BMP6 Treatment Compensates for the Molecular Defect and Ameliorates Hemochromatosis in *Hfe* Knockout Mice

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BACKGROUND & 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 shown 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 wild-type (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, whereas anti-BMP6 antibody administration to *Hfe Tg* mice improved the hepcidin excess and iron 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.

Hereditary hemochromatosis is a genetic iron overload disorder most commonly resulting from mutations in *HFE* (reviewed in Pietrangelo[1]). Because 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 Babitt and Lin[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 multiorgan 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 down-regulating the iron exporter ferroportin on duodenal enterocytes, reticuloendothelial macrophages, and hepatocytes (reviewed in Babitt JL and Lin[2]). Inappropriately low hepcidin expression is characteristic of both mouse models and human patients with *HFE* mutations,[7–11] whereas 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 because of underlying cardiac disease, hypotension, dizziness, fatigue, and vascular access problems.[12] In such circumstances, iron chelation therapy can be considered, but it is otherwise uncommonly

Abbreviations used in this paper: BMP, bone morphogenetic protein; BMP6 Ab, BMP6 antibody; CHB, Children's Hospital Boston; *Hamp*, Hepcidin mRNA; $Hfe^{-/-}$, *Hfe* knockout mice; *Hfe Tg*, *Hfe* transgenic mice; HJV, hemojuvelin; MGH, Massachusetts General Hospital; P-SMAD1/5/8, phosphorylated SMAD1/5/8; TFR1, transferrin receptor 1; TFR2, transferrin receptor 2; WT, wild-type; Tf Sat, transferrin saturation.

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used to treat hemochromatosis because of cost, potential toxicity, and paucity of data that document benefits 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 characteristics of hemochromatosis. This may help explain the observation that nonheme iron absorption is increased by phlebotomy in patients with hemochromatosis.[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 Babitt and Lin[2]). HFE also binds transferrin receptor 2 (TFR2),[15–17] mutations 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 up-regulate hepcidin expression, possibly by 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 Babitt and Lin[2]). BMPs are members of the transforming growth factor-β 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 coreceptor hemojuvelin (HJV),[29,30] and the intracellular signaling molecule SMAD4,[31] 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] whereas 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 1 hour,[33] whereas 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, although hepatic *Bmp6* mRNA is appropriately up-regulated 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.

Mice overexpressing an *Hfe* transgene in the liver under control of the hepatocyte-specific transthyretin promoter (*Hfe Tg*) were generated essentially as previously described[18] with the use of a wild-type (WT) C57BL/6 background. *Hfe Tg* mice and littermate WT mice were housed in CHB and maintained on a 380 ppm iron Prolab RMH 3000 diet. For experiments studying the baseline phenotype of *Hfe Tg* mice, 8-week-old females were killed, and tissues were harvested for analysis. *Hfe Tg* male mice were also examined, and details are given in Supplementary Material. For BMP6 antibody injection experiments, 8- to 10-week-old male and female *Hfe Tg* mice received an intraperitoneal injection of BMP6 antibody (BMP6 Ab) in phosphate-buffered saline at 15 mg/kg (R&D Systems, Minneapolis, MN[27]) or an equal