J Pathol 2014, 184: 2862–2867; http://dx.doi.org/10.1016/j.ajpath.2014.07.008)*

Iron is necessary for life, but in excess it can be toxic. Therefore, iron is tightly regulated. Many of the genes that regulate iron on the systemic level are expressed in the retina and may play a role in local iron regulation.[1] We previously studied double knockout (KO) mice deficient for the iron-exporting ferroxidases ceruloplasmin and hephaestin.[2] These double KO mice have age-dependent retinal iron accumulation and degeneration.[3,4] Double KO mice have retinal iron overload despite systemic iron deficiency, so it is likely that the retina accumulates iron as a result of impaired retinal iron export. We also studied mice with systemic knockout of the iron-regulatory hormone hepcidin (*Hamp*). These mice also have age-dependent retinal iron accumulation with degeneration.[5] Bone morphogenic protein 6 (Bmp6) is known to up-regulate Hamp in the liver and retina,[6] and *Bmp6* KO mice have retinal iron accumulation similar to *Hamp* KO mice.[7] Because *Hamp* and *Bmp6* KO mice have elevated serum iron levels, it is unclear whether the retinal iron accumulation in these mice results from elevated serum iron levels caused by diminished liver Hamp production or from low retinal Hamp levels impairing local iron regulation in the retina.

There are several routes for iron influx yet only one for cellular iron efflux[8–10]; the transmembrane protein ferroportin can export iron out of cells. Ferroportin can be regulated by the 25 amino acid hepcidin peptide, which triggers its internalization and degradation, leading to decreased export of cellular iron.[11] *Hamp* expression in the liver is increased by iron overload, and, after secretion into the bloodstream, limits intestinal iron absorption, macrophage iron recycling, and hepatocyte iron release. In comparison, iron deficiency anemia and tissue hypoxia can inhibit *Hamp* expression to increase the iron supply for the body.[12–14]

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Hamp is expressed in many tissues, including the retina within photoreceptors, Müller cells, and retinal pigment epithelium (RPE).[15] Ferroportin, the target of hepcidin, is localized to RPE, the photoreceptor inner segment, the outer plexiform layer, and the inner limiting membrane.[16] Locally synthesized Hamp may regulate ferroportin in the neural retina (NR). To determine whether retinal hepcidin deficiency contributes to the retinal iron accumulation in *Bmp6* KO mice, we compared Hamp mRNA levels in *Bmp6* KO versus wild-type (WT) mice. We also tested whether WT mice given i.v. injections of iron sucrose would develop retinal iron accumulation despite the presence of Hamp synthesized locally in the retina.

Materials and Methods

Animals

Bmp6 KO mice on the CD1 background were generated as described previously.[17] *Bmp6* KO male mice aged 2.5, 3.5, 5, and 10 months and age-matched WT male CD1 mice were used for the experiments. WT C57BL/6J mice at 2.5 months were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6J mice were treated with or without 1.2 mg of iron sucrose (Venofer; American Regent, Shirley, NY) in 200 mL of 0.9% saline solution via tail vein injection three times (once per week) until sacrifice.

Experimental procedures were performed in accordance with the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmology and vision research. All the protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania (Philadelphia, PA). The eyes were enucleated immediately after death and were fixed overnight in 4% paraformaldehyde for immunofluorescence.

Immunolabeling

Mouse globes fixed in 4% paraformaldehyde were rinsed in phosphate-buffered saline, and the eyecups were dissected. The eyecups were cryoprotected in 30% sucrose solution overnight and then embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek USA Inc., Torrance, CA) and slowly frozen in 2-methylbutane on dry ice. Immunofluorescence was performed on sections 10 μm thick, as described previously.[18] Primary antibodies were rabbit anti−L ferritin (E17) at 1:200 dilution [a gift from Dr. Paolo Arosio (University of Brescia, Brescia, Italy)], rabbit anti-malondialdehyde (MDA) at 1:100 dilution (Alpha Diagnostic, San Antonio, TX), and rabbit anti−4-hydroxy-nonenal (HNE) at 1:100 dilution (Alpha Diagnostic). Primary antibody was detected using fluorophore-labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Control sections were treated identically except that primary antibodies were omitted. Sections were analyzed by fluorescence microscopy with identical exposure parameters using a Nikon TE300 microscope equipped with ImagePro Plus software version 6.1 (Media Cybernetics Inc., Bethesda, MD).

RPE Isolation

We used a recently developed method for the rapid isolation of RPE cells for RNA extraction and analysis.[19] This method uses simultaneous RPE cell isolation and RNA stabilization. Briefly, eyes were enucleated and placed on an ice-cold phosphate-buffered saline−soaked sponge. The periocular tissues were removed, the eye was opened posterior to the limbus using Vannas scissors, and the anterior segment and the retina were removed. The resulting posterior eyecups were quickly dipped into a phosphate-buffered saline−containing microcentrifuge tube to remove any loosely adherent debris. Afterward, the eyecup was immediately transferred to a microcentrifuge tube containing 200 μL of ice-cold RNAprotect cell reagent (Cat. No. 76526; Qiagen Inc., Valencia, CA), with gentle manual tapping of the tube every 1 to 2 minutes to accelerate the release of RPE cells. After 10 minutes, the eyecup was removed from the solution using forceps. RPE cells were then centrifuged (5 minutes at 2750 rpm). The RNA isolation was performed using an RNeasy plus micro kit (Cat. No. 74034; Qiagen Inc.) according to the manufacturer's protocol.

Real-Time Quantitative PCR

Liver, NR, and RPE samples obtained from *Bmp6* KO and WT mice were analyzed using real-time quantitative PCR for gene expression, as described previously.[20] RNA isolation was performed using an RNeasy mini kit (Cat. No. 74106; Qiagen Inc.) according to the manufacturer's protocol. The RNA was quantified using a spectrophotometer and was stored at −80°C. cDNA was synthesized using TaqMan reverse transcription reagents (Cat. No. 808-0234; Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. TaqMan gene expression assays were obtained from Applied Biosystems and were used for PCR analysis. Probes included hepcidin antimicrobial peptide (Mm04231240_s1), transferrin receptor (Tfrc, Mm00441941_m1), L ferritin (Mm03030144_g1), von Willebrand factor homolog (Mm00550376_m1), retinal pigment epithelium 65 (Rpe65, Mm00504133_m1), and collagen, type VI, alpha 1 (Mm00487160_m1). Eukaryotic 18S rRNA (Hs99999901_s1) served as an internal control owing to its constant expression level among the studied sample sets. Real-time TaqMan RT-PCR (Applied Biosystems) was performed using an ABI Prism 7500 detection system and the $\Delta\Delta C_T$ method, which provided normalized expression values. The amount of target mRNA was compared among groups of interest. All reactions were performed in biological (three mice) and technical (three real-time PCR replicates per mouse) triplicates.

Statistical Analysis

The means ± SEM were calculated for each comparison group. Statistical analysis was performed using Student's two-group, two-sided *t*-test. For multiple comparisons, we used one-way analysis of variance with post hoc pairwise comparisons using Bonferroni correction for multiple comparisons. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism software version 5 (GraphPad Software Inc., San Diego, CA).

Results

Regulation of Hamp in NR in Response to Iron Status

Expression of *Hamp* in the liver, a key regulator of systemic iron metabolism, can be up-regulated by Bmp6.[3] Consistent with a previous report,[6] Hamp mRNA levels in the livers of male *Bmp6* KO mice at several ages were diminished compared with those in WT male mice (Figure 1, A–C).

Because the Bmp6 pathway can increase Hamp expression at the systemic level, to determine whether it can also regulate

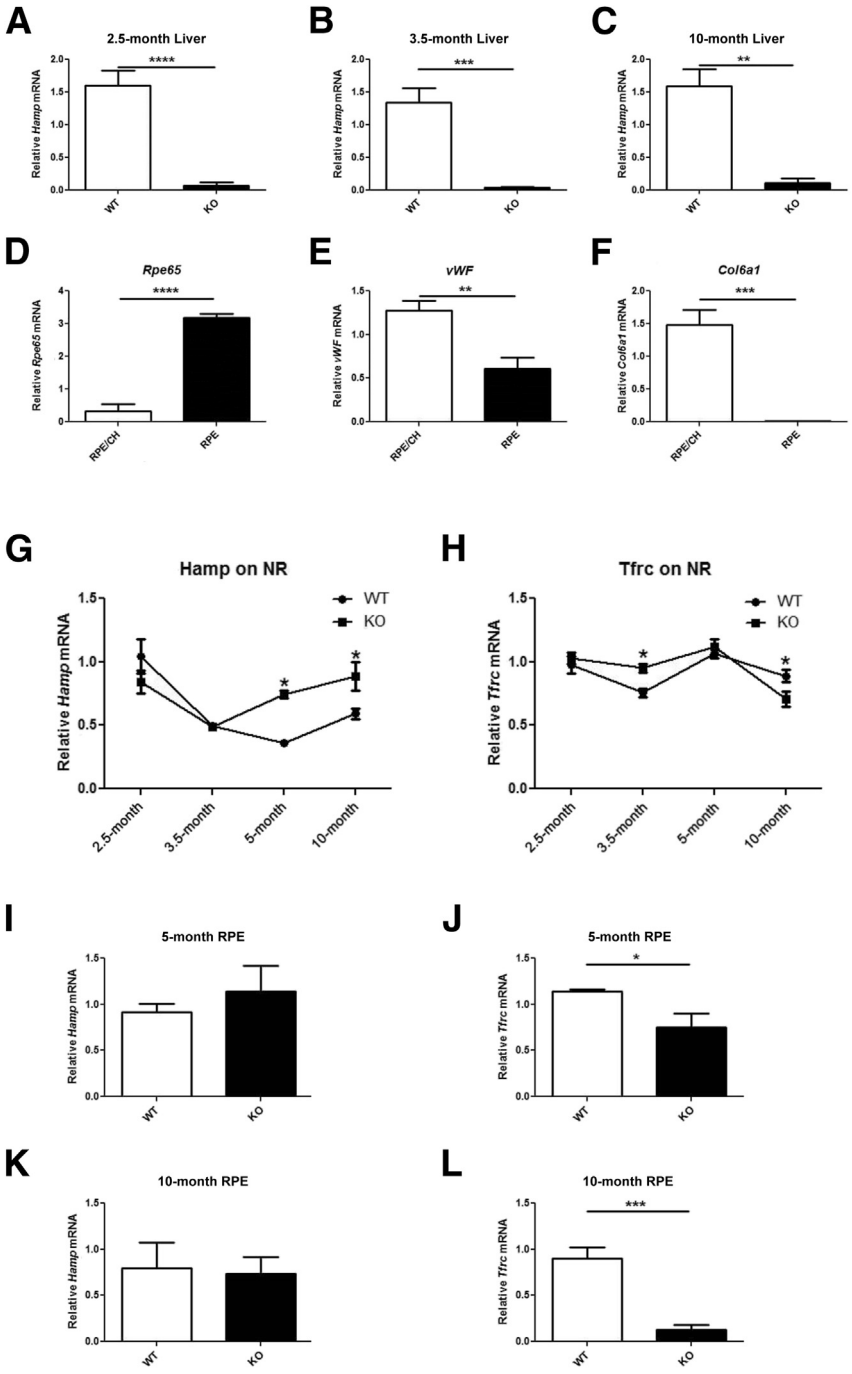

Figure 1 Quantitative PCR results in liver, NR, RPE/choroid (CH), and isolated RPE of Bmp6 KO and WT mice. Bmp6 KO mice had lower Hamp mRNA levels in liver than did age- and sex-matched WT controls at 2.5 months (**A**), 3.5 months (**B**), and 10 months (**C**) of age. Relative mRNA levels of RPE65 (**D**), von Willebrand factor homolog (vWF) (**E**), and collagen VI (Col6a1) (**F**) in RPE/CH versus isolated RPE. RPE samples had enriched RPE65, limited vWF, and barely detectable Col6a1 mRNA levels compared with RPE/CH. Relative mRNA levels of Hamp (**G, I,** and **K**) and Tfrc (**H, J,** and **L**) in NR of *Bmp6* KO and WT mice at different ages. **G:** Hamp mRNA levels in 2.5- and 3.5-month-old Bmp6 KO mice were not different from those in WT controls. However, Hamp mRNA levels were significantly higher in 5- and 10-month-old Bmp6 KO mice compared with WT controls. **H:** Tfrc mRNA levels in Bmp6 KO NR showed no significant difference at 2.5 and 5 months and was decreased at 3.5 and 10 months compared with age- and sex-matched WT mice. **I** and **K:** Hamp mRNA levels in RPE showed no significant difference between *Bmp6* KO mice and WT mice at 5 and 10 months. **J** and **L:** However, Tfrc mRNA levels showed a significant decrease in *Bmp6* KO mice compared with WT controls at 5 and 10 months. Values shown are means ± SEM of $\Delta\Delta C_T$. $N = 3$ biological and technical replicates for all real-time quantitative PCR experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

retinal Hamp we measured Hamp mRNA levels in NR and RPE. First, to test the level of RPE RNA enrichment in isolated RPE cells, we measured mRNA levels of RPE65, an RPE-specific marker, which was almost sixfold higher in purified RPE samples compared with the RPE/choroid samples. In addition, the von Willebrand factor homolog and collagen VI mRNA levels, which are expressed in choroid and sclera, were decreased significantly in purified RPE samples compared with RPE/choroid samples (Figure 1, D–F).

Hamp mRNA levels in NR showed no significant difference between *Bmp6* KO and WT mice at 2.5 and 3.5 months and increased in *Bmp6* KO compared with WT mice aged 5 to 10 months (Figure 1G). Tfrc mRNA levels in *Bmp6* KO NR showed no significant difference at 2.5 months, were decreased at 3.5 months, were unchanged at 5 months, and were decreased at 10 months (Figure 1H) in *Bmp6* KO mice compared with age- and sex-matched WT mice. Decreased Tfrc mRNA levels indicate elevated iron levels because increased intracellular iron prevents IRP1 and IRP2 from stabilizing Tfrc mRNA.[21]

Hamp mRNA levels in RPE showed no significant differences between *Bmp6* KO mice and WT mice at 5 and 10 months (Figure 1, I and K, respectively). Tfrc mRNA levels in RPE showed a significant decrease in *Bmp6* KO mice compared with WT mice (Figure 1, J and L).

Eyes from C57BL/6J Mice Treated with I.V. Iron Have Increased L Ferritin and Oxidative Stress Markers

The levels of the cytosolic iron storage protein ferritin are regulated by labile iron levels through iron regulatory proteins IRP1 and IRP2.[21] Accordingly, when labile iron levels rise, ferritin translation increases, leading to increasing levels of L ferritin and H ferritin proteins.[22] RPE cells showed L ferritin immunoreactivity that was stronger in mice treated with i.v. iron than in age-matched controls (Figure 2, A–D). Iron accumulation–induced lipid peroxidation was assessed by immunofluorescence detection of MDA and HNE. These are reactive intermediates in the formation of advanced lipoxidation end products. Thus, they are frequently measured as indicators of lipid peroxidation and oxidative stress. We detected an increase of MDA and HNE in the inner segment and outer plexiform layers of mice that were injected with iron compared with controls (Figure 2, E–H).

Hamp, L Ferritin, and Transferrin Receptor mRNA Levels in C57BL/6J Mice Treated with I.V. Iron

In C57BL/6J mice treated with three weekly i.v. iron sucrose injections, Hamp and L ferritin mRNA levels were significantly higher in the liver compared with control mice

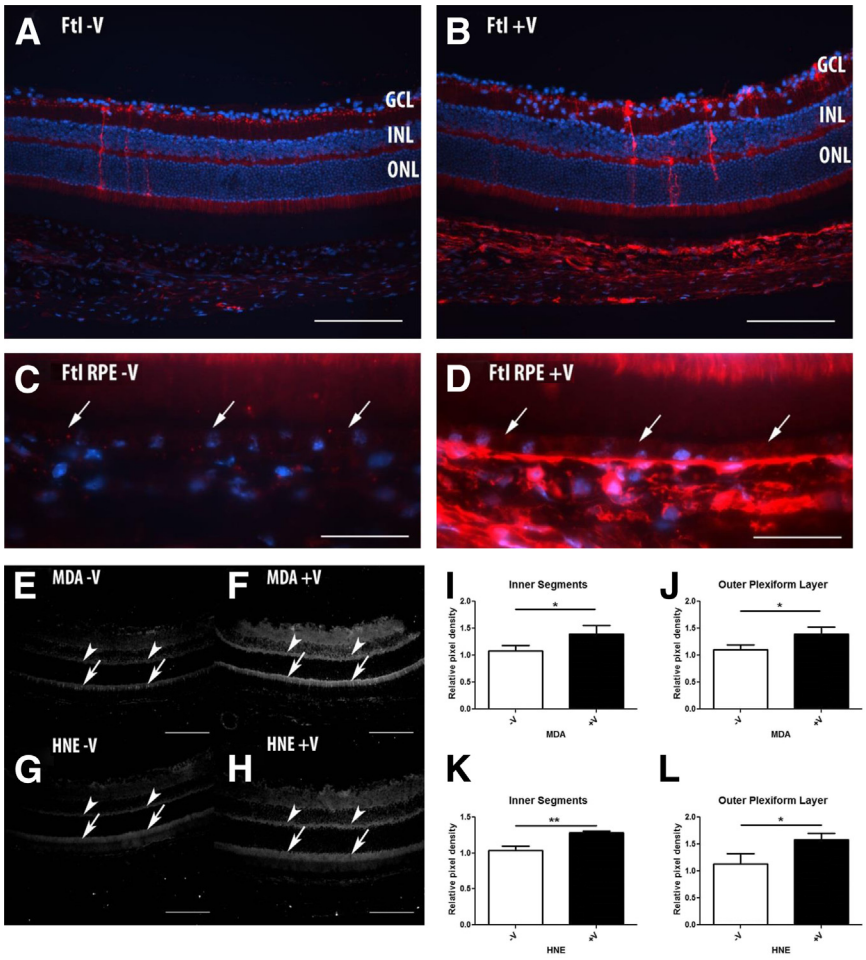

Figure 2 Iron labeling in RPE of i.v. iron sucrose–injected C57BL/6J mice. Iron level as determined by immunolabeling for L ferritin (Ftl; red) was greater in the RPE of iron sucrose (V)–injected mice (**B**) compared with controls (**A**). **C** and **D** show the high magnification (60× objective) of the RPE layer (**arrows**) in **A** and **B**, respectively. Oxidative stress levels determined by immunolabeling for MDA and HNE were greater in inner segments (**arrows**) and outer plexiform layers (**arrowheads**) with iron sucrose injection (**F** and **H**) compared with controls (**E** and **G**). **I–L:** Pixel density quantification of MDA and HNE immunostaining in the inner segments and outer plexiform layer of three mice with iron sucrose injection or controls. Statistical analysis was performed using Student's two-group, two-sided *t*-test. $*P < 0.05$, $**P < 0.01$. Scale bars: 100 μm (**A**, **B**, **E–H**); 33.3 μm (**C** and **D**). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

(Figure 3, A and C), whereas Tfrc mRNA levels were unchanged (Figure 3B). Hamp mRNA levels were not significantly different in NR and RPE of mice treated with or without iron (Figure 3, D and G). Tfrc mRNA levels were significantly lower in NR of iron-treated mice compared with control mice (Figure 3E), whereas L ferritin mRNA levels (a less sensitive measure of iron) were unchanged (Figure 3F). L ferritin mRNA levels were significantly higher in the RPE of iron sucrose–injected mice compared with controls, and Tfrc mRNA levels were significantly lower (Figure 3, H and I).

Discussion

Previous studies showed elevated serum and retinal iron levels in systemic *Hamp* and *Bmp6* KO mice.[5,7] To determine whether the retinal iron accumulation was due to loss of Bmp6-stimulated retinal production of Hamp, we measured Hamp mRNA in NR and RPE by real-time quantitative PCR in *Bmp6* KO mice. These results indicate an important influence of systemic Hamp (and, consequently, serum iron) levels on retinal iron. In *Bmp6* KO mice, liver but not retinal Hamp levels were diminished.

Bmp6 can up-regulate Hamp expression systemically, and we hypothesized that there was a similar local regulatory mechanism in the retina. Despite retinal expression of Bmp6 and its receptors, retinal Hamp expression was not diminished in *Bmp6* KO mice; it increased significantly. These results provide evidence that basal retinal Hamp expression is independent of the Bmp6 pathway. As the retina takes in more iron from the iron-rich blood in *Bmp6* KO mice, this iron seems to up-regulate Hamp expression. As a measure of retinal iron levels, Tfrc mRNA levels at 2.5 months were similar between KO and WT mice. Tfrc levels were decreased at age 3.5 months, indicating elevated retinal iron levels. At 5 months, Hamp expression in NR was increased in *Bmp6* KO mice, possibly diminishing the iron influx, as Tfrc levels were normal. However, at 10 months, Hamp levels were still higher in *Bmp6* KO mice than in age-matched WT mice, but Tfrc levels were lower in KO mice, suggesting that the increased Hamp levels were insufficient to prevent further iron influx.

In RPE, 5- and 10-month-old *Bmp6* KO mice had decreased Tfrc mRNA levels compared with age- and sex-matched WT mice. This reflects increased RPE iron levels caused by high systemic iron levels; a reduction in local retinal Hamp levels was not responsible because there was no difference in RPE Hamp mRNA levels between *Bmp6* KO mice and WT mice.

To further assess the effects of elevated systemic iron levels on retinal iron, we injected mice with i.v. iron sucrose, which is used in patients to treat iron deficiency anemia. After three weekly i.v. injections, we confirmed that liver Hamp mRNA levels increased, reflecting elevated hepatocyte iron levels. Tfrc levels did not change in the liver, suggesting that subpopulations of liver cells did not experience enough of an iron load to down-regulate Tfrc mRNA. In the RPE and NR of iron sucrose–treated mice, Tfrc mRNA levels decreased compared with controls. L ferritin mRNA levels increased in the RPE of iron sucrose–injected mice. These results show that in WT mice, high systemic iron levels caused increased iron levels in the RPE, and, to a lesser extent, NR (because only Tfrc mRNA and

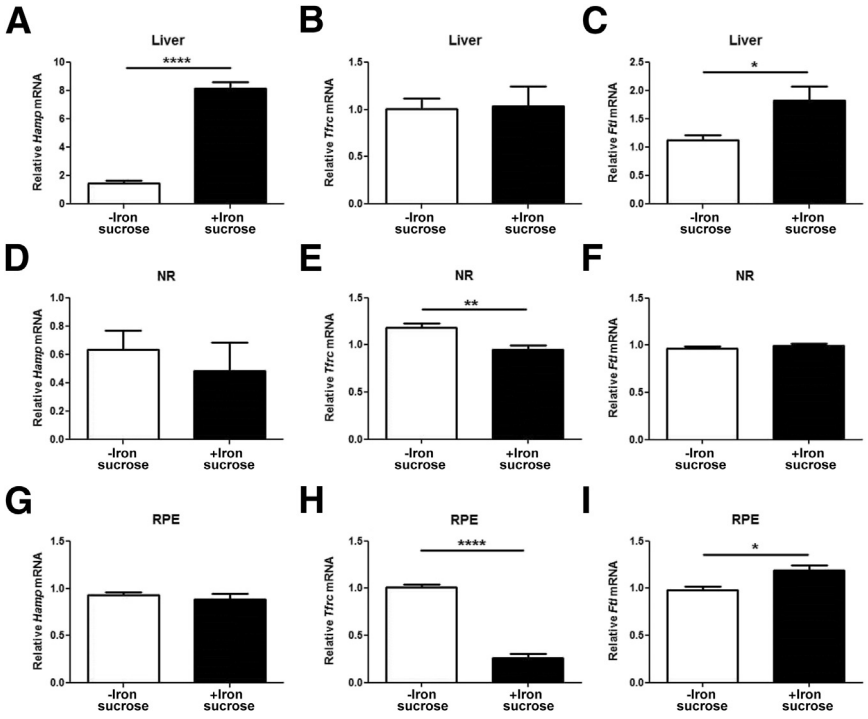

Figure 3 Quantitative PCR results in retina and RPE of i.v. iron sucrose–injected C57BL/6J mice. Relative mRNA levels of Hamp, Tfrc, and L ferritin (Ftl) in the liver (**A**–**C**), NR (**D**–**F**), and RPE (**G**–**I**) of mice with or without iron sucrose injection. Hamp (**A**) and Ftl (**C**) mRNA levels in liver were significantly higher in mice with iron sucrose injection compared with controls. **B:** Tfrc mRNA levels in liver of mice with or without iron sucrose injection were not different. Hamp (**D**) and Flt (**F**) mRNA levels in NR of mice with or without iron sucrose injection were not different. **E:** Tfrc mRNA levels were significantly lower in mice with iron sucrose injection compared with controls. **G:** Hamp mRNA levels in RPE of mice with or without iron sucrose injection were not different. **H:** Tfrc mRNA levels were significantly lower in the RPE of iron sucrose–injected mice compared with mice without iron sucrose injection. **I:** Ftl mRNA levels were significantly higher in the RPE of mice with iron sucrose injection compared with controls. Values shown are means ± SEM of $\Delta\Delta C_T$. $N = 3$ biological and technical replicates for all real-time quantitative PCR experiments. $^*P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$.

not the less sensitive measure L ferritin mRNA changed in the NR). Therefore, Hamp produced locally in the retina is insufficient to prevent retinal iron uptake in the context of increased blood iron levels. Although elevated iron levels in the liver are a strong stimulus for Hamp expression, after iron sucrose injection, Hamp mRNA levels in the NR were unaltered; the acute systemic iron challenge did not cause retinal Hamp up-regulation.

After i.v. iron injection, the oxidative stress markers MDA and HNE increase in the photoreceptor inner segments. The RPE accumulates more iron than the NR, but oxidative stress markers were not elevated in the RPE. It is likely that the RPE has a more robust antioxidant defense system than the photoreceptors.

These studies emphasize the importance of blood iron levels on retinal iron levels; elevated blood iron levels may cause increased retinal iron–induced oxidative stress levels. Elevated systemic iron levels, whether caused by genetics or diet, may increase the risk of age-related retinal disease, including age-related macular degeneration. In support of this possibility, people eating diets high in red meat, which causes high levels of iron absorption by the gut, have an increased risk of age-related macular degeneration.[23]

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