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Endothelial *Bmp2* knockout exacerbates hemochromatosis in *Hfe* knockout mice but not
Bmp6 knockout mice

Short title: Combined ablation of *Bmp2* and *Bmp6* or *Hfe*

Xia Xiao*, Som Dev*, Susanna Canali, Abraham Bayer, Yang Xu, Aneesh Agarwal, Chia-Yu Wang,
and Jodie L. Babitt[#]

Division of Nephrology, Program in Membrane Biology, Massachusetts General Hospital, Harvard
Medical School, Boston, MA

* Contributed equally

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#Contact Information

Jodie L. Babitt MD, Massachusetts General Hospital, 185 Cambridge St., CPZN-8208, Boston, MA 02114, Phone: (617)-643-3181, Fax: (617)-643-3182
Email: Babitt.jodie@mgh.harvard.edu

List of Abbreviations

Bone morphogenetic protein (BMP), knockout (KO), beta 2 microglobulin (B2M), wildtype (WT), phosphorylated SMAD5 (pSMAD5), analysis of variance (ANOVA), hepcidin mRNA (*Hamp*), hemojuvelin (HJV), transferrin (Tf), transferrin receptor 2 (TFR2)

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Conflict of Interest Disclosures

JLB has ownership interest in Ferrumax Pharmaceuticals and has received consulting fees from Keryx Biopharmaceuticals and Disc Medicine. All other authors have nothing to declare.

ABSTRACT

Bone morphogenetic proteins BMP2 and BMP6 play key roles in systemic iron homeostasis by regulating production of the iron hormone hepcidin. HFE also regulates hepcidin through a mechanism that intersects with the BMP-SMAD1/5/8 pathway. However, the relative roles of BMP2 compared with BMP6 and whether HFE regulates hepcidin through a BMP2-dependent mechanism remain uncertain. We therefore examined the iron phenotype of mice deficient for both *Bmp2* and *Bmp6* or both *Bmp2* and *Hfe* compared with single knockout (KO) mice and littermate controls. Eight-week-old double endothelial *Bmp6/Bmp2* KO mice exhibited a similar degree of hepcidin deficiency, serum iron overload, and tissue iron overload compared with single KO mice. Notably, dietary iron loading still induced liver SMAD5 phosphorylation and hepcidin in double *Bmp6/endothelial Bmp2* KO mice, although no other BMP ligand mRNAs were increased in the livers of double KO mice, and only *Bmp6* and *Bmp2* mRNA were induced by dietary iron loading in wildtype mice. In contrast, double *Hfe/endothelial Bmp2* KO mice exhibited reduced hepcidin and increased extrahepatic iron loading compared to single *Hfe* or endothelial *Bmp2* KO mice. Liver phosphorylated SMAD5 and the SMAD1/5/8 target *Id1* mRNA were also reduced in double *Hfe/endothelial Bmp2* KO compared with single endothelial *Bmp2* KO females. Finally, hepcidin and *Id1* mRNA induction by homodimeric BMP2, homodimeric BMP6, and heterodimeric BMP2/6 were blunted in *Hfe* KO primary hepatocytes. **Conclusion:** These data suggest that BMP2 and BMP6 work collaboratively to regulate hepcidin expression, that BMP2- and BMP6-independent SMAD1/5/8 signaling contributes a non-redundant role to hepcidin regulation by iron, and that HFE regulates hepcidin at least in part through a BMP2-independent, but SMAD1/5/8-dependent, mechanism.

Systemic iron homeostasis is regulated by the peptide hormone hepcidin and its receptor ferroportin, which control iron entry into circulation from dietary sources and body stores (1). Iron loading and inflammation stimulate the liver to produce and secrete hepcidin (2-4), which induces ferroportin degradation (5) to prevent iron overload and limit iron availability to pathogens. Iron deficiency and erythropoietic drive inhibit hepcidin production, thereby increasing iron availability for erythropoiesis (3). A failure of iron to appropriately upregulate hepcidin underlies the iron overload disorder hereditary hemochromatosis, whereas excessive hepcidin suppression by ineffective erythropoiesis contributes to the pathogenesis of iron loading anemias such as thalassemia (6, 7). A failure of iron deficiency anemia to appropriately suppress hepcidin underlies iron refractory iron deficiency anemia, whereas excessive hepcidin stimulation by chronic inflammation contributes to the pathogenesis of anemia of inflammation (7, 8).

The bone morphogenetic protein (BMP)-SMAD1/5/8 signaling pathway is a central mechanism for regulating hepcidin production in response to its main physiologic signals, including iron and erythropoietic drive (1, 9-11). Two BMP ligands have been identified to play critical roles in regulating hepcidin expression: BMP6 (12, 13) and BMP2 (14, 15). A key role for BMP6 was first suggested by a microarray analysis in mice demonstrating that liver *Bmp6* mRNA expression was regulated by dietary iron concordantly with hepcidin (16). Global *Bmp6* KO mice were subsequently found to have hemochromatosis, characterized by hepcidin deficiency, serum iron overload, tissue iron overload in the liver, heart, and pancreas, and reduced spleen iron (12, 13). Liver endothelial cells were then recognized as the key source for BMP6 with a hemochromatosis phenotype reported in conditional endothelial *Bmp6* KO mice (17). In humans, heterozygous mutations in the *BMP6* prodomain have also been associated with inappropriately low hepcidin and iron overload (18), although the functional impact of *BMP6* prodomain mutations in humans is still controversial (19). A role for BMP2 was hypothesized when it was observed that the residual ability of dietary iron to induce hepcidin expression in *Bmp6* KO mice was associated with an induction in liver SMAD1/5/8 signaling and was prevented by a neutralizing BMP2/4 antibody (14). A functional role for BMP2 was confirmed by the observation that endothelial *Bmp2* KO mice developed hemochromatosis due to low hepcidin expression (14, 15). Moreover, dietary iron was found to regulate liver *Bmp2* mRNA and circulating BMP2 protein levels concordantly with hepcidin (14, 20). Similar to *Bmp6* KO mice, endothelial *Bmp2* KO mice also exhibited a residual ability for iron to induce SMAD5 phosphorylation and hepcidin expression (20). An association between a common SNP in the *BMP2* gene and serum ferritin has

also been reported in humans with hemochromatosis due to mutations in *HFE* (21), the most common cause of hereditary hemochromatosis (22).

The precise molecular mechanisms by which *HFE* mutations cause hemochromatosis are still not fully understood. *HFE* is thought to regulate hepcidin production in response to iron by intersecting with BMP-SMAD1/5/8 signaling downstream or independent of BMP6 since liver *Bmp6* mRNA was appropriately induced by iron overload, but liver SMAD1/5/8 signaling was inappropriately reduced relative to *Bmp6* mRNA and liver iron levels in *Hfe* KO mice (23-25). Moreover, liver SMAD1/5/8 signaling was induced in parallel with hepcidin in mice overexpressing an *Hfe* transgene in the liver (26). Finally, a functional loss of *HFE* (due to ablation of the *HFE* localizing protein beta-2-microglobulin, B2M) further lowered liver SMAD1/5/8 signaling, worsened hepcidin deficiency, and worsened iron overload in *Bmp6* KO mice, suggesting a BMP6-independent mechanism for *HFE* to intersect with the SMAD pathway to regulate hepcidin (27).

Here, we generated littermate mice with an endothelial KO of *Bmp2* alone, an endothelial or global KO of *Bmp6* alone, both *Bmp2* and *Bmp6*, or neither to determine the relative roles of BMP2 and BMP6 in hepcidin regulation and systemic iron homeostasis. We also tested whether there is any residual pathway for iron-mediated hepcidin induction in mice with a combined global *Bmp6* and endothelial *Bmp2* KO. Finally, we generated littermate mice with an endothelial KO of *Bmp2* alone, a global KO of *Hfe* alone, both, or neither to determine whether *HFE* regulates hepcidin through a BMP2-dependent pathway.

EXPERIMENTAL PROCEDURES

Animals

Animal protocols were approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital. Littermate mice with a single endothelial KO of *Bmp2* (*Bmp2^{fl/fl};Bmp6^{wt/wt};Tek-Cre+*), a single endothelial KO of *Bmp6*, (*Bmp2^{wt/wt};Bmp6^{fl/fl};Tek-Cre+*), a double endothelial KO of *Bmp2* and *Bmp6* (*Bmp2^{fl/fl};Bmp6^{fl/fl};Tek-Cre+*), and controls (*Bmp2^{wt/wt};Bmp6^{wt/wt};Tek-Cre+*) on a mixed C57BL/6, 129S6/SvEv, 129S4/SvJae background were generated by crossing *Bmp2* KO males (*Bmp2^{fl/fl};Tek-Cre+*)(14) with endothelial *Bmp6* floxed females (*Bmp6^{fl/fl};Tek-Cre-*)(17) as described in Figure S1A. Littermate single *Bmp6* KO mice (*Bmp6^{-/-};Bmp2^{fl/fl};Tek-Cre-*) and double global *Bmp6* and endothelial *Bmp2* KO mice (*Bmp6^{-/-};Bmp2^{fl/fl};Tek-*

Cre+) on a mixed C57BL/6, 129S6/SvEv, 129S4/SvJae background were generated by crossing *Bmp2^{fl/fl};Tek-Cre+* males with global *Bmp6* KO females (*Bmp6^{-/-}*) (17) as described in Figure S1B. Littermate mice with a single endothelial KO of *Bmp2* (*Hfe^{wt/wt};Bmp2^{fl/fl};Tek-Cre+*), a single global KO of *Hfe*, (*Hfe^{-/-};Bmp2^{fl/fl};Tek-Cre-*), a double endothelial KO of *Bmp2* and global KO of *Hfe* (*Hfe^{-/-};Bmp2^{fl/fl};Tek-Cre+*), and controls (*Hfe^{wt/wt};Bmp2^{fl/fl};Tek-Cre-*) on a mixed C57BL/6, 129S4/SvJae background were generated by crossing *Bmp2^{fl/fl};Tek-Cre+* males with *Hfe* KO females (*Hfe^{-/-}*) (28) as described in Figure S1C. Mice were genotyped as previously described (14, 17, 28). All mice had *ad libitum* access to water and a house diet (Prolab 5P75 Isopro RMH 3000 containing 380 ppm iron) after weaning unless otherwise indicated.

For dietary iron experiments in *Bmp6^{-/-};Bmp2^{fl/fl};Tek-Cre-* and *Bmp6^{-/-};Bmp2^{fl/fl};Tek-Cre+* mice, rodents received a low iron diet (2-6 ppm iron, Envigo #TD.80396) at weaning for 3 weeks to prevent the iron overload that develops on a standard rodent diet, and then either kept on the low iron diet for 1 more week or switched to the house diet. To examine the regulation of BMP ligands by dietary iron, heterozygous *Bmp6^{wt/-}* mice (17) backcrossed for 10 generations to a C57BL/6N (Taconic) background were intercrossed to generate *Bmp6^{-/-}* knockout and *Bmp6^{wt/wt}* wildtype littermates. Dietary iron challenge experiments were also performed in wildtype (WT) offspring from heterozygous *Hfe^{wt/-}* mice that were originally on a C57BL/6J background (28) but have been breeding in our laboratory for many years, and WT C57BL/6J and C57BL/6NTac mice purchased from Jackson and Taconic respectively. Four- to five-week-old male and female mice were placed on a matched, purified low iron (Low Fe, 2-6 ppm iron, Envigo #TD.80396), iron sufficient (Control, 48 ppm iron, Envigo #TD.80394), or a high iron (High Fe, 2% carbonyl iron, Envigo #TD.08496) diet for 3 weeks.

RNA Isolation, Reverse Transcription, and qRT-PCR

Total liver RNA was isolated using QIAshredder and Purelink RNA mini kits (Invitrogen). First-strand cDNA was synthesized from 1 µg RNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). PCR reactions were performed using the PowerUp SYBR Green Master Mix on the QuantStudio3 Real-Time PCR system (Applied Biosystems) using primers in Supplemental Table S1. Transcript levels were determined as previously described using the standard curve method (10).

Iron Analysis

Serum iron and unsaturated iron binding capacity were measured by colorimetric assay (Pointe Scientific) to calculate transferrin saturation according to manufacturer's instructions. Tissue nonheme iron concentrations (in µg/g wet weight) were determined as described previously (10).

Immunoblot

Liver lysates were prepared and immunoblots performed as described previously (10) using rabbit anti-phosphorylated SMAD5 (pSMAD5; 1:500; ab92698; Abcam), rabbit anti-SMAD5 (1:1000; ab40771; Abcam), or mouse anti-actin (1:20,000 MAB1501; Millipore) antibodies. Immunoreactivity was visualized by chemiluminescence (SuperSignal West Pico, Thermo Scientific) and the G:box mini digital darkroom (Syngene). Quantification was performed using ImageJ 1.46v.

BMP2 ELISA

Serum BMP2 was measured using a BMP2 ELISA Kit (R&D Systems DBP200, lots P178778, P194447, P208993) in 1:1 dilution as previously described (20).

Statistics

Statistical significance was determined by 2-tailed Student's *t*-test or one-way analysis of variance (ANOVA) with Tukey's post hoc test for pairwise multiple comparisons using Prism 7 (GraphPad). Data with unequal variances were log transformed prior to statistical analysis. *P* < 0.05 was considered significant.

RESULTS

Double endothelial KO of *Bmp2* and *Bmp6* does not exacerbate hemochromatosis compared with single KO mice

To determine the relative roles of BMP2 and BMP6 in hepcidin and iron homeostasis regulation, we generated littermate mice with a single endothelial KO of *Bmp2* (*Bmp2*^{fl/fl};*Bmp6*^{wt/wt};*Tek-Cre*+), a single endothelial KO of *Bmp6* (*Bmp2*^{wt/wt};*Bmp6*^{fl/fl};*Tek-Cre*+), a double endothelial KO of *Bmp2* and *Bmp6* (*Bmp2*^{fl/fl};*Bmp6*^{fl/fl};*Tek-Cre*+), and controls (*Bmp2*^{wt/wt};*Bmp6*^{wt/wt};*Tek-Cre*+), and analyzed their hepcidin levels and iron phenotype at 8 weeks of age. Hepcidin and iron parameters were analyzed separately in males and females due to previously established sex differences in mice (14, 17, 29).

All KO mice exhibited reduced liver expression of hepcidin (*Hamp*) mRNA and the BMP-SMAD1/5/8 target transcript *Id1* (Figure 1A, S2A-B), increased serum and liver iron (Figure 1B-C, S2C), and reduced spleen iron (Figure 1D) compared to control mice. Although the hepcidin reduction in female double endothelial *Bmp2/Bmp6* KO mice did not quite reach statistical significance compared to controls by multivariable analysis, this was significantly lower relative to the higher degree of iron loading in these mice (Figure S2A). All male KO mice also exhibited increased extrahepatic iron loading in the pancreas and heart compared to controls, whereas female KO mice had minimal or no increases in extrahepatic iron that reached statistical significance only for pancreas in single KO mice (Figure 1E-F). In general, male KO mice appeared to have lower hepcidin and increased extrahepatic iron overload than female KO mice, consistent with prior reports in single *Bmp6* and endothelial *Bmp2* KO mice (14, 17, 29), although the current study was not powered to compare differences between sexes in addition to genotype. Notably, liver *Hamp*, liver *Id1*, serum iron, liver iron, and extrahepatic iron levels were not significantly different in sex-matched single endothelial *Bmp6* compared with single endothelial *Bmp2* KO mice. Moreover, none of these parameters was significantly different in double KO mice compared to sex-matched single KO mice (Figures 1, S2). Together, these results suggest that under baseline conditions, BMP2 and BMP6 predominantly work together to regulate hepcidin expression and iron homeostasis.

BMP2 is not induced in *Bmp6* KO mice despite iron overload

Total liver *Bmp6* and *Bmp2* mRNA levels were appropriately reduced by 92-96% in the respective single endothelial *Bmp6*, single endothelial *Bmp2*, and double endothelial *Bmp2/Bmp6* KO mice (Figures 2A, 2B left). The reduction in liver *Bmp2* mRNA also corresponded to a reduction in circulating BMP2 protein levels in single and double endothelial *Bmp2* KO mice (Figure 2B right) as previously reported (20). Liver *Bmp6* mRNA levels were induced in single endothelial *Bmp2* KO mice (Figure 2A) as previously reported (14), presumably as a response to iron overload. However, although liver *Bmp2* mRNA was induced by dietary iron loading in wildtype mice (Figure 2D, left), as previously reported (14, 20), liver *Bmp2* mRNA was not increased in the single endothelial or global *Bmp6* KO mice, either on a house or a high iron diet (Figures 2B left, 2D left) despite iron overload (Figures 1, 2C). Surprisingly, in contrast to our previous findings (20), dietary iron failed to regulate serum BMP2 protein levels in multiple cohorts of wildtype mice (Figures 2D right, 2F right), despite a consistent regulation of serum iron, liver iron, liver *Hamp* mRNA, liver *Bmp6* mRNA, and liver *Bmp2* mRNA that were similar to our prior findings (Figures 2C-F, S3) (14, 20). Serum BMP2 protein was

also not changed in endothelial or global *Bmp6* KO mice despite iron overload (Figure 2B right, 2D right). Thus, *Bmp6* appears to be required for iron-mediated liver *Bmp2* mRNA induction, although the impact of dietary iron on liver *Bmp2* mRNA is quite modest compared with *Bmp6*, and dietary iron alone is not sufficient to regulate circulating BMP2 protein levels.

Liver SMAD5 phosphorylation and hepcidin expression are still inducible by iron in mice with a combined global *Bmp6* and endothelial *Bmp2* KO

We previously reported that chronic dietary iron loading still induced liver SMAD phosphorylation and hepcidin expression in single *Bmp6* global KO mice and single *Bmp2* endothelial KO mice, and that BMP neutralizing antibodies inhibited this effect in *Bmp6* KO mice, suggesting a residual pathway for iron-mediated, BMP/SMAD-dependent hepcidin induction in the absence of BMP2 or BMP6 (14, 20). We therefore sought to determine whether there was any residual pathway for iron-mediated hepcidin expression in mice with a combined loss of *Bmp2* and *Bmp6*. Although the endothelial KO mice provide the most balanced comparison of the relative effects of BMP2 and BMP6 (since embryonic lethality precludes utilizing global *Bmp2* KO mice (30)), the 98% recombination efficiency of the Tek-Cre in liver endothelial cells results in low level residual liver BMP ligand expression in this model (17). To minimize this impact, we generated littermate mice with a single global *Bmp6* KO (*Bmp6*^{-/-};*Bmp2*^{fl/fl};*Tek-Cre*-) or a combined global *Bmp6* and endothelial *Bmp2* KO (*Bmp6*^{-/-};*Bmp2*^{fl/fl};*Tek-Cre*+) mice. Analogous to the results in endothelial conditional KO mice, double *Bmp6*^{-/-};*Bmp2*^{fl/fl};*Tek-Cre* mice exhibited similar liver *Hamp* mRNA, serum iron, liver iron, spleen iron, and extrahepatic iron levels in the heart and pancreas compared to single *Bmp6*^{-/-};*Bmp2*^{fl/fl};*Tek-Cre*- mice at 8 weeks of age (Figure S4).

To test the response to iron loading, single *Bmp6*^{-/-};*Bmp2*^{fl/fl};*Tek-Cre*- and double *Bmp6*^{-/-};*Bmp2*^{fl/fl};*Tek-Cre* mice were weaned to a low iron diet (2-6 ppm) to prevent the iron overload induced by a house diet (14). After 3 weeks, mice were either kept on the low iron diet or switched to the house diet containing 380 ppm iron for 1 week. Serum iron and liver iron levels were robustly induced by the switch to the higher iron house diet in both genotypes (Figure 3A-C), along with a slight increase in pancreas iron (Figure S5). Similar to prior reports (14, 31), liver phosphorylated SMAD5 protein and *Hamp* mRNA were both significantly induced by the house diet in single *Bmp6*^{-/-};*Bmp2*^{fl/fl};*Tek-Cre*- mice (Figure 3D-E). Notably, the house diet also significantly induced liver phosphorylated SMAD5 protein and *Hamp* mRNA in double *Bmp6*^{-/-};*Bmp2*^{fl/fl};*Tek-Cre* mice (Figure

3D-E). Similar findings were seen in single *Bmp6*^{-/-};*Bmp2*^{fl/fl};*Tek-Cre*- and double *Bmp6*^{-/-};*Bmp2*^{fl/fl};*Tek-Cre*⁺ mice switched to a matched purified iron sufficient diet (48 ppm iron) where iron is the only dietary component changed compared to the low iron diet (Figure S6).

The ability of dietary iron to induce SMAD5 phosphorylation and hepcidin in double *Bmp6*^{-/-};*Bmp2*^{fl/fl};*Tek-Cre*⁺ mice suggests that another BMP ligand may contribute to iron-mediated hepcidin induction. We therefore examined whether any other BMP ligand was increased in endothelial *Bmp2* and/or *Bmp6* KO mice or was regulated by dietary iron in WT mice. In contrast to liver *Bmp2* and *Bmp6* mRNA, which were consistently reduced by a low iron diet and increased by a high iron diet (Figure 2E-F), liver *Bmp4*, *Bmp5*, *Bmp7*, and *Bmp9* mRNA were not concordantly regulated by dietary iron in WT mice (Figure S7, left). In fact, *Bmp4*, *Bmp5*, and *Bmp9* were reduced by a high iron diet. Similarly, no BMPs were induced despite iron overload in single or double endothelial *Bmp2* and/or *Bmp6* KO mice (Figure S7, right).

Combined global *Hfe* and endothelial *Bmp2* KO lowers hepcidin and worsens extrahepatic iron overload compared with single KO mice

HFE was previously proposed to stimulate hepcidin through a pathway that intersects with the BMP-SMAD signaling cascade, but downstream or independent of BMP6 (23-25, 32, 33). To determine if HFE-mediated hepcidin regulation is dependent on BMP2, we generated littermate mice with a single endothelial KO of *Bmp2* (*Hfe*^{wt/wt};*Bmp2*^{fl/fl};*Tek-Cre*⁺), a single global KO of *Hfe*, (*Hfe*^{-/-};*Bmp2*^{fl/fl};*Tek-Cre*⁻), a double endothelial KO of *Bmp2* and global KO of *Hfe* (*Hfe*^{-/-};*Bmp2*^{fl/fl};*Tek-Cre*⁺), and controls (*Hfe*^{wt/wt};*Bmp2*^{fl/fl};*Tek-Cre*⁻), and analyzed their iron phenotype. *Hamp* mRNA levels were significantly reduced in single endothelial *Bmp2* KO mice compared to controls, whereas *Hamp* mRNA was only reduced in *Hfe* KO mice when analyzed relative to liver iron content (Figure 4A). This was associated with significantly increased serum iron, transferrin saturation, and liver iron in all single KO mice (Figure 4B-C). Spleen iron was significantly reduced in endothelial *Bmp2* KO females, although there was only a trend toward reduction in males and no significant change in female or male *Hfe* KO mice (Figure 4D). Male endothelial *Bmp2* KO mice had significant extrahepatic iron loading in both pancreas and heart, whereas little or no extrahepatic iron loading was seen in *Hfe* KO males or single KO females of either genotype (Figures 4E-F). The overall hemochromatosis phenotype was worse in the single endothelial *Bmp2* KO mice compared with *Hfe* KO mice as evidenced by significantly increased transferrin saturation and liver iron in both sexes (Figure 4B-C) and

significantly lower hepcidin, lower spleen iron, and increased heart and pancreas iron in males (Figures 4A, D-F). Notably, *Hamp* mRNA levels were markedly lower in double *Hfe*/endothelial *Bmp2* KO mice compared to controls (~1/5000 lower in females, ~1/120 in males) or single KO mice (~1/3000 lower in females, ~1/24-1/130 in males) (Figure 4A). Moreover, this was associated with significantly increased iron overload in double KO mice compared with controls and single KO mice, as evidenced by higher transferrin saturation and/or serum iron in double KO mice compared with controls or *Hfe* KO mice (Figure 4B), increased pancreas and heart iron in double KO mice compared with controls or single KO mice (Figure 4E-F), and increased liver iron in double KO compared with control or single KO females (Figure 4C). Liver *Bmp2* mRNA and circulating BMP2 protein were appropriately reduced in the respective KOs (Figure S8). Similar to *Bmp6* KO mice, no increase in liver *Bmp2* mRNA or serum BMP2 protein was seen in *Hfe* KO mice despite iron overload (Figure S8). Together, these data demonstrate a worse hemochromatosis phenotype in double *Hfe*^{-/-};*Bmp2*^{fl/fl};*Tek-Cre*⁺ mice compared with single KO mice, suggesting that HFE can function independent of BMP2 in hepcidin and iron homeostasis regulation. Data from the single KO mice also suggest that BMP2 has a stronger impact on hepcidin and iron homeostasis regulation than HFE.

Interestingly, liver SMAD5 phosphorylation and *Id1* mRNA levels were significantly lower in female double *Hfe*/endothelial *Bmp2* KO mice compared with single KO mice, with similar trends in males (Figure 5). Although SMAD5 phosphorylation was not significantly lower in either single KO strain compared with controls, and *Id1* mRNA was not lower in *Hfe* KO mice compared with controls, both were inappropriately lower relative to the higher body iron burden of these mice (Figure 5) as previously reported (23). Induction of *Hamp* and *Id1* mRNA by BMP6 homodimer, BMP2 homodimer, and BMP2/6 heterodimer were all blunted in *Hfe*^{-/-} primary hepatocytes compared with WT hepatocytes (Figure S9). These data are consistent with a functional role for HFE in inducing hepcidin by enhancing the SMAD1/5/8 signaling response to BMP ligands, and suggest that the BMP2-independent function is still SMAD1/5/8 dependent.

DISCUSSION

BMP2 and BMP6 have key roles in regulating hepcidin expression and systemic iron balance since mice with an endothelial KO of either of these genes exhibit hemochromatosis. However, their relative roles have remained uncertain. Here, we show that littermate mice with a single endothelial KO of *Bmp2* or *Bmp6* alone had similar levels of hepcidin deficiency, serum iron loading and liver iron loading. Surprisingly, a combined KO of endothelial *Bmp2* and *Bmp6* did not significantly worsen the hemochromatosis phenotype of single KO mice. Similarly, a KO of endothelial *Bmp2* did not significantly worsen the hemochromatosis phenotype of global *Bmp6* KO mice. These data suggest that under iron adequate or overload conditions, BMP2 and BMP6 mainly work together to regulate hepcidin expression and systemic iron homeostasis and that neither endogenously expressed BMP2 or BMP6 appreciably compensates for the loss of the other.

The results in double global or endothelial *Bmp6*/endothelial *Bmp2* KO mice are in sharp contrast to double *Hfe*/endothelial *Bmp2* KO mice, where there was a robust exacerbation of hepcidin deficiency and extrahepatic iron overload compared with single *Hfe* or endothelial *Bmp2* KO mice. Single endothelial *Bmp2* KO mice also exhibited a stronger overall hemochromatosis phenotype compared with single *Hfe* KO mice, particularly in males. Additionally, multiple prior studies have demonstrated a clear exacerbating impact on hepcidin deficiency and extrahepatic iron overload in mice with a double KO of *Bmp6* and/or other hemochromatosis genes including *Hfe* or its chaperone *B2m*, *Tfr2* (encoding transferrin receptor 2), and *Hjv* (encoding hemojuvelin) compared to the respective single KO mice on a standard diet (25, 27, 34). These findings demonstrate the feasibility of detecting an exacerbating hemochromatosis phenotype in mice with a global *Bmp6* KO or endothelial *Bmp2* KO on a standard rodent diet. Moreover, these results suggest that whereas BMP2 and BMP6 predominantly function together in hepcidin regulation, BMP2 and HFE can function independently. Previous publications also support an at least partially independent role for BMP6 and HFE, TFR2, and HJV (27, 34).

A limitation of our study is that we focused on mice receiving a standard rodent diet at 8 weeks of age. It is therefore possible that more subtle differences between double and/or single endothelial *Bmp2* and/or *Bmp6* KO mice might be uncovered in younger or older mice, under different iron loading or deficiency conditions, or in response to other treatments. Indeed, there was a slight tendency for increased extrahepatic iron overload in double endothelial or global *Bmp6/Bmp2* KO mice compared with sex-matched single KO mice (Fig. 1E-F, S4E-F). There was also a tendency for lower hepcidin expression and higher serum and liver iron levels in mice with a combined global

Bmp6/endothelial *Bmp2* KO compared with single *Bmp6* KO mice maintained on a low iron diet (Figure 3). We also previously reported that when maintained on a low iron diet and then challenged with acute oral iron gavage that increased serum iron without increasing liver iron, endothelial *Bmp2* KO mice had a modest residual ability to induce hepcidin, whereas endothelial or global *Bmp6* KO mice failed to induce hepcidin (20). This prior study was limited by the use of mice with different mixed background strains, which may have contributed to the apparent differences reported (20). A potential complicating feature of the *Bmp2* and *Bmp6* endothelial KO models is the small residual BMP ligand expression due to the 98% efficiency of Tek-Cre-mediated recombination (14, 17), an unavoidable limitation due to embryonic lethality of global *Bmp2* KO mice (30). However, total liver *Bmp6* and *Bmp2* mRNA levels were markedly reduced by ≥ 92 -95% in the corresponding Tek-Cre mice, leading to a >60 -95% reduction in total liver and circulating BMP2 protein levels (14, 17, 20). Moreover, we previously reported that the phenotype of endothelial *Bmp6* KO mice was only slightly less severe than global *Bmp6* KO mice (17), and in the current study, we obtained similar results in double endothelial *Bmp6/Bmp2* KO mice and double global *Bmp6*/endothelial *Bmp2* KO mice. Importantly, the robust worsening of hepcidin deficiency and extrahepatic iron overload in the double *Hfe*/endothelial *Bmp2* KO mice compared with single *Hfe* or endothelial *Bmp2* KO mice demonstrate the feasibility of detecting an exacerbating hemochromatosis phenotype in mice with an endothelial ablation of *Bmp2*, even if it is not a full knockout. Taken together, our findings in the double KO mice demonstrate clear distinctions in the relative roles of BMP2 and BMP6, which predominantly work together in hepcidin regulation, and BMP2 and HFE, which can function independently; however, we cannot rule out a more subtle ability of BMP2 and/or BMP6 to work independently from the other to regulate hepcidin under some conditions.

Data in the double *Bmp6*/endothelial *Bmp2* KO mice reveal the existence of another mechanism for iron-mediated hepcidin induction in the absence of both BMP6 and BMP2. We previously hypothesized that BMP2 accounts for the residual ability of iron to induce hepcidin in single *Bmp6* knockout mice since a neutralizing BMP2/4 antibody inhibited hepcidin induction by iron in *Bmp6* KO mice and endothelial *Bmp2* KO mice exhibit hemochromatosis (14). However, we previously found that the neutralizing BMP2/4 antibody was not specific, but inhibited all BMPs aside from BMP9 (14). Moreover, in the current study, the additional deletion of endothelial *Bmp2* in *Bmp6* KO mice did not impact the ability of iron to induce hepcidin compared to single *Bmp6* KO mice (Figure 3D). Although this is not a full *Bmp2* KO, endothelial *Bmp2* KO removes the majority of liver *Bmp2* mRNA, liver

BMP2 protein, and circulating BMP2 protein as discussed above, which is sufficient to cause hepcidin deficiency and iron overload on its own, and exacerbate hepcidin deficiency and hemochromatosis in *Hfe* KO mice. The lack of any impact of endothelial *Bmp2* KO on iron-mediated hepcidin induction in *Bmp6* KO mice makes it unlikely that the low level of residual BMP2 expression in the double KO mice accounts for the robust residual iron-mediated hepcidin induction. The most likely mechanism involves another BMP ligand or ligands given the strong SMAD pathway activation by iron in the double *Bmp6*/endothelial *Bmp2* KO mice and the complete inhibition of iron-mediated hepcidin induction in *Bmp6* KO mice by the pan-neutralizing BMP2/4 antibody (14). In contrast to BMP2 and BMP6, no other BMP ligand mRNAs were increased by dietary iron loading in wildtype mouse livers. We also did not find increased BMP ligand mRNAs in the livers of iron overloaded endothelial *Bmp2* and/or *Bmp6* KO mice. This does not preclude a role for these other BMPs in hepcidin and iron homeostasis regulation since BMP ligand expression and/or activity can be regulated at many other levels. Future studies will be needed to identify which other BMP ligands(s) contribute to hepcidin and iron homeostasis regulation and how their expression or activity is modified by iron.

Interestingly, combined *Hfe* and endothelial *Bmp2* ablation reduced liver SMAD5 phosphorylation and *Id1* mRNA expression compared with endothelial *Bmp2* ablation alone. Although liver SMAD5 phosphorylation and *Id1* mRNA levels in *Hfe* KO mice were not significantly different from controls, these were inappropriately low relative to the degree of iron overload as previously reported (23), since chronic dietary iron loading normally induces liver SMAD1/5/8 phosphorylation and *Id1* mRNA expression in wildtype mice (9, 16). A reduction in liver phosphorylated SMAD5 expression has also been reported for mice with a double KO of *Bmp6* and the HFE-localizing protein *B2m* compared with single *Bmp6* KO mice (27). These data support the hypothesis that HFE regulates hepcidin by intersecting with the SMAD pathway, even in the absence of BMP2 or BMP6, potentially by modulating signaling by another BMP ligand. Prior studies using *in vitro* overexpression systems have suggested that this functional interaction might involve a physical interaction between HFE and the BMP type I receptor ALK3 (35) and/or the BMP co-receptor hemojuvelin/HJV (36). These models await further validation *in vivo*. Notably, although HFE has some ability to regulate hepcidin independent of BMP2 and BMP6, it remains possible that HFE also contributes to hepcidin regulation by BMP2 and BMP6 when they are available. Indeed, hepcidin induction by homodimeric BMP6,

homodimeric BMP2, and heterodimeric BMP2/6 was impaired in *Hfe*^{-/-} primary hepatocytes compared to wildtype hepatocytes, similar to a prior report for BMP6 (23).

Although liver *Bmp2* mRNA failed to be appropriately induced despite iron overload in both *Bmp6* and *Hfe* KO mice, it is unlikely that this plays a major role in the pathogenesis of hemochromatosis in these mice. *Bmp2* mRNA upregulation by dietary iron is quite modest (~30%) compared with *Bmp6* (~5-fold). Additionally, although we previously found that serum BMP2 protein was upregulated in mice fed a high iron diet (20), the high iron diet failed to upregulate serum BMP2 protein in multiple cohorts of wildtype mice in the current study despite consistent increases in serum iron, liver iron, liver *Hamp*, *Bmp6*, and *Bmp2* mRNA. Although the reason for the different findings in these studies is unclear, the current study demonstrates that changes in dietary iron alone are not sufficient to regulate serum BMP2 levels. Importantly, prior quantitative copy number analysis showed that *Bmp2* mRNA is much more highly expressed than *Bmp6* in liver endothelial cells both under control and high iron conditions, at least in mice (14). Thus, if BMP2 and BMP6 predominantly work together, as suggested by the findings in the double endothelial *Bmp6* and *Bmp2* KO compared with single KO mice, BMP6 expression would be the limiting factor, and a modest change in *Bmp2* mRNA expression would be unlikely to have major functional significance.

An unresolved question is why BMP2 and BMP6 predominantly function together to regulate hepcidin. An intriguing possibility is that BMP2 and BMP6 must act as heterodimeric proteins for optimal function. Indeed, BMPs function as dimeric proteins and there are some biologic contexts where heterodimeric BMP proteins are required for function whereas homodimeric proteins are not able to compensate (37, 38). However, exogenously added homodimeric BMP2 and BMP6 ligand can stimulate hepcidin expression both *in vitro* and *in vivo* (12, 39). Future studies will be needed to understand why endogenous BMP6 and BMP2 fail to appreciably compensate for the loss of the other in endothelial *Bmp2* and *Bmp6* KO mice respectively, and whether endogenous heterodimeric BMP2/6 ligand has an important function role in hepcidin regulation and systemic iron homeostasis.

In summary, our results demonstrate that BMP2 and BMP6 predominantly work together to regulate hepcidin expression and that HFE regulates hepcidin at least in part through a BMP2-independent, but SMAD-dependent, pathway. Our results also support the existence of additional BMP ligand(s) that play a role in iron-mediated hepcidin regulation to maintain systemic iron homeostasis. These

findings provide new insights into how iron regulates hepcidin production and how this process is impaired in hereditary hemochromatosis.

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FIGURE LEGENDS

Figure 1: Combined endothelial *Bmp6* and *Bmp2* KO does not worsen hepcidin deficiency or iron overload compared to single KO mice.

Eight-week-old littermate female (closed symbols) and male (open symbols) control (*Bmp2*^{wt/wt};*Bmp6*^{wt/wt};*Tek-Cre*⁺), single endothelial *Bmp2* KO (*Bmp2*^{fl/fl};*Bmp6*^{wt/wt};*Tek-Cre*⁺), single endothelial *Bmp6* KO (*Bmp2*^{wt/wt};*Bmp6*^{fl/fl};*Tek-Cre*⁺), and double endothelial *Bmp2* and *Bmp6* KO (*Bmp2*^{fl/fl};*Bmp6*^{fl/fl};*Tek-Cre*⁺) mice were analyzed for (A) liver hepcidin (*Hamp*) relative to *Rpl19* mRNA by qRT-PCR. The average of the control female mice was set to 1. (B) Serum transferrin saturation and tissue iron levels in the (C) liver, (D) spleen, (E) pancreas, and (F) heart were analyzed by colorimetric assay. For all graphs, individual data points are shown and bars represent mean $\pm$ SEM. Female and male mice were analyzed separately by one-way ANOVA with Tukey's post-hoc test. Means without a common superscript differ significantly ($P < 0.05$).

Figure 2: Liver *Bmp6* mRNA is increased in endothelial *Bmp2* KO mice, but *Bmp2* mRNA and protein are not increased in endothelial *Bmp6* KO mice.

(A-B) Eight-week-old littermate female (black closed symbols) and male (black open symbols) control (*Bmp2*^{wt/wt};*Bmp6*^{wt/wt};*Tek-Cre*⁺), single endothelial *Bmp2* KO (*Bmp2*^{fl/fl};*Bmp6*^{wt/wt};*Tek-Cre*⁺), single endothelial *Bmp6* KO (*Bmp2*^{wt/wt};*Bmp6*^{fl/fl};*Tek-Cre*⁺), and double endothelial *Bmp2* and *Bmp6* KO (*Bmp2*^{fl/fl};*Bmp6*^{fl/fl};*Tek-Cre*⁺) mice were analyzed for (A) liver *Bmp6* and (B, left) *Bmp2* relative to *Rpl19* mRNA by qRT-PCR. The average of the control mice was set to 1. (B, right) Serum BMP2 protein levels were quantitated by ELISA. (C-D) Four-week-old female (black closed symbols) and male (black open symbols) global *Bmp6*^{-/-} and littermate WT mice (*Bmp6*^{wt/wt}) were fed a matched, purified iron sufficient (Control, 48 ppm iron) or high iron (High Fe, 2% carbonyl iron) diet for 3 weeks, followed by analysis of (C) liver iron, (D, left) liver *Bmp2* relative to *Rpl19* mRNA by qRT-PCR, and (D, right) serum BMP2 protein levels by ELISA. For qRT-PCR analysis, the average of the control *Bmp6*^{wt/wt} mice was set to 1. (E-F) Four- to five-week-old WT C57BL/6 male and female mice from four independent cohorts (grey symbols: ○ male *Hfe*^{wt/wt} littermates; ■ □ female and male *Bmp6*^{wt/wt} littermates; ▲ Jackson female WT; ◇ Taconic male WT) were fed a matched, purified low iron (Low Fe, 2-6 ppm iron), iron sufficient (Control, 48 ppm iron), or high iron (High Fe, 2% carbonyl iron) diet for 3 weeks, followed by analysis of (E) liver *Bmp6* and (F, left) *Bmp2* relative to *Rpl19* mRNA by qRT-PCR. The average of the control diet-fed mice was set to 1. (F, right) Serum BMP2 protein levels were quantified by ELISA. For all graphs, individual data points are shown and bars

represent mean $\pm$ SEM. Results were analyzed by one-way ANOVA with Tukey's post-hoc test. Means without a common superscript differ significantly ($P < 0.05$).

Figure 3: Dietary iron loading induces liver SMAD5 phosphorylation and hepcidin expression in double global *Bmp6*/endothelial *Bmp2* KO mice.

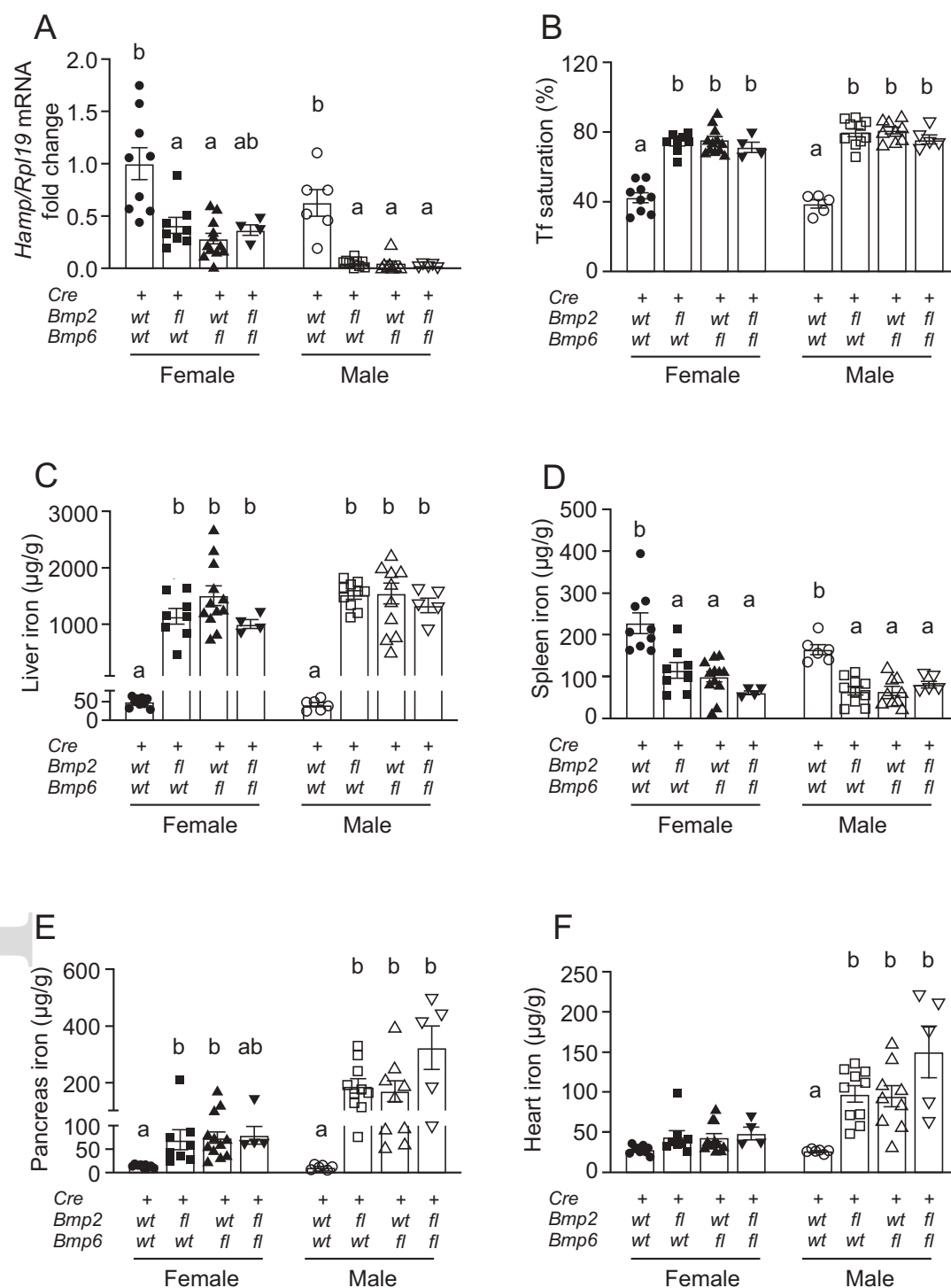
Littermate female (closed symbols) and male (open symbols) single global *Bmp6* KO (*Bmp6*^{-/-}; *Bmp2*^{fl/fl}; *Tek-Cre*⁻) and double global *Bmp6* and endothelial *Bmp2* KO (*Bmp6*^{-/-}; *Bmp2*^{fl/fl}; *Tek-Cre*⁺) mice were placed on a low iron diet (2-6 ppm iron) at weaning for 3 weeks to prevent the iron overload induced by the house diet. Mice were either maintained on the low iron diet (Low Fe) or switched to the house diet for 1 more week. Mice were analyzed for (A) serum iron, (B) serum transferrin saturation, and (C) liver iron by colorimetric assay. (D) Livers were analyzed for *Hamp* relative to *Rpl19* mRNA by qRT-PCR. The average of the single *Bmp6* KO mice on the low iron diet was set to 1. (E) Livers were analyzed for phosphorylated SMAD5 (pSMAD5) relative to total SMAD5 and Actin protein by immunoblot and chemiluminescence quantitation. For all graphs, individual data points are shown and bars represent mean $\pm$ SEM. ** $P < 0.01$, *** $P < 0.001$ relative to low iron diet mice of the same genotype by Student's *t*-test.

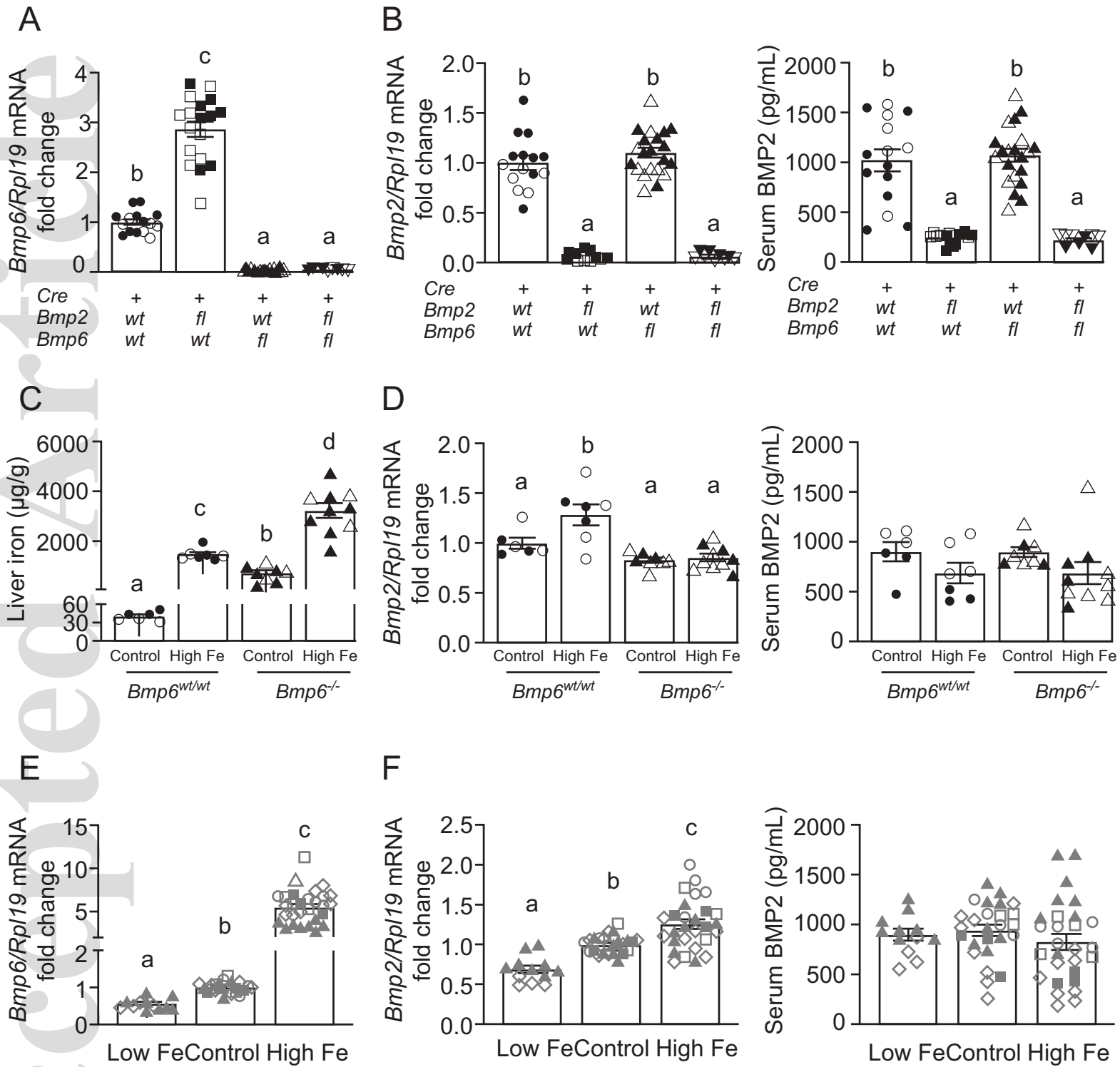
Figure 4: Combined global *Hfe* and endothelial *Bmp2* KO worsens hepcidin deficiency and iron overload compared to single KO mice.

Eight-week-old littermate female (closed symbols) and male (open symbols) control (*Hfe*^{wt/wt}; *Bmp2*^{fl/fl}; *Tek-Cre*⁻), single endothelial *Bmp2* KO (*Hfe*^{wt/wt}; *Bmp2*^{fl/fl}; *Tek-Cre*⁺), single global *Hfe* KO (*Hfe*^{-/-}; *Bmp2*^{fl/fl}; *Tek-Cre*⁻), and double endothelial *Bmp2* and global *Hfe* KO (*Hfe*^{-/-}; *Bmp2*^{fl/fl}; *Tek-Cre*⁺) mice were analyzed for (A) liver *Hamp* relative to *Rpl19* mRNA by qRT-PCR. The average of the control female mice was set to 1. *Hamp/Rpl19* mRNA was divided by liver iron content to normalize for the degree of iron overload in the right panel. (B) Serum iron, serum transferrin saturation and tissue iron levels in the (C) liver, (D) spleen, (E) pancreas, and (F) heart were analyzed by colorimetric assay. For all graphs, individual data points are shown and bars represent mean $\pm$ SEM. Female and male mice were analyzed separately by one-way ANOVA with Tukey's post-hoc test. Means without a common superscript differ significantly ($P < 0.05$).

Figure 5: Combined global *Hfe* and endothelial *Bmp2* KO reduces liver SMAD5 phosphorylation and *Id1* mRNA compared to single KO mice.

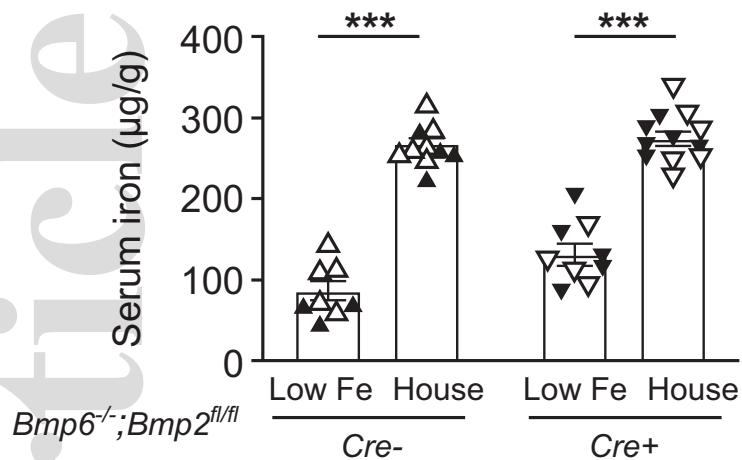
Eight-week-old littermate female (closed symbols) and male (open symbols) control (*Hfe*^{wt/wt};*Bmp2*^{fl/fl};*Tek-Cre*⁻), single endothelial *Bmp2* KO (*Hfe*^{wt/wt};*Bmp2*^{fl/fl};*Tek-Cre*⁺), single global *Hfe* KO (*Hfe*^{-/-};*Bmp2*^{fl/fl};*Tek-Cre*⁻), and double endothelial *Bmp2* and global *Hfe* KO (*Hfe*^{-/-};*Bmp2*^{fl/fl};*Tek-Cre*⁺) mice were analyzed for (A) *Id1* relative to *Rpl19* mRNA by qRT-PCR and (B) phosphorylated SMAD5 (pSMAD5) relative to total SMAD5 and Actin protein by immunoblot and chemiluminescence quantitation. The average of the control female mice was set to 1. *Id1/Rpl19* mRNA and pSMAD5/SMAD5 were divided by liver iron content to normalize for the degree of iron overload in the right-sided panels. For all graphs, individual data points are shown and bars represent mean ± SEM. Female and male results were analyzed separately by one-way ANOVA with Tukey's post-hoc test. Means without a common superscript differ significantly ($P < 0.05$). One representative immunoblot each for female and male mice are shown.



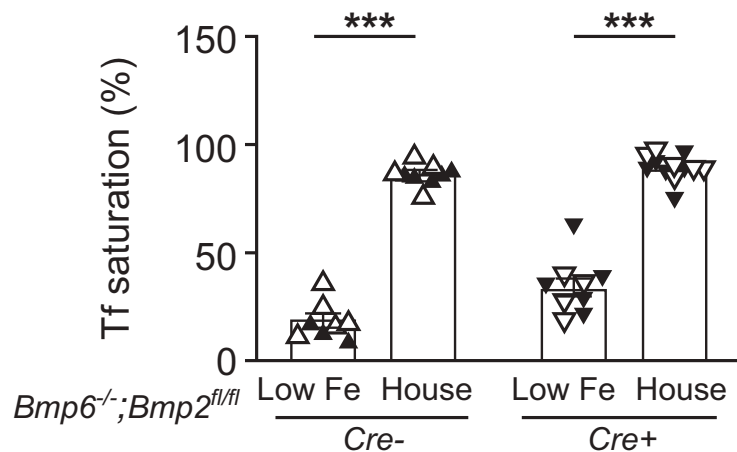


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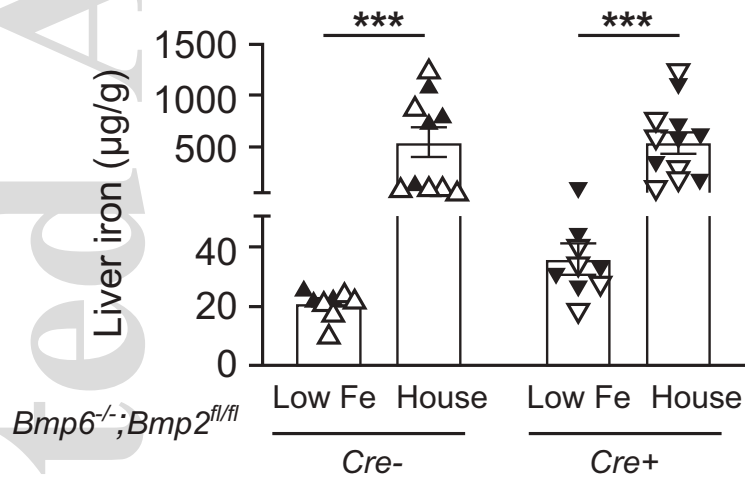
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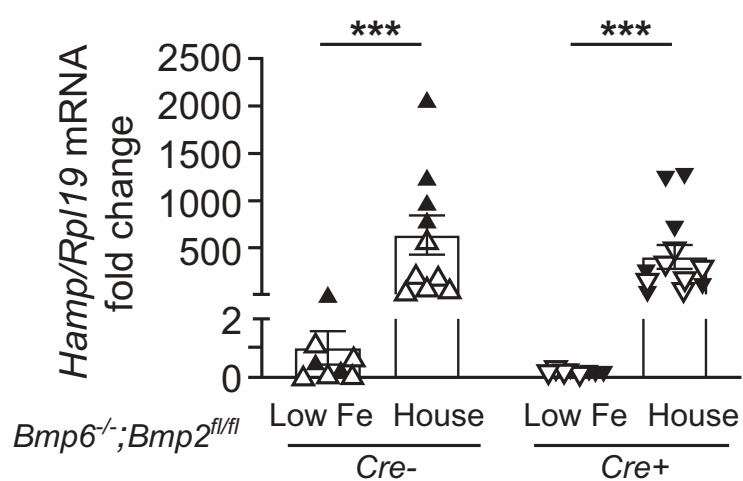
B



C



D



E

