Modulation of bone morphogenetic protein signaling in vivo regulates systemic iron balance

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Systemic iron balance is regulated by hepcidin, a peptide hormone secreted by the liver. By decreasing cell surface expression of the iron exporter ferroportin, hepcidin decreases iron absorption from the intestine and iron release from reticuloendothelial stores. Hepcidin excess has been implicated in the pathogenesis of anemia of chronic disease, while hepcidin deficiency has a key role in the pathogenesis of the iron overload disorder hemochromatosis. We have recently shown that hemojuvelin is a coreceptor for bone morphogenetic protein (BMP) signaling and that BMP signaling positively regulates hepcidin expression in liver cells in vitro. Here we show that BMP-2 administration increases hepcidin expression and decreases serum iron levels in vivo. We also show that soluble hemojuvelin (HJV.Fc) selectively inhibits BMP induction of hepcidin expression in vitro and that administration of HJV.Fc decreases hepcidin expression, increases ferroportin expression, mobilizes splenic iron stores, and increases serum iron levels in vivo. These data support a role for modulators of the BMP signaling pathway in treating diseases of iron overload and anemia of chronic disease.

Introduction
Anemia of chronic disease, also known as anemia of inflammation, is prevalent in patients with many systemic diseases including autoimmune disorders, malignancy, and chronic kidney disease (1). In these patients intestinal iron absorption is impaired and iron remains sequestered in reticuloendothelial cells, leading to hypoferremia and anemia (1). Research over the last several years implicates hepcidin excess in the pathogenesis of this disease (1–6). A key regulator of systemic iron homeostasis (7), hepcidin is secreted by the liver (2, 8, 9) and induces internalization and degradation of the iron exporter ferroportin in absorptive enterocytes and reticuloendothelial cells, thereby inhibiting iron absorption from the intestine and iron release from reticuloendothelial cell stores (10). Hepcidin expression is inhibited by anemia and hypoxia, thus increasing iron availability when needed for erythropoiesis (3). Conversely, hepcidin expression is induced by iron loading, thus providing a feedback mechanism to limit further iron absorption (2–4). Hepcidin expression is also induced by inflammatory cytokines, and this is thought to be the mechanism underlying the impaired intestinal iron absorption, reticuloendothelial cell iron sequestration, and hypoferremia characteristic of anemia of chronic disease (1–6).

While hepcidin excess has a role in anemia of chronic disease, inadequate hepcidin expression appears to be a common pathogenic mechanism for the iron overload disorder hereditary hemochromatosis as a result of mutations in the genes encoding hepcidin (HAMP), hemojuvelin (HFE2), HFE, or transferrin receptor 2 (TFR2) (11–21). In patients and animal models with iron overload due to mutations in these genes, hepcidin levels are low, thereby leading to ferroportin overactivity, increased intestinal iron absorption, increased reticuloendothelial cell iron release, elevated serum iron levels, and abnormal tissue iron deposition (11–21). Although the mechanisms by which mutations in HFE and TFR2 lead to low hepcidin levels remain unclear, emerging evidence suggests that hemojuvelin functions as a coreceptor for bone morphogenetic protein (BMP) signaling (22) and that BMP/TGF-β superfamily signaling has a role in regulating hepcidin expression and systemic iron balance (22–24).

Members of the BMP/TGF-β superfamily, including BMPs, TGF-βs, growth and differentiation factors (GDFs), and activins, initiate an intracellular signaling cascade by binding to a complex of type I and type II serine threonine kinase receptors (25). The activated receptor complex phosphorylates intracellular Smad proteins, which then complex with common mediator Smad4. Smad complexes translocate to the nucleus where they modulate gene transcription. In general, BMPs and GDFs signal via one set of Smad proteins (Smad1, Smad5, and Smad8), while TGF-βs and activins signal via another set (Smad2 and Smad3).

Hemojuvelin (also known as RGMc) is a member of the repulsive guidance molecule (RGM) family, which includes RGMa and DRAGON (also known as RGMb) (13, 26, 27). We have recently demonstrated that, similar to RGMa and DRAGON (28, 29), hemojuvelin functions as a BMP coreceptor that binds directly to BMP-2 and BMP-4 and enhances cellular responses to BMP, but not TGF-β, ligands (22). Furthermore, BMP-2 positively regulates hepcidin expression (22, 24), and hemojuvelin increases hepcidin induction in response to BMP-2 (22). Hemojuvelin mutants associated with juvenile hemochromatosis have impaired BMP signaling ability, and hepatocytes from Hjfofe2–/– mice demonstrate blunted hepcidin induction in response to BMP-2 (22). This suggests that the mechanism for iron overload in patients with hemojuvelin mutations is a result of decreased BMP signaling in the liver leading to decreased hepcidin expression.

Further evidence supporting a role for BMP signaling in regulating hepcidin expression and iron metabolism in vivo comes from...
mice with a conditional liver-specific knockout of Smad4. These mice have low hepcidin levels and develop iron overload (23). In that study, both BMP-4 and TGF-β1 were shown to induce hepcidin expression in liver cells in vitro (23). Hepcidin induction by BMP-9 has also been described (24).

Here we test a wide array of TGF-β superfamily members to compare their relative abilities to regulate hepcidin expression in vitro. We also investigate the ability of BMP-2 to positively regulate hepcidin expression and reduce serum iron levels in vivo. Finally, we generate soluble hemojuvelin, comprised of the extracellular domain of hemojuvelin fused to the Fc portion of human IgG (HJV.Fc) and examine the ability of soluble hemojuvelin to inhibit hepatic BMP signaling, decrease hepcidin expression, increase ferroportin expression, mobilize reticuloendothelial iron stores, and increase serum iron levels.

Results

Selective regulation of hepcidin by BMP/TGF-β superfamily members. TGF-β superfamily members were tested for their ability to regulate hepcidin using both a hepcidin promoter reporter assay (Figure 1A) and quantitative real-time RT-PCR (Figure 1B) in Hep3B hepatoma-derived cells. Relative concentrations of BMP/TGF-β superfamily ligands used are similar to those previously used by others to compare responses among superfamily ligands (23, 30, 31). BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, and BMP-9 robustly increased hepcidin promoter luciferase activity 20- to 100-fold over baseline and increased hepcidin mRNA expression by 160- to 1,100-fold. In contrast, TGF-β1, -β2, and -β3 increased hepcidin expression by only 1.5- to 3-fold over baseline by both methods. BMP-3, BMP-11, GDF-5, GDF-6, and GDF-7 showed no or comparatively little hepcidin induction by both methods. Activin A increased hepcidin promoter relative luciferase activity by 10-fold but increased hepcidin mRNA expression to a lesser extent relative to BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, and BMP-9 as analyzed by real-time RT-PCR. Biologic activity of all ligands was verified by luciferase assay using a BMP-responsive firefly luciferase reporter (30) and a TGF-β/activin-responsive firefly luciferase reporter (31). Results using both methods correlated well with each other, suggesting that the hepcidin promoter luciferase assay is a good surrogate for hepcidin mRNA expression by quantitative real-time RT-PCR. Thus, many TGF-β superfamily members can positively regulate hepcidin expression in vitro; however, BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, and BMP-9 are much more potent regulators of hepcidin compared with other superfamily members, including all 3 TGF-β ligands.

BMP-2 administration in vivo increases hepcidin expression and decreases serum iron. We next investigated whether BMP-2 regulates hepcidin expression and iron metabolism in vivo. Purified BMP-2 at 1 mg/kg was injected retroorbitally into mice, followed by determination of serum iron levels and hepatic hepcidin mRNA expression 4 hours after injection. BMP-2 administration increased hepatic hepcidin mRNA expression 1.8-fold over mice injected with vehicle alone (Figure 2A; P = 0.02). BMP-2 administration also decreased serum iron levels from 170 μg/dl to 114 μg/dl (Figure 2B; P = 0.02). This is consistent with a role for BMP-2 as a positive regulator of hepcidin expression in vivo.

Soluble HJV.Fc selectively inhibits BMP signaling in vitro. Soluble receptors such as the soluble TNF receptor etanercept have previously been used to inhibit ligand activity in vitro and in vivo, presumably by binding to ligands and preventing their interaction with membrane-bound receptors (32). Interestingly, soluble hemojuvelin has been detected in human sera and has been shown to inhibit hepcidin expression in cultured cells, although the mechanism for this inhibition was not investigated (33). We therefore generated purified soluble HJV.Fc (Figure 3A), the murine homolog of which...
we have previously shown can bind to BMP-2 and BMP-4 ligands (22). We then investigated whether HJV.Fc inhibited basal hepcidin expression and BMP induction of hepcidin expression in vitro. In hepatoma-derived HepG2 cells, which have higher basal hepcidin expression, HJV.Fc inhibited basal hepcidin mRNA expression by 80% (Figure 3B; \(P = 0.03\)). These results are consistent with a prior report using soluble hemojuvelin that does not contain an Fc tail (33), suggesting that the Fc tail does not affect the function of soluble hemojuvelin. HJV.Fc also inhibited BMP-2 induction of hepcidin expression (Figure 3C; \(P = 0.009\)) and BMP-2–induced activation of the hepcidin promoter in a dose-dependent fashion (Figure 3D). HJV.Fc inhibition of BMP ligands was selective: HJV.Fc inhibited more than 90% of hepcidin promoter activation induced by BMP-2, BMP-4, BMP-5, and BMP-6 but did not inhibit BMP-9 even at lower ligand concentrations (Figure 3D). There was a trend toward low-level inhibition of BMP-7 (Figure 3D).

We have previously demonstrated that BMP-2 and BMP-4 are endogenously expressed in HepG2 cells (22), and we hypothesized that inhibition of these endogenous BMP ligands was the mechanism by which HJV.Fc decreased basal hepcidin expression in HepG2 cells. We therefore used RT-PCR to investigate whether other BMP ligands are endogenously expressed in HepG2. We then tested whether siRNA inhibition of these endogenous BMP ligands inhibited basal hepcidin expression in a manner similar to HJV.Fc. BMP-2, BMP-4, and BMP-6 were endogenously expressed in HepG2 cells, with BMP-4 being the most abundant (Figure 4A). BMP-2, BMP-4, and BMP-6 siRNA each selectively and significantly reduced endogenous ligand expression in HepG2 cells by 65%, 90%, and 55%, respectively, as measured by real-time RT-PCR (Figure 4B). BMP-2, BMP-4, and BMP-6 siRNA each significantly inhibited basal hepcidin expression in HepG2 cells by approximately 10% \((P = 0.012)\), 35% \((P = 0.0027)\), and 15% \((P = 0.0026)\), respectively, as measured by real-time RT-PCR (Figure 4B).
Western blot analysis of liver lysates from these mice showed decreased phosphorylated Smad1, Smad5, and Smad8 expression relative to total Smad1 expression. The relative ability of each ligand to inhibit basal hepcidin correlated with the relative mRNA abundance of the ligand and the strength of siRNA inhibition of ligand expression.

Quantitative real-time RT-PCR analysis revealed a 10-fold decrease in hepcidin expression relative to GAPDH mRNA expression as an internal control. BLAST search and sequence analysis confirmed the identity of the amplified fragment for HJV.Fc-treated mice compared with controls.

Discussion
Hepcidin deficiency is the common pathogenic mechanism for both juvenile and adult forms of the genetic iron overload disorder hereditary hemochromatosis as a result of mutations in HAMP, HFE2, TFR2, and HFE (11–21). We have previously shown that hemojuvulin acts as a coreceptor for BMP signaling and that BMP-2 signaling induces hepcidin expression in vitro (22). Here we show that BMP-2 administration in mice increases hepcidin expression and reduces serum iron levels in vivo. These data support our in vitro data that BMP-2 can positively regulate hepcidin expression in vivo. The modest induction of hepcidin expression in response to BMP-2 in vivo compared with our in vitro findings is likely multifactorial. First, the mice were maintained on a standard diet, where dietary iron is replete, basal hepcidin levels are generally high, and hepcidin induction by well established regulators such as iron and LPS have been reported to be absent or less robust compared with mice maintained on an iron-deficient diet (4). Indeed, the degree of hepcidin induction by BMP-2 in our study was similar to the 1.8-fold induction reported.
Whether the liver is in fact the source of the endogenous BMP superfamily ligands is an important role for TGF-β signaling (23); however, we found that BMP-4 and BMP-9 were much more efficient to the liver. Nevertheless, the decrease in serum iron after LPS administration in mice fed on a standard diet (34). Further, BMPs typically act in an autocrine or paracrine manner in vivo, while i.v. administered BMP-2 has been documented to be rapidly eliminated from the systemic circulation ($t_{1/2} = 16$ min) (35). Thus, it is likely that the systemically administered BMP-2 dose was not delivered efficiently to the liver. Nevertheless, the decrease in serum iron suggests that the BMP-2–induced increase in hepcidin expression was physiologically relevant and presumably reflects decreased iron export from reticuloendothelial cells and duodenal enterocytes due to hepcidin-induced internalization and degradation of ferroportin. Although treatment with systemic BMP-2 itself may be impractical due to its high cost and rapid elimination from the systemic circulation, our data suggest that therapies that enhance hepatic BMP signaling may provide alternative treatment strategies for managing iron overload in patients with hereditary hemochromatosis.

Liver-specific conditional Smad4 knockout mice have reduced hepcidin expression and total body iron overload, underscoring the important role for TGF-β/BMP superfamily members in regulating hepcidin expression and iron metabolism in vivo (23). While our data demonstrate that BMP-2 can positively regulate hepcidin expression in vivo, it remains unknown which superfamily ligands are the endogenous regulators of hepcidin expression, since Smad4 is the common downstream mediator for all superfamily members. Indeed, many superfamily members are endogenously expressed in the liver, including BMP-2, BMP-4, BMP-5, BMP-6, BMP-9, and all 3 TGF-β ligands (36–40 and J.L. Babitt, unpublished observations). Whether the liver is in fact the source of the endogenous BMP superfamily ligands, however, remains uncertain. Our previous data showing that hemojuvelin is a coreceptor for BMP, but not TGF-β, signaling (22) suggests that members of the BMP subfamily are more important than members of the TGF-β subfamily for regulating iron metabolism in vivo. Here we show that many members of the TGF-β superfamily can induce hepcidin mRNA expression in vitro. However, our results suggest that a subset of BMP ligands including BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, and BMP-9 are much more potent inducers of hepcidin expression than other ligands tested, including all 3 TGF-β ligands. Our results showing 3-fold induction of hepcidin expression by TGF-β1 are consistent with prior findings (23); however, we found that BMP-4 and BMP-9 were much more potent inducers of hepcidin expression compared with prior studies (23–24). This may be in part related to differences in ligand concentration (2- to 5-fold higher in our study) and/or differences in cell lines used. Interestingly, although BMP-9 is expressed in the liver (40) and can robustly increase hepcidin mRNA expression in vitro, HJV.Fc was unable to inhibit BMP-9 activation of the hepcidin promoter. HJV.Fc also had reduced ability to inhibit BMP-7 compared with BMP-2, BMP-4, BMP-5, and BMP-6 ligands, consistent with our prior findings (22). The ability of HJV.Fc to inhibit hepcidin expression and increase serum iron in vivo therefore suggests that BMP-2, BMP-4, BMP-5, and/or BMP-6 are good candidates for endogenous regulators of hepcidin expression, while BMP-9, BMP-7, and TGF-β ligands may not be important endogenous regulators of hepcidin. Future research will be needed to definitively determine which TGF-β/BMP superfamily ligands function as endogenous regulators of hepcidin expression and systemic iron balance, as well as determine the source of these ligands in vivo.

Anemia of chronic disease is associated with hypoferremia and reticuloendothelial cell iron sequestration. Inflammatory cytokines are potent inducers of hepcidin expression, and hepcidin excess is believed to have a key role in the pathogenesis of anemia in these patients (1–6). Presumably, inhibitors of hepcidin expression would allow for increased availability of iron from the diet and increased mobilization of iron from the spleen, thereby improving red blood cell production and ameliorating anemia. Here we provide in vivo evidence showing that soluble HJV.Fc inhibits BMP signaling in the liver, inhibits hepcidin expression, increases ferroportin protein expression, decreases splenic iron stores, and increases serum iron levels. This suggests that HJV.Fc is a potential new treatment for anemia associated with hepcidin excess.

We hypothesize that inhibition of hepatic BMP signaling is the predominant mechanism by which HJV.Fc inhibits hepcidin expression and regulates systemic iron balance in vivo. Indeed, inhibition of endogenous BMP signaling in HepG2 cells using BMP siRNAs had a similar effect on decreasing hepcidin expression as treatment with HJV.Fc. Furthermore, loss of TGF-β/BMP superfamily signaling in the liver is sufficient to reduce hepcidin expression and generate iron overload, as demonstrated in mice with a liver-specific conditional knockout of Smad4 (23). However, hemojuvelin has been shown to bind to the receptor neogenin, a member of the deleted in colon cancer (DCC) receptor group, which has been reported to have a role in diverse functions including cell survival, axonal guidance, and cellular iron uptake (41). Whether this or other mechanisms contribute to HJV.Fc inhibition of hepcidin and alterations of systemic iron balance remains to be determined. Treatment with HJV.Fc in our study did not appear to have any other adverse effects on mice. Indeed, regulation of hepcidin expression and iron metabolism appears to be the principal role for TGF-β/BMP superfamily signaling in the adult liver in vivo, since iron overload was the predominant phenotype of liver-specific conditional Smad4 knockout mouse (23).

Recent studies suggest that inflammatory mediators such as IL-6 regulate hepcidin expression through STAT3 (42–44). Although the relationship between the IL-6/STAT3 and BMP/SMAD signaling pathways in the regulation of hepcidin expression is still poorly understood, mice with a liver-specific conditional knockout of Smad4 demonstrate attenuated hepcidin induction in response to IL-6 (23). This suggests that BMP/TGF-β superfamily signaling is also necessary for hepatic excess in inflammatory states and that inhibition of BMP signaling with HJV.Fc might attenuate hepcidin excess induced by inflammatory states. Here we show that HJV.Fc inhibits hepcidin induction in response to...
the inflammatory cytokine IL-6, consistent with prior reports for recombinant soluble hemojuvelin (33). Taken together, ouresults suggest that HJV.Fc or other inhibitors of BMP signaling
may prove to be viable treatments for anemia of chronic disease
caused by inflammation.

Methods

cDNA subcloning. To generate cDNA encoding HJV.Fc, an upstream frag-
ment of human hemojuvelin (containing a preprotrypsin leader sequence
and FLAG tag) was digested SpeI/BstEII from the plasmid FLAG-HJV (22).
A downstream fragment of human hemojuvelin that does not include the
glycophosphatidylinositol domain was amplified by PCR from the plasmid
FLAG-HJV using the primers 5'-AGAAGGTGTATCAGGCTGAGGTGG-3' and
5'-CAGCTCAGTGAAGGGAAGAATGCAGCTTTCTC-3', followed by BstEII/XhoI digestion. Both fragments were ligated into the SpeI/XhoI sites of Signal pIg plus (R&D Systems) in-frame with the Fc portion of
human IgG. Sequences were verified by bidirectional sequencing at the
Animal Care and Use Committee at the Massachusetts General Hospi-
tal.

Purification of HJV.Fc. HEK293 cells (catalog no. CRL-1573; ATCC) cul-
tivated in RPMI medium 1640 (Invitrogen) supplemented with 1-glutamine (Invitrogen) and 10% FBS (Atlanta Biologicals) were stably transfected
with CDNA encoding HJV.Fc using Lipofectamine 2000 (Invitrogen)
according to the manufacturer's instructions. Stably transfected cells
were collected and cultured in 1 mg/ml geneticin (Meditech Inc.). HJV.Fc
was purified from the conditioned media of stably transfected cells by Bio
Express Cell Culture Services.

Animals. All animal protocols were approved by the Institutional
Animal Care and Use Committee at the Massachusetts General Hospital
or Children's Hospital Boston. Six- to 8-week-old 129S6/SvEvTac mice
(Taconic) were housed in the Massachusetts General Hospital or
Children's Hospital Boston rodent facilities and fed on either the Prolab
Animal Diet or Children's Hospital Boston. Six- to 8-week-old 129S6/SvEvTac
mice (Taconic) were housed in the Massachusetts General Hospital
or Children's Hospital Boston. Six- to 8-week-old 129S6/SvEvTac
mice (Taconic) were housed in the Massachusetts General Hospital
or Children's Hospital Boston.

Somatic mutation of hepcidin to generate knock-in mice involved
knock-in of a 3 x 105bp fragment containing the human hepcidin promoter.

Transplantation. All animal protocols were approved by the Institutional
Animal Care and Use Committee at the Massachusetts General Hospital
or Children's Hospital Boston. Six- to 8-week-old 129S6/SvEvTac mice
(Taconic) were housed in the Massachusetts General Hospital or
Children's Hospital Boston. Six- to 8-week-old 129S6/SvEvTac
mice (Taconic) were housed in the Massachusetts General Hospital
or Children's Hospital Boston.

Somatic mutation of hepcidin to generate knock-in mice involved
knock-in of a 3 x 105bp fragment containing the human hepcidin promoter.

Assay System (Promega) as previously described (22) with the following
modifications: for BMP/TGF-β stimulation assays, cells transfected with
the hepcidin promoter luciferase reporter and control Renilla luciferase
vector (pRL-TK) were serum starved in α-MEM with 1-glutamine (Invitrogen)
supplemented with 1% FBS for 6 hours, followed by stimulation
with 50 ng/ml BMP-2, BMP-4, BMP-6, or BMP-7 ligands, 50 ng/ml TGF-β
ligands (R&D Systems) for 16 hours. Relative concentrations of BMP/
TGF-β superfamily ligands were similar to those previously used by oth-
ers to compare superfamily ligand responses (23, 30–31). For HJV.Fc
inhibition assays, cells transfected with the hepcidin promoter luciferase
reporter and pRL-TK were serum starved as above and incubated with
25 ng/ml BMP-2, BMP-4, BMP-6, or BMP-7 ligands, 50 ng/ml BMP-5, or
5 ng/ml BMP-9 either alone or with 0.5–25 μg/ml of HJV.Fc for 16 hours. Relative concentrations of BMP ligands were chosen to elicit
similar degrees of hepcidin promoter relative luciferase activity. Experi-
ments using equal concentrations of ligands were also carried out and
had similar results (data not shown).

RT-PCR. Total RNA was isolated from HepG2 or Hep3B cells and was
analyzed for BMP2, BMP4, BMP5, BMP6, and BMP9 expression as previ-
ously described (29) using the primers forward, reverse, BMP-6 forward and
reverse, and BMP-9 forward and reverse (see Supplemental Table 1 for primer sequences; supplemental data available online with this article; doi:10.1172/31342DS1).

Quantitative real-time RT-PCR. Hep3B or HepG2 cells were serum
starved for 6 hours in α-MEM supplemented with 1% FBS and treated
for 16 hours with varying amounts of BMP/TGF-β superfamily ligands
or 100 ng/ml IL-6 in the absence or presence of 25 μg/ml purified HJV.
Fc. For BMP siRNA experiments, HepG2 cells were plated in 24-well
plates and transfected with 200 ng pcDNA3 (Invitrogen) and 40 nM
BMP-2, BMP-4, BMP-6, BMP-7, or control scramble siRNA (Ambion;
see Supplemental Table 2 for siRNA sequences) in α-MEM using Lipo-
fectamine 2000 (Invitrogen) according to the manufacturer’s instruc-
tions. Cells were serum starved overnight in α-MEM supplemented with
0.1% BSA. Total RNA was isolated from treated cells, and real-time quan-
tification of hepcidin relative to β-actin mRNA transcripts was performed
using 2-step quantitative real-time RT-PCR as previously described (22).
For BMP siRNA experiments, real-time quantitation of BMP2, BMP4, and
BMP6 relative to β-actin mRNA transcripts was also performed as
described above using the primers qBMP-2 forward, qBMP-2 reverse,
qBMP-4 forward, qBMP-4 reverse, qBMP-6 forward, and qBMP-6 reverse
(see Supplemental Table 3 for primer sequences). For mouse livers, total
RNA was isolated using the Illustra RNAse Mini Kit (GE Healthcare)
according to the manufacturer’s instructions. Real-time quantification of hepcidin (Hamp1) relative to Gapdh mRNA transcripts was performed
as described above using primers Hamp1 forward (6) Hamp1 reverse
(6), Gapdh forward, and Gapdh reverse (see Supplemental Table 3 for primer sequences).

Western blot. Western blot of purified HJV.Fc using anti-hemojuvelin
antibody (22) and anti-Flag antibody (Jackson ImmunoResearch Labo-
ratories) was performed as previously described (22). Western blot of
liver lysates for phosphorylated Smad1, Smad5, and Smad8 expression
relative to total Smad1 and β-actin expression was performed as previ-
ously described (22). Chemiluminescence was quantitated using IPLab
Spectrum software version 3.9.5 r2 (Scanalytics). For ferroportin assays,
spleen membrane preparations were prepared as previously described
(51). Protein concentrations were determined by BCA assay (Pierce). After
solubilization in 1x Laemmli buffer for 30 minutes at room temperature,
35 μg of protein per sample were separated by SDS-PAGE using pre-cast
NuPAGE Novex 4–12% Bis-Tris gels (Invitrogen) and transferred onto
PDVF membranes. Western blot was performed using anti-ferroportin

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antibody (kindly donated by Francois Canonne-Hergaux) as previously described (52). Blots were stripped and reprobed for β-actin expression as a loading control as previously described (22).

**Statistical**. A 2-tailed Student’s t test with P < 0.05 was used to determine statistical significance.

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