nature genetics

BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism

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Juvenile hemochromatosis is an iron-overload disorder caused by mutations in the genes encoding the major iron regulatory hormone hepcidin (*HAMP*)[1] and hemojuvelin (*HFE2*)[2]. We have previously shown that hemojuvelin is a co-receptor for bone morphogenetic proteins (BMPs) and that BMP signals regulate hepcidin expression and iron metabolism[3,4]. However, the endogenous BMP regulator(s) of hepcidin *in vivo* is unknown. Here we show that compared with soluble hemojuvelin (HJV.Fc), the homologous DRAGON.Fc is a more potent inhibitor of BMP2 or BMP4 but a less potent inhibitor of BMP6 *in vitro*. *In vivo*, HJV.Fc or a neutralizing antibody to BMP6 inhibits hepcidin expression and increases serum iron, whereas DRAGON.Fc has no effect. Notably, *Bmp6*-null mice have a phenotype resembling hereditary hemochromatosis, with reduced hepcidin expression and tissue iron overload. Finally, we demonstrate a physical interaction between HJV.Fc and BMP6, and we show that BMP6 increases hepcidin expression and reduces serum iron in mice. These data support a key role for BMP6 as a ligand for hemojuvelin and an endogenous regulator of hepcidin expression and iron metabolism *in vivo*.

Secreted by the liver[5], hepcidin inhibits intestinal iron absorption and macrophage iron release by decreasing cell surface expression of the iron exporter ferroportin[6]. Hepcidin is upregulated by iron administration[5,7,8] and inhibited by anemia[7]. Hepcidin deficiency and unchecked ferroportin activity are the common pathogenic mechanisms underlying the genetic iron-overload disorder hereditary hemochromatosis[9]. Hepcidin is also upregulated by inflammatory cytokines, and hepcidin excess is implicated in the pathogenesis of anemia of inflammation[5,7,8,10–12].

Recently, a role for the BMP signaling pathway in hepcidin regulation was discovered[3,4,13]. BMPs are members of the TGF-β superfamily, which comprises over 40 ligands[14]. BMP–TGF-β superfamily ligands initiate an intracellular signaling cascade by binding to a complex of type I and type II serine-threonine kinase receptors. The activated receptor complex phosphorylates intracellular Smad proteins, which translocate to the nucleus to modulate gene expression. Reduction of hepatic BMP signaling by a liver-specific conditional knockout of the common BMP–TGF-β intracellular mediator *Smad4* (ref. 13), or by mutations in the BMP co-receptor *HFE2* (refs. 2,3,15,16), is associated with inappropriately low hepcidin expression and iron overload. We and others have shown that BMP signals positively increase hepcidin expression at the transcriptional level *in vitro*[3,4,13,17,18]. We have also shown that iron administration *in vivo* increases hepatic BMP signaling[19] and that BMP administration *in vivo* increases hepcidin expression and reduces serum iron[4]. Conversely, inhibition of endogenous BMP signaling with soluble hemojuvelin (HJV.Fc) or with the small-molecule BMP inhibitor dorsomorphin inhibits hepcidin expression and increases serum iron *in vivo*[4,19]. Presumably, the mechanism by which HJV.Fc inhibits hepcidin is by binding to endogenously secreted BMP ligands and preventing their interaction with cell surface signaling receptors[4].

Hemojuvelin (also known as RGMc) is a member of the repulsive guidance molecules (RGM) family, including RGMa and DRAGON (RGMb). RGM family members share 50–60% amino acid identity[20]. Like hemojuvelin, RGMa[21] and DRAGON[22] also bind BMP ligands and function as co-receptors for the BMP signaling pathway. To explore other BMP inhibitors as potential hepcidin-lowering agents, we tested whether purified soluble DRAGON fused to the Fc portion of human IgG1 (DRAGON.Fc) inhibited BMP induction of hepcidin expression in human hepatoma–derived Hep3B cells in a manner similar to HJV.Fc[4]. DRAGON.Fc significantly inhibited hepcidin promoter induction by BMP2 or BMP4 but was less effective in inhibiting BMP5, BMP6 or BMP7 and did not inhibit BMP9 (**Fig. 1a**). In comparison with HJV.Fc, DRAGON.Fc was significantly more potent against BMP2 (**Fig. 1b**) and BMP4 (**Fig. 1c**) but less potent against BMP6 (**Fig. 1d**). DRAGON.Fc also inhibited endogenous *HAMP* mRNA expression in hepatoma-derived HepG2 cells, in

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Received 3 November 2008; accepted 26 January 2009; published online 1 March 2009; doi:10.1038/ng.335

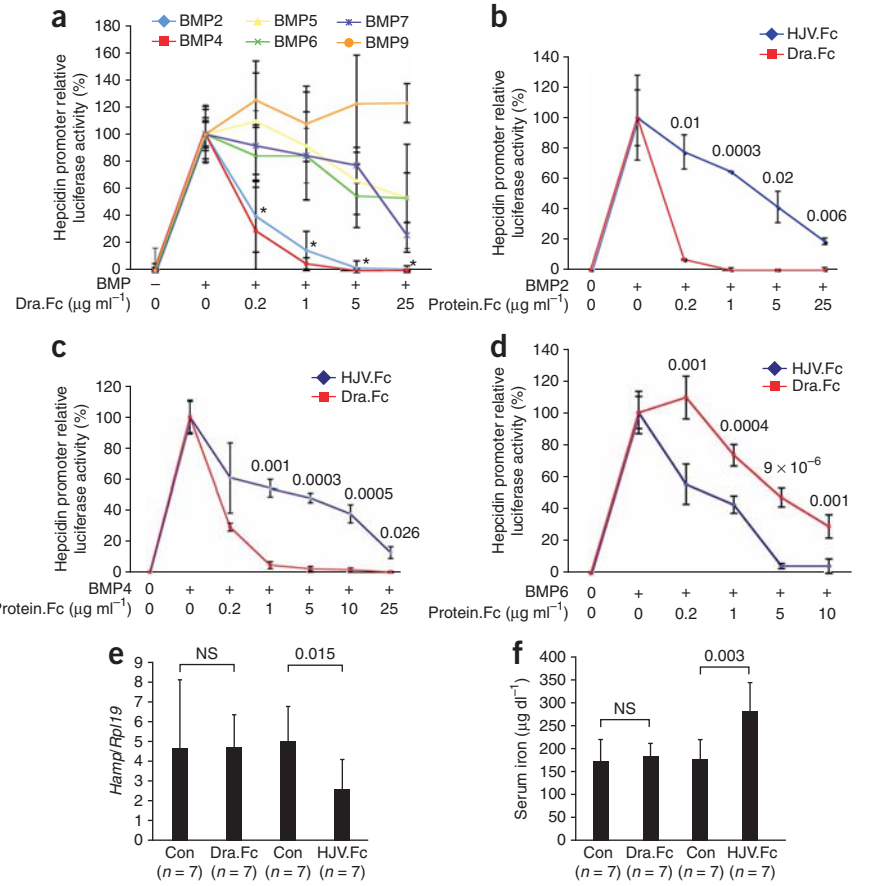

Figure 1 DRAGON.Fc selectively inhibits BMP induction of hepcidin expression *in vitro* but does not affect hepcidin expression or iron metabolism *in vivo*. (**a**–**d**) Effects of DRAGON.Fc (Dra.Fc) on hepcidin promoter induction by BMP ligands in Hep3B cells as measured by luciferase assay. Results are reported as the mean ± s.d. of the percentage decrease in relative luciferase activity for cells treated with BMP ligands in combination with Dra.Fc or HJV.Fc, compared with cells treated with BMP ligands alone, $n = 6$–11 per group. (**a**) Effects of Dra.Fc on BMP2, BMP4, BMP5, BMP6, BMP7 and BMP9 ligands. For the indicated cells treated with BMP2 or BMP4 in combination with all concentrations of Dra.Fc, $^*P < 0.001$ compared with cells treated with BMP2 or BMP4 alone, and $^*P \leq 0.02$ compared with cells stimulated with all other BMPs and treated with identical concentrations of Dra.Fc. (**b**–**d**) Head-to-head comparison of Dra.Fc and HJV.Fc for inhibition of BMP2 (**b**), BMP4 (**c**) and BMP6 (**d**). Exact *P* values are shown. (**e**,**f**) Effects of Dra.Fc and HJV.Fc administration in mice on hepatic *Hamp* (hepcidin) mRNA expression and serum iron. Control mice (Con) were treated with an equal volume of isotonic saline. (**e**) *Hamp* mRNA abundance, relative to *Rpl19* mRNA as an internal control, in total liver RNA as analyzed by quantitative real-time RT-PCR. (**f**) Serum iron. Results in **e**,**f** are reported as the mean ± s.d, $n = 7$ per group. Exact *P* values are shown.

which basal hepcidin expression is dependent in part on endogenous BMP2, BMP4 and BMP6 ligands[4] (**Supplementary Fig. 1**).

We then tested whether DRAGON.Fc administration affected hepcidin expression and iron metabolism *in vivo*. HJV.Fc at a similar dose was used as a positive control. In contrast to HJV.Fc, DRAGON.Fc had no effect on hepatic *Hamp* mRNA (**Fig. 1e**), splenic ferroportin (**Supplementary Fig. 2a**,**b**), serum iron (**Fig. 1f**), serum transferrin saturation, or liver or spleen iron content (**Supplementary Fig. 2c**,**e**) compared with those in mock-treated control mice. Anti-BMP2 activity in the serum of DRAGON.Fc-treated mice was confirmed by the ability of this serum to inhibit BMP2 induction of hepcidin promoter activity *in vitro* compared with serum from mock-treated mice (**Supplementary Fig. 2f**).

Because DRAGON.Fc—despite its higher potency *in vitro* as an inhibitor of BMP2 and BMP4 compared with HJV.Fc—had no effect *in vivo* and because DRAGON.Fc was less potent than HJV.Fc at inhibiting BMP6, we hypothesized that the BMP6–inhibiting properties of HJV.Fc are important for its effects as a hepcidin-lowering agent *in vivo*. We therefore tested whether a neutralizing antibody to BMP6 (anti-BMP6) affected hepcidin expression and serum iron levels in mice. The antibody selectively inhibited BMP6–induced hepcidin promoter activity *in vitro* but had no significant effect on BMP2, BMP4 or BMP9 (**Fig. 2a**). Anti-BMP6 showed some inhibitory activity against BMP7, and to a lesser extent BMP5, at higher concentrations, but significantly less than with BMP6 (**Fig. 2a**). This cross-reactivity is not surprising because BMP6, BMP7 and BMP5 have 71–80% amino acid identity and form a subfamily within the BMP ligands[23]. To minimize the effects of this cross-reactivity, we used

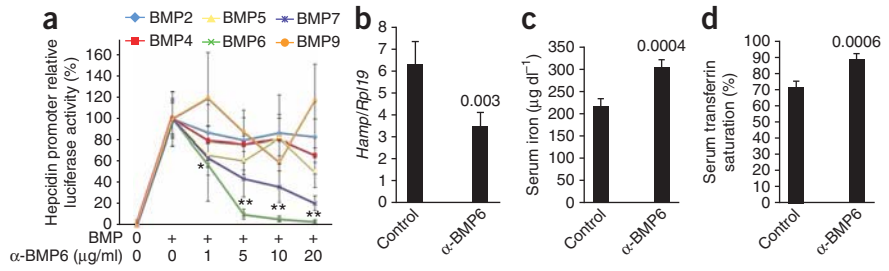

Figure 2 Neutralizing BMP6 inhibits hepatic hepcidin expression and increases serum iron and transferrin saturation *in vivo*. (**a**) Effects of a neutralizing anti-BMP6 on hepcidin promoter induction by BMP ligands in Hep3B cells as measured by luciferase assay ($n = 5$ per group). (**b**–**d**) Effects of anti-BMP6 administration in mice (at 10 mg per kg body weight daily for 3 d) on hepatic *Hamp* (hepcidin) mRNA expression and serum iron parameters. Control mice were treated with an equal volume of isotonic saline ($n = 4$ per group). (**b**) *Hamp* mRNA abundance, relative to *Rpl19* mRNA as an internal control, in total liver RNA as analyzed by quantitative real-time RT-PCR. (**c**) Serum iron. (**d**) Serum transferrin saturation. All results are expressed as the mean ± s.d. In **a**, $^*P = 0.036$ for cells treated with 0.2 μg ml^{-1} anti-BMP6 in combination with BMP6 ligand compared with cells treated with BMP6 alone; $^{**}P \leq 0.01$ for all other concentrations of anti-BMP6 compared with cells treated with BMP6 alone and compared with cells stimulated with all other BMPs and treated with identical concentrations of anti-BMP6. In **b**–**d**, exact *P* values are shown.

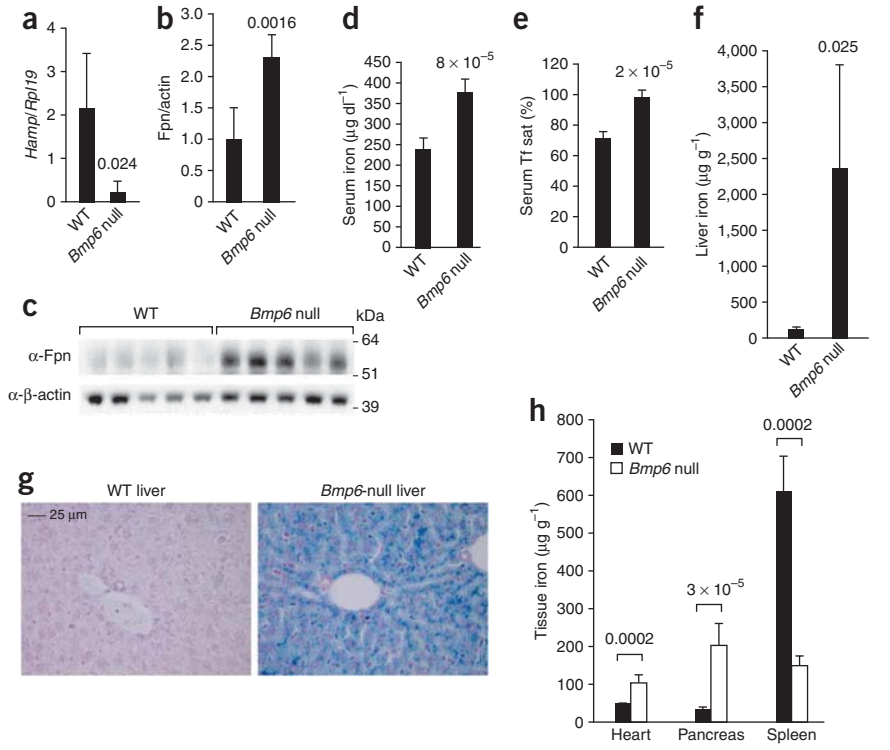

Figure 3 *Bmp6*-null mice show reduced hepatic hepcidin expression, increased spleen ferroportin expression, increased serum iron and transferrin saturation, increased liver, heart, and pancreas iron content, and reduced spleen iron content. (**a**–**g**) Results of analyses of 8-week-old male *Bmp6*-null mice ($n = 5$) and strain-matched wild-type control mice (WT, $n = 5$) for (**a**) *Hamp* (hepcidin) mRNA expression relative to *Rpl19* mRNA expression by quantitative real-time RT-PCR; (**b**,**c**) ferroportin (Fpn) expression relative to β-actin expression by protein blotting (**c**) followed by quantification using IPLab Spectrum software (**b**); (**d**) serum iron; (**e**) serum transferrin saturation (Tf sat); (**f**) quantitative liver iron content; (**g**) Perls Prussian blue staining of liver iron (original magnification ×40); and (**h**) quantitative heart, pancreas and spleen iron content. Results are expressed as mean ± s.d. Exact *P* values are shown.

the lowest effective dose of anti-BMP6 for subsequent *in vivo* experiments. Cross-reactivity with BMP7 was also less of a concern because BMP7 is not a ligand for hemojuvelin, is not expressed in the liver and is not thought to be an important endogenous hepcidin regulator[4,23]. Treatment with anti-BMP6 at 10 mg per kg body weight for 3 d significantly reduced hepatic *Hamp* mRNA expression, by ∼50%, and increased serum iron and transferrin saturation compared with those in mock-treated control mice (**Fig. 2b–d**). Anti-BMP6 treatment also caused a trend toward reduced hepatic mRNA expression of *Id1*—another expected target of BMP6 (ref. 21) that we assessed as a control—by ∼60% (**Supplementary Fig. 3**). Similar results were found with a lower dose of anti-BMP6, although this dose was less effective (**Supplementary Fig. 4**)

To validate the importance of endogenous BMP6 in regulating hepcidin expression and iron metabolism *in vivo*, we examined 4- and 8-week-old *Bmp6*-null mice previously generated by other researchers, which had been found to have some mild delays in bone formation during development but no other overt defects[24]. Compared with wild-type mice, 8-week-old *Bmp6*-null mice showed significantly (tenfold) reduced hepatic *Hamp* mRNA expression, increased splenic ferroportin expression, and increased serum iron, with serum transferrin saturation approaching 100% (**Fig. 3a–e**). Liver iron content was significantly increased in the *Bmp6*-null mice, by 6-fold at 4 weeks and 20-fold at 8 weeks (**Supplementary Fig. 5a** and **Fig. 3f**,**g**). Significant iron accumulation was also evident in the hearts and pancreases of *Bmp6*-null mice, whereas spleen iron content was reduced (**Fig. 3h** and **Supplementary Fig. 5b**). The degree of iron overload in the livers of 8-week-old *Bmp6*-null mice appears comparable to that reported in *Hfe2*$^{-/-}$ mice at a similar age[15,16]. Thus, *Bmp6*-null mice have a phenotype that resembles mouse models of juvenile hemochromatosis as a result of the loss of the BMP co-receptor hemojuvelin.

To further explore whether BMP6 is a ligand for hemojuvelin, purified BMP6 alone, HJV.Fc alone or the combination of BMP6 and HJV.Fc were incubated in solution, and complexes containing HJV.Fc were pulled down with protein A beads. Protein blotting of eluates with anti-BMP6 showed that an 18-kDa protein corresponding to the predicted size of BMP6 monomer was pulled down in the presence of HJV.Fc (**Fig. 4**, arrow). No 18-kDa band was seen on the blots of pull-downs from the solutions containing BMP6 alone or HJV.Fc alone (**Fig. 4**). These data demonstrate a direct interaction between BMP6 and HJV.Fc.

Next, we examined the ability of exogenous BMP6 to regulate hepcidin expression and iron metabolism *in vivo*. Mice were injected intraperitoneally (i.p.) with a single dose of exogenous BMP6 at 250 or 1,000 μg per kg body weight. BMP6 administration significantly increased hepatic expression of *Id1* mRNA, which we assessed as a

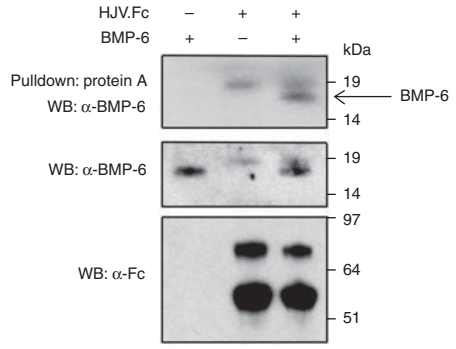

Figure 4 BMP6 interacts with HJV.Fc. Above, protein A pulldown of solutions containing BMP6 alone, HJV.Fc alone or BMP6 + HJV.Fc, followed by protein blotting (WB) with anti-BMP6 under reducing conditions. The band migrating at ∼18 kDa present only in the lane containing BMP6 + HJV.Fc corresponds to the predicted size of the BMP6 monomer (arrow). Below, as a control to demonstrate input proteins, solution aliquots from before protein A pulldown were also analyzed by protein blotting with anti-Fc and anti-BMP6 under reducing conditions. Control blots revealed the predicted bands migrating at 18 kDa for BMP6 monomer and 75 and 60 kDa for HJV.Fc[4]. A background band at ∼19 kDa was present in the lanes containing HJV.Fc probed with anti-BMP6, suggesting that this antibody has nonspecific cross-reactivity with a component of the HJV.Fc solution.

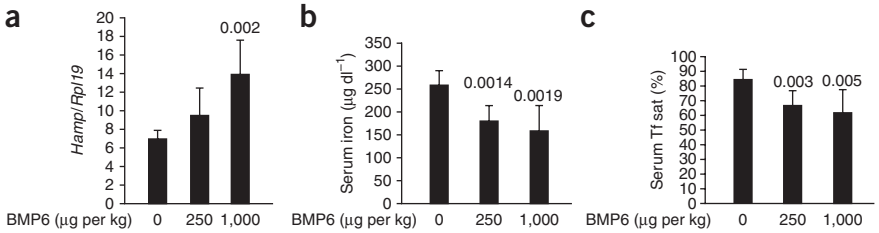

Figure 5 BMP6 administration in mice increases *Hamp* (hepcidin) mRNA expression and reduces serum iron. Effects of BMP6 administration at 250 ($n = 6$) or 1,000 μg per kg body weight ($n = 7$) in mice on hepatic *Hamp* mRNA expression and serum iron. Control mice were injected with an equal volume of vehicle alone ($n = 6$). (**a**) *Hamp* mRNA abundance, as determined by quantitative real-time RT-PCR relative to *Rpl19* mRNA as an internal control, in total liver RNA of these mice. (**b**) Serum iron. (**c**) Serum transferrin saturation (Tf sat). Results are reported as the mean ± s.d. Exact *P* values are shown where significant.

positive control (**Supplementary Fig. 6**). BMP6 administration also significantly increased hepatic *Hamp* mRNA expression (**Fig. 5a**) and caused dose-dependent reductions in serum iron (**Fig. 5b**) and serum transferrin saturation (**Fig. 5c**).

Together, these data suggest that BMP6 is a ligand for hemojuvelin and that BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism *in vivo*. Numerous other BMP ligands have been shown to regulate hepcidin when added exogenously *in vitro* or *in vivo*, including BMP2, BMP4, BMP5, BMP7 and BMP9 (refs. 3,4,13,17). We have also previously shown that endogenous BMP2, BMP4 and BMP6 all contribute to basal hepcidin expression in HepG2 cells[4]. Thus, it is not surprising that inhibition of BMP2 and BMP4 by DRAGON.Fc inhibits hepcidin expression in these cells. However, it is well established that cell-based assays do not accurately mirror the *in vivo* regulation of hepcidin by iron, as iron increases hepcidin expression *in vivo* but decreases hepcidin expression *in vitro* under most conditions[5,10,25]. One reason for this discrepancy may be that the complement of BMP ligands and receptors that regulate hepcidin expression *in vitro* is different from those that regulate hepcidin expression in response to iron *in vivo*. We hypothesize that HJV.Fc and anti-BMP6 inhibit hepcidin expression *in vivo* by binding to endogenously secreted BMP6 and preventing its interaction with cell-surface signaling receptors. We hypothesize that DRAGON.Fc does not affect hepcidin expression *in vivo* because (i) it is a less potent inhibitor of BMP6 than HJV.Fc and does not significantly inhibit BMP6 at the doses used in this study, and (ii) in contrast to the *in vitro* experiments, endogenous BMP2 and BMP4 ligands do not significantly contribute to hepcidin regulation *in vivo*. Indeed, the liver is presumably the source of the endogenous BMPs that regulate hepcidin, and a recent study reported that hepatic *Bmp6* mRNA levels vary concordantly with *Hamp* mRNA levels in response to dietary iron content, whereas *Bmp2* mRNA levels are only slightly upregulated under extreme iron overload and *Bmp4* mRNA levels are not modulated by dietary iron[26]. We also did not find changes in hepatic *Bmp2* and *Bmp4* mRNA levels in *Bmp6*-null mice despite significant iron overload (**Supplementary Fig. 7**). Notably, *Bmp2* and *Bmp4* mRNA abundance were increased 2.5- to 6-fold in the bone of *Bmp6*-null mice (P. Simic, I. Orlic, V. Kufner and S.V., unpublished data), suggesting that BMP2 and BMP4 are differentially regulated in different tissues and may compensate for the loss of BMP6 function in some tissues but do not compensate for the lack of BMP6 to regulate hepcidin expression and iron metabolism.

The *Bmp6*-null mice contain a neomycin cassette, raising the possibility that expression of neighboring genes might also be affected, as described in other mouse models[27]. The closest neighboring gene to *Bmp6* is that encoding thioredoxin domain–containing protein 5 (*Txndc5*), 547 bp away. Expression of *Txndc5* mRNA was similar in *Bmp6*-null and wild-type mice as measured by RT-PCR (**Supplementary Fig. 7**). Although we cannot rule out small changes in *Txndc5* mRNA levels by this technique, our interventional data showing that BMP6 administration increases hepcidin and reduces serum iron, while endogenous BMP6 inhibition reduces hepcidin expression and increases serum iron, lend further support to the hypothesis that it is the loss of BMP6 that is the main cause of the iron-overload phenotype in *Bmp6*-null mice.

Our data suggest that selective BMP6 inhibitors may be effective agents for treating anemia of inflammation due to hepcidin excess. The lack of any other notable phenotype in *Bmp6*-null mice suggests that a more selective inhibitor may be better tolerated, with fewer off-target effects. Additionally, BMP6-like agonists may provide an alternative treatment strategy for managing iron-overload disorders in patients resistant to current therapies. Although no human patients with *BMP6* mutations have yet been described, our data also suggests that *BMP6* mutations or *BMP6* gene variants may function as another cause of hereditary hemochromatosis or a modifier of disease penetrance.

METHODS

cDNA. cDNA encoding codon-optimized DRAGON.Fc was generated by GenScript Corp., based on the extracellular domain of human DRAGON protein sequence upstream of the predicted glycosylphosphatidylinositol anchor (UniProtKB/Swiss-Prot accession code Q6NW40, amino acids 1–409) and the human IgG1 Fc sequence (from the Signal pIg plus vector (R&D Systems) and GenBank AF150959).

Production and purification of DRAGON.Fc and HJV.Fc. cDNA encoding DRAGON.Fc was transfected using 239fectin (Invitrogen) into Freestyle 293-F cells (Invitrogen) according to the manufacturer's instructions. Transfected cells were cultured in GIBCO Freestyle 293 Expression medium (Invitrogen) shaking at 110 r.p.m. in a humidified 8% CO_2 incubator at 37 °C. Seven days after transfection, cells were pelleted by centrifugation, and DRAGON.Fc was purified from the medium via one-step protein A affinity chromatography using Hi-Trap rProtein A FF columns (Amersham Biosciences) as previously described[21]. HJV.Fc was produced as previously described[4]. To determine purity and to quantify protein concentration, DRAGON.Fc and HJV.Fc were subjected to reducing SDS-PAGE followed by Bio-safe Coomassie blue staining (Bio-Rad) as well as protein blotting with anti-hemojuvelin[3], anti-DRAGON[20] and goat anti-human Fc (Jackson ImmunoResearch Laboratories) as previously described[3,20]. Protein concentration was also quantified by the bovine serum albumin protein assay (Pierce).

Production of BMP6. Purified recombinant human BMP6 was prepared as previously described[28]. Lyophilized BMP6 was dissolved in 20 mM sodium acetate, 5% mannitol solution, pH 4.0, for animal injections.

Luciferase assay. Hepcidin promoter–luciferase reporter assays in hepatoma-derived Hep3B cells were carried out using the Dual-Luciferase Reporter Assay System (Promega) as previously described[3,4] with the following modifications. Hep3B cells transfected with the hepcidin promoter–luciferase reporter[3] and control *Renilla* luciferase vector (pRL-TK) were serum starved in α-MEM with L-glutamine (Invitrogen) supplemented with 1% FBS for 6 h, followed by stimulation with 25 ng ml^{-1} BMP2 (kindly provided by V. Rosen, Harvard School of Dental Medicine), BMP4, BMP6, or BMP7, 50 ng ml^{-1} BMP5 or

5 ng ml^{-1} BMP9 (R&D Systems) either alone or with 0.2–25 μg ml^{-1} of DRAGON.Fc, HJV.Fc or anti-BMP6 for 16 h. Relative concentrations of BMP ligands were chosen to elicit similar degrees of hepcidin promoter–luciferase activity, as previously described[4]. Relative luciferase activity was calculated as the ratio of firefly to *Renilla* luciferase to control for transfection efficiency. The increase over baseline was generally between 40- and 150-fold for all BMP ligands for most experiments, although there was some variability. All experiments were performed at least three times. Some repetitions used a mouse homolog of DRAGON.Fc[20], which yielded results similar to those for the human DRAGON.Fc homolog.

Animals. All animal protocols were approved by the Institutional Animal Care and Use Committee at the Massachusetts General Hospital and the Institutional Animal Care Committee and the Ministry of Science and Technology at the University of Zagreb School of Medicine. Eight-week-old 129S6/SvEvTac mice (Taconic) were housed in the Massachusetts General Hospital rodent facility and fed on the Prolab 5P75 Isopro RMH 3000 diet with 380 p.p.m. iron. *Bmp6*-null mice on a mixed 129Sv/C57 background[24], kindly provided by E.J. Robertson, were housed at the University of Zagreb School of Medicine and maintained on standard GLP diet (4RF21, Mucedola) with 180 mg iron per kg.

For DRAGON.Fc and HJV.Fc experiments, 8-week-old 129S6/SvEvTac mice (Taconic) received an i.p. injection of DRAGON.Fc at doses of 5 or 10 mg per kg, HJV.Fc at doses of 5 or 7 mg per kg or an equal volume of isotonic saline three times per week for 3 weeks. Twenty-four hours after the last injection, mice were killed and blood and livers were harvested for measurement of iron parameters and hepcidin expression. Results for both DRAGON.Fc and HJV.Fc doses were similar and were therefore combined into one group.

For anti-BMP6 injection experiments, 8-week-old 129S6/SvEvTac mice received an i.p. injection of monoclonal anti-human BMP6 (R&D Systems) at 10 mg per kg daily for 3 d or at 5 mg per kg three times weekly for 3 weeks. Control mice received an i.p. injection of an equal volume of isotonic saline using the same dosing regimen. Based on the assumption that anti-BMP6 would function by a mechanism similar to that of HJV.Fc protein, by binding and sequestering endogenously produced BMP6 ligand, we chose the expected minimal effective dose of anti-BMP6 for these *in vivo* experiments based on our *in vitro* data obtained using this antibody and our experience with HJV.Fc protein. Twelve hours after the last injection, mice were killed and blood and livers were harvested for measurement of iron parameters and hepcidin expression.

For BMP6 injection experiments, 8-week-old 129/SvEvTac mice received an i.p. injection of BMP6 at 250 or 1,000 μg per kg or an equal volume of vehicle alone (20 mM sodium acetate, 5% mannitol solution, pH 4.0). Six hours after injection, mice were killed and blood, livers and spleens were harvested for measurement of iron parameters and hepcidin expression. A time point of 6 h was chosen to capture both an increase in hepcidin expression and a resulting decrease in serum iron based on prior *in vitro*[3] and *in vivo*[4] data about the effects of BMP2 on hepcidin expression and serum iron, as well as preliminary dose-curve and time-curve data on the effects of BMP6 on hepcidin expression and serum iron *in vivo* (data not shown).

For *Bmp6*-null mouse experiments, 4-week-old and 8-week-old *Bmp6*-null mice and wild-type control mice of a similar age were killed and blood, livers, hearts, pancreases and spleens were harvested for measurements of iron parameters and hepcidin expression.

Quantitative real-time RT-PCR and RT-PCR. Total RNA was isolated from mouse livers using the RNeasy kit (Qiagen) according to the manufacturer's instructions. Real-time quantification of *Hamp1* mRNA transcripts relative to *Rpl19* was performed using two-step quantitative real-time RT-PCR as previously described[3,4,29]. Real-time quantification of *Id1* mRNA was performed using primers summarized in **Supplementary Table 1**. Real time quantification of *Bmp2*, *Bmp4* and *Bmp6* mRNA from livers of *Bmp6*-null versus wild-type mice was performed using previously described primers[26]. *Txndc5* mRNA was amplified from the livers of *Bmp6*-null and wild-type mice using primers summarized in **Supplementary Table 1**.

Protein blotting. For ferroportin assays, spleen membrane preparations were prepared as previously described[4]. Protein concentrations were determined by BCA assay (Pierce). After solubilization in 1× Laemmli buffer for 30 min at room temperature, 20 μg of protein per sample were resolved by reducing SDS-PAGE using pre-cast NuPAGE Novex 4–12% Bis-Tris gels (Invitrogen) and transferred onto PDVF membranes (liquid transfer method). The blots were saturated with 10% nonfat dry milk in TBS-T) (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) and probed overnight at 4 °C with 2.5 μg ml^{-1} anti-ferroportin (diluted in TBS-T with 5% nonfat milk)[30]. Following a wash with TBS-T, the blots were incubated with 1:5,000-diluted peroxidase-coupled goat anti–rabbit IgG (Sigma) for 1 h. Detection was performed with the enhanced chemiluminescence ECL method (Perkin Elmer). Blots were stripped and re-probed for β-actin expression as a loading control as previously described[3]. Chemiluminescence was quantified using IPLab Spectrum software version 3.9.5 r2 (Scanalytics).

Serum and tissue iron measurements. Serum was collected and analyzed for iron concentration and unsaturated iron-binding capacity as previously described[4]. Total iron-binding capacity and transferrin saturation were calculated as previously described[4]. Quantitative measurement of nonheme iron was performed on liver, spleen, heart and pancreas as previously described[4].

Histology. Tissues from *Bmp6*-null and wild-type mice were fixed in 2% paraformaldehyde followed by 2% ethanol and embedded in paraffin. Sections were cut at 5 μm, deparaffinized in xylene and hydrated to distilled water. Sections were then placed in staining solution, consisting of equal volumes of 2% potassium ferrocyanide (Electron Microscopy Sciences) and 2% hydrochloric acid, for 60 min at room temperature. The sections were rinsed in distilled water, counterstained in 0.2% safranin O (Electron Microscopy Sciences) for 2 min and washed in 1% acetic acid, before being dehydrated in 95% alcohol and then absolute alcohol, cleared in xylene and mounted in DPX medium.

Pulldown assay. BMP6 (1 μg, R & D Systems) alone, HJV.Fc (5 μg) alone, or BMP6 (1 μg) in combination with HJV.Fc (5 μg) were incubated at 4 °C overnight in 500 μl of 50 mM Tris-HCl, 150 mM NaCl, 0.2% Tween-20, pH 7.4. The solutions were then incubated for 3 h at 4 °C with protein A beads (Pierce Biotechnology) that had been blocked with 1% BSA. The beads were washed and proteins were eluted with reducing 1× Laemmli sample buffer. Eluted protein was separated by SDS-PAGE and subjected to protein blot analysis under reducing conditions using a rabbit polyclonal anti-BMP6 (1:500)[28]. Protein blot analysis of the above solutions before protein A pulldown was also performed under reducing conditions using rabbit polyclonal anti-BMP6 (1:500) as above and goat anti–human Fc (Jackson ImmunoResearch Laboratories) as previously described[3].

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

Accession numbers. *HAMP*, hepcidin (*Homo sapiens*), NM_021175, GeneID 57817; *Hamp1*, hepcidin (*Mus musculus*), NM_032541, GeneID 84506; *HFE2* transcript variant a, hemojuvelin (*H. sapiens*), NM_213653, GeneID 148738; *Id1*, inhibitor of DNA binding (*M. musculus*), NM_010495, GeneID 15901; *Bmp6*, bone morphogenetic protein 6 (*M. musculus*), NM_007556, GeneID 12161; *Bmp2*, bone morphogenetic protein 2 (*M. musculus*), NM_007553, GeneID 12156; *Bmp4*, bone morphogenetic protein 4 (*M. musculus*), NM_007554, GeneID 12159; *Txndc5*, thioredoxin domain–containing 5 (*M. musculus*), NM_145367, GeneID 105245; *Rpl19*, ribosomal protein L19 (*M. musculus*), NM_009078, GeneID 19921.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We thank V. Rosen for kindly providing BMP2 for our study. We thank L.M. Russo for assistance with animal work. E.C. was supported in part by the Associazione Modenese per le Malattie del Fegato. S.V. was supported in part by the Croatian Ministry of Science, Education, and Sport. H.Y.L. was supported in part by US National Institutes of Health grants RO1 DK-69533 and RO1 DK-071837. J.L.B. was supported in part by National Institutes of Health grant K08 DK-075846 and by a Claflin Distinguished Scholar Award from the Massachusetts General Hospital.

AUTHOR CONTRIBUTIONS

B.A., E.C., Y.X., H.Y.L. and J.L.B. designed experiments. B.A., E.C., Y.X., S.A.F., S.C., L.G. and J.L.B. performed experiments. B.A., E.C., Y.X., S.A.F. and J.L.B. analyzed data. M.D.K., A.P., S.V. and H.Y.L. contributed vital resources and reagents. B.A., E.C., Y.X., A.P., S.V., H.Y.L. and J.L.B. wrote or edited the paper. J.L.B. conceived and oversaw the entire project.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturegenetics/.

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