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BMP6 treatment compensates for the molecular defect and ameliorates hemochromatosis in *Hfe* knockout mice

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Abstract

BACKGROUND AND AIMS—Abnormal hepcidin regulation is central to the pathogenesis of *HFE* hemochromatosis. Hepatic bone morphogenetic protein 6 (BMP6)-SMAD signaling is a main regulatory mechanism controlling hepcidin expression, and this pathway was recently demonstrated to be impaired in *Hfe* knockout (*Hfe*^{−/−}) mice. To more definitively determine whether HFE regulates hepcidin expression through an interaction with the BMP6-SMAD signaling pathway, we investigated whether hepatic *Hfe* overexpression activates the BMP6-SMAD pathway to induce hepcidin expression. We then investigated whether excess exogenous BMP6 administration overcomes the BMP6-SMAD signaling impairment and ameliorates hemochromatosis in *Hfe*^{−/−} mice.

METHODS—The BMP6-SMAD pathway and the effects of neutralizing BMP6 antibody were examined in *Hfe* transgenic mice (*Hfe* *Tg*) compared with wildtype (WT) mice. *Hfe*^{−/−} and WT mice were treated with exogenous BMP6 and analyzed for hepcidin expression and iron parameters.

RESULTS—*Hfe* *Tg* mice exhibited hepcidin excess and iron deficiency anemia. *Hfe* *Tg* mice also exhibited increased hepatic BMP6-SMAD target gene expression compared with WT mice, while anti-BMP6 antibody administration to *Hfe* *Tg* mice improved the hepcidin excess and iron

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deficiency. In *Hfe*^{-/-} mice, supraphysiologic doses of exogenous BMP6 improved hepcidin deficiency, reduced serum iron, and redistributed tissue iron to appropriate storage sites.

CONCLUSIONS—HFE interacts with the BMP6-SMAD signaling pathway to regulate hepcidin expression, but HFE is not necessary for hepcidin induction by BMP6. Exogenous BMP6 treatment in mice compensates for the molecular defect underlying *Hfe* hemochromatosis, and BMP6-like agonists may have a role as an alternative therapeutic strategy for this disease.

Keywords

hemochromatosis; HFE; bone morphogenetic protein

INTRODUCTION

Hereditary hemochromatosis is a genetic iron overload disorder most commonly due to mutations in *HFE* (reviewed in ¹). Since there is no regulated mechanism for iron removal from the body, systemic iron balance is maintained by tight regulation of iron absorption from the diet and iron recycling from body stores in the liver and in reticuloendothelial macrophages (reviewed in ²). *HFE* hemochromatosis is characterized by a failure to prevent excess iron release into the circulation, leading to progressive tissue iron accumulation with the potential for multi-organ damage and disease, including cirrhosis, diabetes, cardiomyopathy, hypogonadism, arthritis, skin pigmentation, and increased risk of cancer.¹ Mouse models with either a global or hepatocyte-specific disruption of the *Hfe* gene have an iron overload phenotype similar to human patients with this disease, suggesting that the liver is the predominant organ for HFE action in iron homeostasis.^{3–6} The liver is also the key site for the production of hepcidin, the central iron regulatory hormone that blocks iron release into the bloodstream by downregulating the iron exporter ferroportin on duodenal enterocytes, reticuloendothelial macrophages, and hepatocytes (reviewed in ²). Inappropriately low hepcidin expression is characteristic of both mouse models and human patients with *HFE* mutations^{7–11}, while constitutive expression of hepcidin in *Hfe*^{-/-} mice prevents iron overload.⁸ These data suggest that impaired regulation of hepcidin expression by HFE plays a central role in the pathogenesis of HFE hemochromatosis.

The current mainstay of therapy for *HFE* hemochromatosis is phlebotomy to remove excess iron. Although effective, phlebotomy is contraindicated or poorly tolerated in some patients due to underlying cardiac disease, hypotension, dizziness, fatigue, and vascular access problems.¹² In such circumstances, iron chelation therapy can be considered, but is otherwise uncommonly used to treat hemochromatosis due to cost, potential toxicity, and paucity of data documenting benefit in this patient population.¹² Importantly, existing therapies for hemochromatosis do not target the pathogenic mechanisms underlying this disease. In fact, phlebotomy has been shown to inhibit hepcidin expression^{11,13}, which may potentially exacerbate the hepcidin deficiency, dietary iron over-absorption, and tissue iron maldistribution characteristic of hemochromatosis. This may help explain the observation that non-heme iron absorption is increased by phlebotomy in hemochromatosis patients.^{12, 14} Understanding the molecular mechanisms by which *HFE* mutations impair hepcidin regulation may lead to novel treatments for this disorder.

HFE is an atypical major histocompatibility class I-like protein that requires $\beta 2$ microglobulin for appropriate cell surface localization, and HFE competes with transferrin for binding to transferrin receptor 1 (TFR1) (reviewed in ²). HFE also binds transferrin receptor 2 (TFR2)^{15–17}, mutations in which also lead to adult-onset hereditary hemochromatosis.¹ It has been postulated that TFR1 in the liver sequesters HFE, and that when serum iron levels increase, iron-saturated transferrin displaces HFE from TFR1,¹⁸

thereby freeing HFE to upregulate hepcidin expression, possibly via an interaction with TFR2.^{15,17–18} However, the precise molecular mechanism by which HFE (either alone or in complex with TFR2) affects hepcidin expression is still unknown.

We and others have recently shown that *Hfe*^{−/−} mice exhibit an impairment in the bone morphogenetic protein (BMP)-SMAD signaling pathway^{19–20}, which is a central regulator of hepcidin expression (reviewed in ²). BMPs are members of the TGF-beta superfamily of ligands that bind to complexes of type I and type II serine threonine kinase receptors to induce phosphorylation of intracellular SMAD proteins, which translocate to the nucleus to modulate gene expression such as *ID1* and *SMAD7*.^{21–22} Hepcidin is a target gene that is directly transcriptionally regulated by the BMP-SMAD pathway.^{23–26} Mutations in the genes encoding the ligand BMP6^{27–28}, the BMP co-receptor hemojuvelin (HJV)^{29–30}, and the intracellular signaling molecule SMAD4³¹, all result in inappropriately suppressed hepcidin expression and tissue iron overload, supporting the central importance of the BMP6-HJV-SMAD signaling pathway in hepcidin regulation and iron homeostasis. Furthermore, pharmacologic modulators of the BMP6-HJV-SMAD signaling pathway alter hepcidin expression and systemic iron balance *in vivo*.^{27,32–33} For example, BMP ligand administration increases hepcidin expression and decreases serum iron^{27,32}, while BMP inhibitors decrease hepcidin expression, increase ferroportin expression, mobilize reticuloendothelial cell iron stores, and increase serum iron in mice.^{27, 32–33} The BMP6-HJV-SMAD signaling pathway appears to be an important mechanism by which iron regulates hepcidin expression. Acute iron administration increases phosphorylation of hepatic SMAD1/5/8 proteins within one hour³³, while chronic iron administration increases hepatic *Bmp6* mRNA, with a strong correlation between liver iron concentration and hepatic *Bmp6* mRNA levels.^{19,34} Importantly, the ability of acute iron administration to induce hepcidin expression is inhibited by BMP6-HJV-SMAD pathway inhibitors.^{33, 35}

Recently, we and others have shown in *Hfe*^{−/−} mice that, while hepatic *Bmp6* mRNA is appropriately upregulated relative to their iron overload, hepatic phosphorylated SMAD1/5/8 protein (P-SMAD1/5/8) and *Id1* mRNA levels are not appropriately increased relative to iron burden and BMP6 levels.^{19–20} Furthermore, we demonstrated that hepcidin induction by low doses of BMP6 ligand is impaired in *Hfe*^{−/−} primary hepatocyte cultures.¹⁹ These data provide indirect evidence that HFE may regulate hepcidin expression through an interaction with the BMP6-SMAD pathway. Notably, higher concentrations of BMP ligands were able to induce hepcidin expression in *Hfe*^{−/−} primary hepatocytes^{19,36}, suggesting that although HFE may be important to optimize downstream SMAD signaling induced by BMP ligands, HFE may not be necessary for BMP-SMAD signal transduction.

Here, we investigated the BMP6-SMAD signaling pathway in mice overexpressing an *Hfe* transgene in the liver to more definitively determine whether HFE regulates hepcidin expression through an interaction with the BMP6-SMAD pathway. We also tested whether supraphysiologic levels of exogenous BMP6 can ameliorate the hepcidin deficiency and hemochromatosis phenotype in an *Hfe*^{−/−} mouse model.

MATERIALS AND METHODS

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at the Massachusetts General Hospital (MGH), Children's Hospital Boston (CHB), or the University Hospital of Modena (UHM).

Mice overexpressing an *Hfe* transgene in the liver under control of the hepatocyte-specific transthyretin promoter (*Hfe Tg*) were generated essentially as previously described¹⁸ using a

wildtype C57BL/6 background. *Hfe Tg* mice and littermate WT mice were housed in CHB and maintained on a 380ppm iron Prolab RMH 3000 diet. For experiments studying the baseline phenotype of *Hfe Tg* mice, 8-week-old females were sacrificed and tissues harvested for analysis. *Hfe Tg* male mice were also examined in Supplementary Material. For BMP6 antibody injection experiments, 8–10-week-old male and female *Hfe Tg* mice received an intraperitoneal (IP) injection of BMP6 antibody in PBS at 15 mg/kg (BMP6 Ab, R&D Systems²⁷) or an equal volume of PBS alone (Mock) daily for 10 days (genders were matched between groups)

Hfe^{-/-} mice (kindly provided by Dr. Nancy Andrews, Duke University Medical Center⁴) on a 129S6/SvEvTac or C57BL/6 background were used for BMP6 injection experiments. For single BMP6 injection experiments, 8- to 10-week-old male and female WT or *Hfe*^{-/-} mice on a 129S6/SvEvTac background housed at UHM and maintained on a 330 ppm iron 2018 Harlan Teklad diet received BMP6 at 100 or 250 µg/kg or an equal volume of vehicle (20 mM sodium acetate, 5% mannitol solution, pH 4.0) alone IP (genders were matched between groups). For chronic BMP6 injection experiments, 8-week-old male WT or *Hfe*^{-/-} mice on a C57BL/6 background housed at MGH and maintained on a 380 ppm Prolab 5P75 Isopro RMH 3000 diet received BMP6 at 500 µg/kg or an equal volume of vehicle alone (Mock) IP twice daily for 10 days. Mice were sacrificed and tissues harvested for analysis six hours after the last injection. BMP6 was produced as previously described.²⁷

Hematologic and iron analysis

Blood was obtained by retro-orbital bleed or cardiac puncture at the time of sacrifice as previously described.^{18, 32} Complete blood count was analyzed using a Drew Scientific HEMAVET 950FS machine at the Clinical Pathology Laboratory in the Center for Comparative Medicine at MGH, or an Advia 120 analyzer (Bayer) at CHB Clinical Core Laboratories. Serum iron and transferrin saturation were determined as previously described.^{19, 32} Duodenum was fixed in 10% buffered formalin and then embedded in paraffin. Tissue iron staining was performed as previously described.²⁷

Quantitative real-time RT-PCR

Total RNA was isolated from mouse livers and real-time quantification of *Hamp* (also known as *Hamp1*), *Id1*, *Smad7*, and *Bmp6* mRNA relative to *Rpl19*, or *Hamp* relative to *Gapdh* mRNA were performed using two-step quantitative real-time RT-PCR as previously described.^{19,27,34}

Statistics

A two-tailed Student's *t*-test with *P* < 0.05 was used to determine statistical significance.

RESULTS

Hepatic overexpression of an *Hfe* transgene causes hepcidin excess and iron deficiency anemia, and activates the BMP6-SMAD signaling pathway

It has recently been shown that *Hfe*^{-/-} mice overexpressing an *Hfe* transgene in the liver (*Hfe*^{-/-} *Hfe Tg* mice) exhibit increased hepatic hepcidin mRNA expression, iron deficiency, and anemia.¹⁸ We examined whether hepatic overexpression of an *Hfe* transgene in WT C57BL/6 mice (*Hfe Tg*) produced a similar phenotype. Compared with littermate WT mice, *Hfe Tg* mice expressed 22-fold higher hepatic *Hfe* mRNA (Supplementary Methods and Supplementary Fig. 1), and exhibited a microcytic hypochromic anemia, reduced serum iron and transferrin saturation (Tf Sat), and reduced tissue iron in liver, heart and pancreas (Tables 1–2 and Supplementary Table 1). *Hfe Tg* mice also exhibited 4–5-fold increased hepatic hepcidin (*Hamp*, also known as *Hamp1*) mRNA expression relative to WT

littermates (Fig. 1A). Notably, iron deficiency and anemia normally inhibit hepcidin expression^{13,19,34}, suggesting that the hepcidin increase in *Hfe Tg* is even more striking when considered in the context of the iron deficiency anemia phenotype of these animals. The inappropriately elevated hepcidin levels presumably accounts for the iron deficient and anemic phenotype of *Hfe Tg* mice.

Although it was recently shown that *Hfe*^{-/-} mice exhibit an impairment in the BMP6-SMAD signaling pathway¹⁹⁻²⁰, this provides only indirect evidence of a role for HFE in BMP6-SMAD regulation of hepcidin. To determine more definitively whether HFE positively regulates hepcidin expression through an interaction with the BMP6-SMAD pathway, we measured BMP6-SMAD pathway components in *Hfe Tg* mice and WT littermate controls. *Hfe Tg* mice had significantly elevated hepatic *Id1* and *Smad7* mRNA levels (target transcripts upregulated by BMP6^{19,22,34}) compared with WT mice (Fig. 1B–C). A similar increase in hepatic *Id1* mRNA expression was also found in *Hfe*^{-/-} *Hfe Tg* mice (PJ Schmidt and MD Fleming, unpublished data). Although there was a trend toward increased P-Smad1/5/8 protein levels in *Hfe Tg* mice compared with WT mice, this did not reach statistical significance (Supplementary Fig. 3).

Notably, BMP6-SMAD pathway signaling mediators and target genes were previously shown to be decreased by an iron deficient diet¹⁹⁻²⁰, suggesting that like hepcidin, *Id1* and *Smad7* mRNA are even more inappropriately increased when considered in the context of the iron deficiency phenotype of the *Hfe Tg* mice. Although it is difficult to generate an experimental model in WT mice that precisely mirrors the chronic iron deficiency anemia phenotype of the *Hfe Tg* mice, we treated C57BL/6 WT mice with an iron deficient diet and phlebotomy to achieve a similar degree of iron deficiency anemia as the *Hfe Tg* mice (Anemic WT mice, Supplementary Methods and Supplementary Table 2). Although an imperfect model for a direct quantitative comparison with *Hfe Tg* mice, anemic WT mice exhibited 10-fold less *Hamp*, 57% reduced *Id1*, and a trend toward reduced *Smad7* mRNA expression compared with non-anemic WT mice (Supplementary Fig. 2). Thus, the absolute increase in *Hamp*, *Id1*, and *Smad7* mRNA in *Hfe Tg* mice compared with normal WT mice are even more notable when considered in the context of their iron deficiency anemia phenotype.

Hepatic *Bmp6* mRNA was not significantly changed in *Hfe Tg* mice compared with WT mice, nor was hepatic *Bmp6* mRNA changed in iron deficient and anemic WT mice compared with control WT mice (Fig. 1D and Supplementary Fig. 2D). Similar results were also found in *Hfe*^{-/-} *Hfe Tg* mice (PJ Schmidt and MD Fleming, unpublished data). Together, these data are consistent with a role for HFE in optimizing downstream signals of BMP6.

Hepcidin excess and iron deficiency in *Hfe Tg* mice is improved by neutralizing anti-BMP6 antibody treatment

To further confirm whether hepcidin overexpression in *Hfe Tg* mice depends on BMP6-SMAD signaling pathway activity, we explored whether a neutralizing anti-BMP6 antibody (BMP6 Ab)²⁷ reversed the hepcidin excess and iron deficient phenotype of *Hfe Tg* mice. Similar to previously published results in WT mice²⁷, BMP6 Ab significantly inhibited hepatic *Id1* mRNA expression in *Hfe Tg* mice (Supplementary Fig. 4), demonstrating the effectiveness of this antibody as an inhibitor of BMP6 signaling in vivo. BMP6 Ab administration for ten days significantly decreased hepatic *Hamp* mRNA (Fig. 2A) and increased serum iron and Tf Sat to levels seen in WT mice (Fig. 2B–C, compare results to Table 2) compared with administration of vehicle alone (Mock). BMP6 Ab also significantly increased reticulocyte hemoglobin content, hemoglobin, and hematocrit in *Hfe Tg* mice (Fig. 2D–F).

Supraphysiologic doses of exogenous BMP6 significantly increase hepcidin mRNA expression and reduce serum iron in *Hfe*^{-/-} mice

We previously showed that low doses of exogenous BMP6 ligand have impaired ability to induce *Hamp* mRNA expression in *Hfe*^{-/-} primary hepatocyte cultures.¹⁹ Notably, at higher concentrations, BMP6 was able to significantly induce *Hamp* mRNA expression in *Hfe*^{-/-} hepatocytes, albeit to a lesser extent than WT hepatocytes, suggesting that although HFE is important to optimize downstream hepcidin induction by BMP6, HFE may not be necessary for hepcidin induction by BMP6.¹⁹ We therefore examined the ability of a single supraphysiologic dose of exogenous BMP6 to regulate hepcidin expression and iron metabolism in *Hfe*^{-/-} versus WT mice on a 129S6/SvEvTac background. Exogenous BMP6 significantly increased hepatic *Hamp* mRNA (Fig 3A) and decreased serum iron (Fig. 3B) in both WT and *Hfe*^{-/-} mice in a dose dependent fashion, similar to prior studies in WT mice^{27,32}. It has been hypothesized that the relatively lower potency of exogenous BMP ligands for inducing hepatic hepcidin expression *in vivo* compared with *their in vitro* effects may be related to their short elimination half-life and/or the presence of circulating BMP inhibitors.³² At these relatively high exogenous doses of BMP6, *Hfe*^{-/-} mice had similar or greater -fold changes in *Hamp* mRNA and serum iron compared WT mice. At a dose of 250 µg/kg, BMP6 restored appropriate levels of hepatic hepcidin expression and serum iron in *Hfe*^{-/-} mice compared with mock treated WT mice.

Exogenous BMP6 administration increases hepatic *Hamp* mRNA, reduces serum iron and transferrin saturation, and increases iron retention in the spleen and duodenum in *Hfe*^{-/-} mice

Next, we tested whether longer-term exogenous BMP6 administration ameliorated the hemochromatosis phenotype of *Hfe*^{-/-} mice. A different background strain of *Hfe*^{-/-} mice (C57BL/6) was used for multiple dosing experiments due to animal availability. A dosing regimen of 500 µg/kg IP twice daily for 10 days was chosen based on additional dosing and pharmacokinetic studies (Supplementary Fig. 5) with the goal to use the minimum effective dose of BMP6 to increase hepcidin expression and modulate systemic iron balance, but to avoid overtreatment that might cause iron deficiency and anemia, and to minimize potential bone-inducing and other off-target effects of BMP6. A comparison of the magnitude of the effect of acute and chronic BMP6 administration is therefore limited by the different experimental conditions (different background strains, different BMP6 dose). As controls for effectiveness and specificity, BMP6 treatment in *Hfe*^{-/-} mice caused a trend toward increased hepatic *Id1* mRNA expression by 2.4-fold (similar to prior studies in WT mice²⁷), but had no effect on the inflammatory marker *Crp* mRNA (Supplementary Fig. 6). BMP6 treatment significantly increased hepatic *Hamp* mRNA expression (Fig. 4A), and reduced serum iron and Tf Sat (Fig. 4B–C) compared with vehicle treatment (Mock) in *Hfe*^{-/-} mice. The CBC parameters remained within normal limits in BMP6 treated *Hfe*^{-/-} mice, although there was a trend toward slightly lower hemoglobin and mean cell volume, and a small but significantly reduced mean corpuscular hemoglobin compared with mock treated *Hfe*^{-/-} mice (Table 3). A specific feature of hemochromatosis is the iron-depleted status of reticuloendothelial macrophages that normally store iron and duodenal enterocytes, with inappropriate iron deposition in other tissues.¹ BMP6 treated *Hfe*^{-/-} mice exhibited increased iron retention in the spleen (Fig. 4D) and increased duodenal iron staining (Fig. 4E) compared with mock treated *Hfe*^{-/-} mice. However, we did not detect a significant reduction in the iron content of other tissues that tend to be iron loaded in hemochromatosis including the liver, heart, or pancreas in this 10-day timeframe (Supplementary Fig. 7). Longer term treatment of *Hfe*^{-/-} mice with BMP6 was limited by the development of peritoneal calcifications after about 2 weeks (data not shown).

DISCUSSION

To investigate the mechanism by which HFE regulates hepcidin expression and to determine more definitively whether this occurs through an interaction with the BMP6-SMAD pathway, we quantitated BMP6-SMAD signaling pathway intracellular mediators (P-Smad1/5/8) and target gene expression (*Id1*, *Smad7*) in mice with hepcidin excess due to hepatic overexpression of an *Hfe* transgene (*Hfe Tg* mice). We demonstrated that *Hfe Tg* mice have increased hepatic expression of BMP6-SMAD pathway target transcripts including *Id1* and *Smad7* compared with littermate WT mice. The increases in hepatic *Id1* and *Smad7* mRNA in *Hfe Tg* mice are even more notable when considered in the context of the iron deficiency anemia of these mice, since it has previously been demonstrated that an iron deficient diet decreases hepatic *Bmp6* mRNA, P-Smad1/5/8 protein, *Id1* mRNA, and *Smad7* mRNA expression.^{19,34} Indeed, we found that WT mice with a similar degree of anemia and a more mild iron deficiency than the *Hfe Tg* mice (induced by an iron deficient diet and phlebotomy) had decreased *Id1* mRNA and a trend toward decreased *Smad7* mRNA compared with WT mice on a normal iron diet. Although we saw a trend toward increased P-Smad1/5/8 in *Hfe Tg* mice, this did not reach statistical significance. This likely reflects the faster kinetics and more transient nature of SMAD phosphorylation in response to BMP ligand compared with downstream mRNA regulation, making it more difficult to observe when looking at the chronic effects of *Hfe* overexpression, particularly since the degree of BMP6-SMAD pathway activation was relatively modest at the level of *Hfe* overexpression achieved in this model. Indeed, changes in Smad1/5/8 phosphorylation were less pronounced than changes in downstream target gene expression in previous studies.^{19–20,34} Together, our data demonstrate that the BMP6-SMAD signaling pathway is more active in *Hfe Tg* mice, and are consistent with prior publications demonstrating that hepatic P-Smad1/5/8 protein and *Id1* mRNA are inappropriately reduced relative to the degree of iron overload in *Hfe*^{-/-} mice.^{19–20} These data further support the hypothesis that HFE activates hepcidin expression through an interaction with the BMP6-SMAD signaling pathway.

We did not see a significant increase in hepatic *Bmp6* mRNA expression in *Hfe Tg* mice. These data suggest that HFE activates the BMP6-SMAD signaling pathway downstream of BMP6. Interestingly, previous studies have demonstrated that hepatic *Bmp6* mRNA expression correlates with liver iron content.^{19,34} In the current study, hepatic *Bmp6* mRNA levels were not lower in *Hfe Tg* mice despite their iron deficiency. However, the liver iron content was very low to begin with in this C57BL/6 strain (55 µg/g wet weight) and was only reduced by about 25–30% in the *Hfe Tg* mice. It is therefore possible that the *Bmp6* levels had already neared their nadir and could not be suppressed any further. We also did not see any significant change in hepatic *Bmp6* mRNA in the anemic WT mice, where similar reductions in liver iron content were achieved. Our data are consistent with prior studies demonstrating that hepatic *Bmp6* mRNA expression is appropriately increased relative to the increased iron burden in *Hfe*^{-/-} mice^{19–20}, suggesting that HFE is not involved in the regulation of BMP6 by iron.

The ability of a neutralizing BMP6 antibody to decrease hepcidin expression, normalize serum iron and Tf Sat, and improve anemia in *Hfe Tg* mice suggests that the BMP6-SMAD pathway activation in *Hfe Tg* mice is necessary for the hepcidin overexpression and iron deficient phenotype of these mice. These data further support an interaction between HFE and the BMP6-SMAD signaling pathway in regulating hepcidin expression and systemic iron balance. However, it is difficult to rule out the possibility that HFE may also regulate hepcidin through a separate pathway that is less dominant than or is dependent on an intact BMP6-SMAD pathway.

Although HFE appears to interact with the BMP6-SMAD signaling pathway to regulate hepcidin expression, HFE is not necessary for hepcidin induction by BMP6. A single IP dose of BMP6 at 250 $\mu\text{g/kg}$ was able to significantly increase hepatic hepcidin expression and decrease serum iron in *Hfe*^{-/-} mice in a similar manner to WT mice. This is consistent with prior *in vitro* data showing that while lower doses of exogenous BMP6 $\leq 1\text{ng/mL}$ had impaired ability to induce hepcidin expression in *Hfe*^{-/-} compared with WT primary hepatocytes, a higher dose of exogenous BMP6 at 2 ng/mL was able to significantly induce hepcidin expression in *Hfe*^{-/-} hepatocytes, albeit to a lesser extent than in WT hepatocytes.¹⁹ Significantly higher doses of other exogenous BMP ligands including BMP2, BMP4, and BMP9 have also been shown to induce hepcidin expression equivalently in both WT and *Hfe*^{-/-} hepatocytes³⁶, and it remains unknown whether the ability of HFE to optimize downstream SMAD signaling to BMP ligands is specific for BMP6. We hypothesize that at the low physiologic concentrations of BMP6 endogenously present at the hepatocyte membrane *in vivo*, HFE is important to optimize downstream SMAD signals and hepcidin induction in response to iron. However, supraphysiologic doses of exogenous BMP6 bypass the need for HFE to optimize downstream SMAD signaling and hepcidin induction in response to iron.

Our data raise the possibility that activators of the BMP6-SMAD signaling pathway may provide a new treatment strategy for HFE hemochromatosis that addresses the underlying pathophysiologic mechanism of this disease. Here, we showed that longer-term administration of exogenous BMP6 for 10 days improved hepcidin deficiency and biochemical iron overload in the serum of *Hfe*^{-/-} mice. Importantly, spleen iron content was increased in BMP6 treated *Hfe*^{-/-} mice demonstrating that BMP6 treatment leads to appropriate iron retention in reticuloendothelial macrophages. Iron staining was also increased in the duodenum of BMP6 treated *Hfe*^{-/-} mice, suggesting that iron release into the bloodstream from the diet is being appropriately suppressed. Presumably, the increase in spleen and duodenal iron content is due to decreased ferroportin activity caused by the increased hepcidin expression, based on the well-described function of hepcidin to bind to ferroportin and induce its internalization and degradation (reviewed in ²). However, we were not able to detect quantitative differences in ferroportin expression by Western blot (Supplementary Fig. 8A). Notably, we also did not detect quantitative differences in ferroportin expression in *Hfe*^{-/-} mice compared with WT mice by Western blot (Supplementary Fig. 8B) consistent with prior studies.³⁷ The most likely explanation is that the change in ferroportin protein expression is not robust enough to be seen by Western blot quantitation due to the relatively modest change in hepcidin expression between *Hfe*^{-/-} mice and WT mice, and between mock-treated and BMP6-treated *Hfe*^{-/-} mice (≤ 2 -fold in our models). It is also possible that the physiologic explanation for iron redistribution in *Hfe*^{-/-} mice can be attributed to another hepcidin-dependent but ferroportin-independent mechanism. Together, these data demonstrate that BMP6 administration improves hepcidin deficiency, reduces serum iron overload, and redistributes iron to appropriate tissue storage sites in *Hfe*^{-/-} mice.

We did not see any significant reduction in liver iron content in *Hfe*^{-/-} mice. One possible explanation is that ferroportin is also expressed in hepatocytes³⁸, and hepcidin induction by BMP6 may decrease hepatocyte ferroportin expression, thereby inhibiting liver iron export. Interestingly, treatment with exogenous hepcidin for 2 months or hepatic overexpression of an hepcidin transgene for three weeks in *Hfe*^{-/-} mice failed to reduce total liver nonheme iron content³⁹⁻⁴⁰, similar to the results in our study. However, tissue iron staining suggested that although total liver iron content was unchanged in the hepcidin transgenic *Hfe*^{-/-} mice, there was an altered distribution of cellular iron from predominantly in hepatocytes in *Hfe*^{-/-} mice to predominantly in macrophages (Kupffer cells) in hepcidin transgenic *Hfe*^{-/-} mice.³⁹ We were not able to detect any changes in the cellular distribution of iron in the

liver in our study (Supplementary Fig. 7B), possibly due to the shorter period of treatment and/or the less robust hepcidin induction achieved. Interestingly, ferroportin expression was decreased in Kupffer cells in hepcidin transgenic *Hfe*^{-/-} mice compared with *Hfe*^{-/-} mice, but was unchanged in hepatocytes, leading the authors to speculate that hepatocyte iron export may occur through an hepcidin-ferroportin independent pathway.³⁹ We were unable to detect differences in ferroportin expression in the liver in our study (Supplementary Fig. 9 and data not shown). Another possible explanation for the inability of BMP6 to decrease liver iron content in our study is that the animals were not treated long enough. Attempts to treat *Hfe*^{-/-} mice for longer periods of time with exogenous BMP6 were unsuccessful because the animals developed peritoneal calcifications, consistent with the known bone-inducing properties of BMP6. Thus exogenous BMP6 administration in its current form is not a viable therapy. However, these data provide proof of concept that activators of the BMP6-SMAD signaling pathway might be useful for treating hemochromatosis if more specific therapies can be developed without significant off-target effects.

In summary, our data provide more definitive proof that HFE interacts with the BMP6-SMAD signaling pathway, downstream of BMP6, to regulate hepcidin expression, but that HFE is not necessary for hepcidin induction by BMP6. Future studies will be needed to determine the precise molecular mechanisms by which HFE intersects with the BMP6-SMAD signaling pathway. Our data also provide one of the first demonstrations in mice of a pharmacologic treatment for *HFE* hemochromatosis that targets the hepcidin deficiency underlying this disease. Future studies will be needed to determine the feasibility of developing BMP6-SMAD activators as an alternative treatment strategy for some human patients with HFE hemochromatosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BMP	bone morphogenetic protein
<i>Hfe</i>^{-/-}	<i>Hfe</i> knockout mice
<i>Hfe</i> Tg	<i>Hfe</i> transgenic mice
WT	wildtype
TFR1	transferrin receptor 1
TFR2	transferrin receptor 2
HJV	hemojuvelin
P-SMAD1/5/8	Phosphorylated SMAD1/5/8

CBC	complete blood count
Tf Sat	transferrin saturation
<i>Hamp</i>	Hepcidin mRNA
BMP6 Ab	BMP6 antibody

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Figure 1. Hepcidin (*Hamp*), *Id1*, and *Smad7* mRNA are increased in *Hfe Tg* mice relative to WT mice

Eight-week-old female *Hfe Tg* mice ($N = 6$) versus littermate WT mice ($N = 6$) were analyzed for hepatic *Hamp*, *Id1*, *Smad7*, and *Bmp6* mRNA relative to *Rpl19* mRNA by quantitative real-time RT-PCR. Results are reported as the mean $\pm$ SD for the fold change from WT mice. Exact P values (or NS if not significant) are shown for the comparison with WT mice.

Figure 2. Neutralizing anti-BMP6 antibody decreases hepcidin expression and increases serum iron, transferrin saturation, reticulocyte mean cell hemoglobin, hemoglobin and hematocrit in *Hfe Tg* mice

Eight to ten-week-old *Hfe Tg* mice received an IP injection of anti-BMP6 antibody (BMP6 Ab, $N = 5$ [3 males, 2 females]) or vehicle alone (Mock, $N = 5$ [2 males, 3 females]) once daily for ten days. Tissues were analyzed for A) hepatic *Hamp* relative to *Rpl19* mRNA by quantitative real-time RT-PCR, B) serum iron, C) serum transferrin saturation (Tsats), D) reticulocyte mean cell hemoglobin, E) hemoglobin, and F) hematocrit. Results are reported as the mean $\pm$ SD. Exact P values are shown for the comparison with mock treatment.

Figure 3. Supraphysiologic doses of exogenous BMP6 increase hepcidin expression and decrease serum iron in *Hfe*^{-/-} mice in a manner similar to WT mice

Eight to ten week-old male and female *Hfe*^{-/-} mice and WT mice received a single IP injection of BMP6 at 100 µg/kg or 250 µg/kg or vehicle alone (Control) as indicated (*N* = 6–10 per group, genders were matched between groups). Six hours after the injection, tissues were harvested and analyzed for A) hepatic *Hamp* relative to *Gapdh* mRNA by quantitative real-time RT-PCR and B) serum iron. Results are reported as the mean ± SD. Exact *P* values (or NS if not significant) are shown.

Figure 4. Exogenous BMP6 increases hepcidin expression, reduces serum iron and transferrin saturation, and increases spleen and duodenal iron content in *Hfe*^{-/-} mice

Eight-to-ten week-old male *Hfe*^{-/-} mice were treated with BMP6 at 500 μg/kg or vehicle alone (Mock) IP twice daily for 10 days (*N* = 8 per group). Tissues were analyzed for A) hepatic *Hamp* relative to *Rpl19* mRNA by quantitative real-time RT-PCR, B) serum iron, C) serum Tsat, and D) spleen iron content. Results are reported as the mean ± SD. Exact *P* values are shown for the comparison with mock treatment. E) Perls Prussian blue staining of duodenal iron (original magnification x40).

Table 1

Hematologic features of *Hfe* *Tg* versus WT mice

Genotype	n	Hgb (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	RDW (%)	CHr (pg)	Retic (%)
WT	6	15.3 ± 0.3	47.9 ± 4.5	52.1 ± 1.7	16.8 ± 0.9	13.0 ± 0.3	15.4 ± 0.2	3.0 ± 0.5
<i>Hfe</i> <i>Tg</i>	6	12.7 ± 0.4 *	43.8 ± 3.7 ‡	40.3 ± 2.3 *	11.7 ± 0.4 *	19.7 ± 0.4 *	11.9 ± 0.5 *	4.5 ± 0.3 *

The red blood cell parameters hemoglobin (Hgb), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin (MCH), red cell distribution width (RDW), reticulocyte count (Retic), and Reticulocyte mean cell hemoglobin (CHr) were measured in 8-week-old female WT or *Hfe* *Tg* mice on a C57BL/6 background. Data are presented as the mean ± SD.

* *P* < 0.001 for *Hfe* *Tg* versus WT mice.

‡ *P* not significant from WT.

Table 2Iron parameters of *Hfe Tg* versus WT mice

Genotype	n	Serum iron (µg/dL)	Tf Sat (%)	LIC (µg/g)
WT	6	149 ± 16	61 ± 5	55 ± 12
<i>Hfe Tg</i>	6	82 ± 4 **	36 ± 2 **	40 ± 2 **

Serum iron, transferrin saturation (Tf Sat), and liver iron content (LIC) were measured in 8-week old female *Hfe Tg* versus littermate WT mice on a C57BL/6 background. Data are presented as the mean ± SD.

**
 $P \leq 0.002$ for *Hfe Tg* versus WT mice.

Table 3

Hematologic features of Mock versus BMP6 treated *Hfe*^{-/-} mice.

Treatment	n	WBC (K/ μ L)	Hgb (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	PLT (K/ μ L)
Mock	8	5.9 $\pm$ 3.1	14.5 $\pm$ 1.2	38.4 $\pm$ 3.7	44.5 $\pm$ 1.7	16.8 $\pm$ 0.3	332 $\pm$ 68
BMP6	8	4.3 $\pm$ 1.2	13.5 $\pm$ 0.5	35.6 $\pm$ 1.5	42.9 $\pm$ 1.2	16.3 $\pm$ 0.4*	340 $\pm$ 92

The red blood cell parameters white blood cell count (WBC), hemoglobin (Hgb), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin (MCH), and platelet count (PLT) were measured in vehicle treated (Mock) or BMP6 treated *Hfe*^{-/-} mice. Data are presented as the mean $\pm$ SD.

* *P* = 0.01 for BMP6 treatment versus Mock group. Other *P* values were not significant (*P* $\geq$ 0.05).