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# Exogenous BMP7 corrects the plasma iron overload and bone loss in *Bmp6*<sup>-/-</sup> mice

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## **Abstract**

*Purpose* Iron overload accelerates bone loss in mice lacking bone morphogenetic protein 6 (*Bmp6*) gene which is the key endogenous regulator of hepcidin, iron homeostasis gene. Here we investigated involvement of other BMPs in the prevention of hemochromatosis and subsequent osteopenia in *Bmp6*<sup>-/-</sup> mice.

*Methods* Iron-treated WT and *Bmp6*<sup>-/-</sup> mice were analyzed for hepcidin mRNA, tissue and blood BMP levels by qRT-PCR, immunohistochemistry, Western blot, ELISA and Proximity Extension Assay. BMPs labeled with technetium 99m were used in pharmacokinetic studies.

*Results* In WT mice, 4 hours following iron challenge liver *Bmp6* and hepcidin expression were increased, while expression of other *Bmps* was not affected. In parallel, we provided the first evidence that BMP6 circulates in WT mice and that iron increased the BMP6 serum level and the specific liver uptake of <sup>99m</sup>Tc-BMP6. In *Bmp6*<sup>-/-</sup> mice, iron challenge lead to blunted activation of liver Smad signaling and hepcidin expression with a delay of 24 hours, associated with increased *Bmp5* and *Bmp7* expression, and an increased expression of *Bmp2*, 4, 5 and 9 in the duodenum. Liver *Bmp7* expression and increased circulating BMP9 eventually contributed to the late hepcidin response. This was further supported by exogenous BMP7 therapy resulting in an effective hepcidin expression followed by a rapid normalization of plasma iron values and restored osteopenia in *Bmp6*<sup>-/-</sup> mice.

*Conclusion* In *Bmp6*<sup>-/-</sup> mice iron activated endogenous compensatory mechanisms of other BMPs that were not sufficient for preventing hemochromatosis and bone loss. Administration of exogenous BMP7 was effective in correcting the plasma iron level and bone loss indicating that BMP6 is an essential but not exclusive *in vivo* regulator of iron homeostasis.

## Introduction

Osteoporosis is a frequent problem in disorders characterized by iron overload, but mechanisms leading to bone loss are not well understood. Recent studies have clearly shown that iron overload accelerates bone loss with decreased bone mineral density (BMD) in patients with hemochromatosis, particularly in males [1,2]. In addition, in iron treated mice an increase in bone resorption and osteoclast number has been found, without a change in the osteoblast number and bone formation parameters [3]. Furthermore, iron overload is associated with osteopenia and osteoporosis as evidenced in *Bmp6*<sup>-/-</sup> mice [4] and in humans. Whether this is caused by a direct effect of iron on bone or mutations in hemochromatosis related genes, including *Bmp6*, remains to be determined.

Systemic iron balance is regulated by the rate of iron entry from the diet and iron release from hepatocyte stores and from macrophages that recycle iron from aged or damaged erythrocytes. Our understanding of the iron metabolism has advanced in the past decade, mainly as a result of the discovery of hepcidin (*Hamp*), a key regulator of systemic iron homeostasis [5]. Secreted by the liver, hepcidin induces the internalization and lysosomal degradation of iron exporter ferroportin present on the surface of enterocytes, macrophages and hepatocytes, and thereby inhibits intestinal iron absorption and macrophage iron release [6]. Hepcidin expression is induced by dietary [5] or parenteral iron loading [7], suggesting its compensatory role in iron homeostasis. Conversely, hepcidin expression is suppressed in conditions of iron deficiency and tissue hypoxia thus increasing the iron availability for erythrocyte production [8].

Recent studies have demonstrated a critical role for bone morphogenetic protein (BMP) signaling in the regulation of hepcidin expression, which involves interaction with the BMP co-receptor hemojuvelin (HJV) [9,10], and translocation of Smad complexes into the nucleus [11,12].

Support for the involvement of the BMP signaling pathway in the regulation of hepcidin also comes from the observation that the liver-specific *Smad4* knockout mouse manifests nearly complete deficiency of hepcidin and a systemic iron overload [13]. Additionally, it has been demonstrated that a loss of *Bmp6* function in mice leads to a reduced hepatic hepcidin expression and iron overload, suggesting that BMP6 is the key endogenous regulator of hepcidin and iron metabolism [14,15]. While BMP6 administration increases hepcidin expression and reduces serum iron levels *in vivo* [16], BMP inhibitors reduce hepatic hepcidin expression, mobilize reticuloendothelial iron cell stores, and increase the serum iron [10,14,17].

Although BMP6 is the key endogenous regulator of hepcidin expression and iron metabolism, several studies have shown that other BMPs also stimulate hepcidin expression *in vitro* [9,10,13,18]. Additionally, recent comparative studies of four hereditary hemochromatosis mouse models, including *Bmp6*<sup>-/-</sup> mice, observed the presence of distinct pathways of hepcidin regulation which raises the possibility of other BMP involvement in iron homeostasis [19]. It is, therefore, unknown whether other BMPs can regulate hepcidin expression *in vivo*, and why they do not fully correct for the iron overload in *Bmp6*<sup>-/-</sup> mice, since the BMP redundancy has been well documented [20,21].

Systemic challenge with iron rapidly induces Smad1/5/8 phosphorylation and the expression of *Bmp6* and *Hamp* [17,22], but the mechanisms by which BMP6 “senses” the iron status is not understood, and whether liver is the exclusive origin of endogenous BMP6 has been debated. Recently, it has been demonstrated that following an iron administration in mice with a 129Sv/Ev background, *Bmp6* expression was upregulated in the small intestine [23]. In several subsequent studies, it was suggested that the liver and not duodenum was the main

source of iron-induced *Bmp6* expression either by an iron-enriched diet or by inactivation of the *Hfe* or *Hfe2* gene [24,25,26]. Furthermore, it is not known whether BMP6 is the sole mediator of the iron-hepcidin axis.

Here, we found that iron overload induced hepatic *Bmp6* mRNA, which was accompanied by an increase of circulating BMP6. In the absence of BMP6, iron stimulated hepatic *Bmp5* and *7* mRNA and other *Bmps* in the duodenum which resulted in a modest and delayed hepcidin expression. Administration of exogenous BMP7 normalized the hepcidin expression and corrected for bone loss, suggesting that upregulated endogenous BMP7 in *Bmp6*<sup>-/-</sup> mice was not sufficient for an adequate iron regulation and bone metabolism.

## Materials and methods

### Animals

Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All experiments were approved by the Institutional Animal Care Review and Ethics Committee, University of Zagreb, School of Medicine. Animals were maintained on standard GLP diet (4RF21, Mucedola, Italy; 180 mg/kg iron). For iron treatment experiments three months old *Bmp6*<sup>-/-</sup> mice with a mixed 129Sv/C57 background (kindly provided by Elizabeth Robertson) [27] and WT mice matched for the background strain (n = 5/group; genders were matched between groups) received an i.v. dose of iron-dextran (200 mg iron/kg body weight; ferric hydroxide dextran complex, Sigma-Aldrich). Animals were sacrificed and tissues harvested at 4, 8 and 24 hours after injection. For measuring BMPs in the serum, blood was collected at 12, 24 and 48 hours after iron application. For single BMP injection experiments, 3 months old WT and *Bmp6*<sup>-/-</sup> mice received an i.v. injection of BMP7 at the dose of 500 µg/kg (n = 5/group). Serum and livers were harvested for analysis 6 hours after injection. In additional experiment, 10 weeks old *Bmp6*<sup>-/-</sup> mice were ovariectomized (n = 14) and 15 days later divided into following treatment groups: 1) *Bmp6*<sup>-/-</sup> + vehicle (n = 7); 2) *Bmp6*<sup>-/-</sup> + BMP7 (10 µg/kg, 3x/week; n = 7). Sham *Bmp6*<sup>-/-</sup> operated mice served as a positive control (n = 7). Vehicle injection was prepared as acetate buffer (pH = 4.5) with 5% mannitol. The same buffer has been used for dissolving lyophilized BMP7 (Creative Biomolecules). After 4 months, these mice were sacrificed. Bone mineral density (BMD) was measured by Dual X-Ray Absortometry (DXA) (Hologic 600), using company's small animal software.

### Serum analyses

Serum was analyzed for iron concentration and unsaturated iron-binding capacity (UIBC) by standard spectrophotometric method using the Olympus AU2700 (Beckman-Coulter-Olympus).

### Production of the BMP6 monoclonal antibody

BMP6 monoclonal antibody 9F2 was produced using a standard protocol. In ELISA, less than 10% cross-reactivity with recombinant mature BMP7 was observed.

### Immunohistochemical staining

Tissues from *Bmp6*<sup>-/-</sup> and WT mice were fixed in 10% formalin and were paraffin embedded. Sections were cut at 5 µm, deparaffinized in xylene and hydrated in distilled water. Immunocytochemistry was performed using the immunoperoxidase detection system (Zymed, San Francisco, CA). Tissues were incubated with monoclonal BMP6 antibody 9F2 and hepcidin (56-Z) Antibody (1:50; Santa Cruz Biotechnology). BMP7 was detected immunohistochemically as described previously [28].

### Gene expression analyses

Total RNA was isolated from tissues and primary hepatocytes using TRIzol (Invitrogen). The cDNA was generated by reverse-transcription of 1 µg adjusted RNA using Super Script III First-Strand Synthesis System (Invitrogen). Gene expression of interest was measured by using a LightCycler FastStart DNA Master SYBR

Green kit in a LightCycler instrument (Roche Diagnostics), as described [29]. Results are represented as a fold change of the comparative expression level. The list of primers used is shown in Table 1.

#### SDS gel electrophoresis and Western blot analyses

Tissues were disrupted with a manual homogenizer in RIPA buffer. Serum (5  $\mu$ l), duodenum (50  $\mu$ g) and liver lysates (150  $\mu$ g) were subjected to SDS-PAGE and transferred to the nitrocellulose membrane. The blots were saturated with 2% BSA in Tris buffered saline containing 0.05% Tween (TBS-T) and probed overnight at 4°C with primary antibodies against BMP6 (1:400; School of Medicine, Rijeka), BMP9 (1:1000; R&D Systems), phosphorylated Smad1/5/8 (1:1000; Cell Signaling), Smad1/5/8 (1:200; Santa Cruz Biotechnology) or  $\beta$ -actin (1:1000; Millipore). Immunolabeling was detected using alkaline phosphatase-conjugated secondary antibodies and BCIP/NBT substrate (Sigma) and the proteins were quantified using ImageJ software (NIH). Results are presented as the mean of duplicates.

#### Mass spectrometry

After Coomassie staining, each of the gel lanes was sliced in 15 pieces and the corresponding pieces were combined. The pieces were then subjected to in-gel reduction, alkylation and trypsin digestion. Tryptic peptides were analyzed by a liquid chromatography-mass spectrometry (LC-MS). Easy-nLC nanoflow HPLC system (Proxeon Biosystems) was coupled to a LTQ-Orbitrap mass spectrometer (Thermo Scientific) using a nano-electrospray LC-MS interface (Proxeon Biosystems). Peptides were loaded on a home-made 75  $\mu$ m C18 HPLC column in solvent "A" (0.5% acetic acid in Milli-Q water) and eluted with a 70-minute segmented linear gradient of 10-60% solvent "B" (80% acetonitrile, 0.5% acetic acid in Milli-Q water) at a flow rate of 250 nL/min as described [30].

#### Serum measurement of BMP6, BMP7 and BMP9

Serum samples were tested for BMP7 and BMP9 by using BMP7 ELISA kit [31] (R&D Systems) and BMP9 ELISA kit [32] (AdipoBiosciences) according to the manufacturer's instructions. For sensitive BMP6 detection and quantification we employed the Proximity Extension Assay technology using the Proseek Assay Development kit (Olink Bioscience). Mouse BMP6 antibodies (AF6325; R&D Systems) which showed approximately 5% cross-reactivity with recombinant mouse BMP5 and BMP7 were conjugated with a pair of DNA oligonucleotides and upon binding to the target protein in 1  $\mu$ l of serum sample, a DNA duplex is formed and extended in real-time PCR according to manual's instructions.

#### Pharmacokinetics and biodistribution of $^{99m}\text{Tc}$ -BMP6 and $^{99m}\text{Tc}$ -BMP7

For technetium labeling we used the Isolink Kit Mallinckrodt (Covidien Pharmaceuticals; kind gift of Dr. Hector H. Knight). A  $^{99m}\text{Tc}$ -pertechnetate solution eluted from the Technetium-99m generator was added to the IsoLink carbonyl labeling agent (DRN4335; Mallinckrodt), and the mixture was incubated in a boiling water bath for 20 min. The obtained solution of  $[\text{}^{99m}\text{Tc}(\text{H}_2\text{O})_3(\text{CO})_3]^+$  (1 mL) was neutralized with 0,1 N HCl solution and mixed with a solution of BMP6 in PBS and incubated at 60°C for 30 minutes. Four months old Sprague-Dawley rats (n=4/group; females) received a single i.v. injection of 10  $\mu$ g/kg  $^{99m}\text{Tc}$ -BMP6 with the activity of 45  $\mu$ Ci in a volume of 300  $\mu$ l. Another group of rats (n=4/group) was treated with 200 mg/kg iron at 24 hours prior to

receiving  $^{99m}\text{Tc}$ -BMP6. Animals were sacrificed 3 hours following injection of  $^{99m}\text{Tc}$ -BMP6. For comparison, BMP7 was also labeled with  $^{99m}\text{Tc}$ -BMP6 and used similarly. Blood and organs were isolated and the radioactivity was measured in a gamma counter, and expressed as a percentage (%) of the applied dose in counts per minute (cpm). All values were corrected for the half-life of  $^{99m}\text{Tc}$ .

#### Data analyses

Results are reported as the mean  $\pm$  SEM. Changes in gene expression and serum parameters were evaluated using the 2-tailed Student *t* test. The results were considered significant when *P* was  $< 0.05$ .

## Results

#### $^{99m}\text{Tc}$ -BMP6 accumulated in liver following iron administration

Four hours after iron administration *Bmp6* expression in the liver was increased by 4.7-fold (Fig. 1a), accompanied by an increase in hepatic hepcidin mRNA by 4.9-fold (Fig. 1b). In the duodenum, in parallel, *Bmp6* mRNA expression was decreased following iron injection, while in the jejunum it was unchanged (Fig. 1a). This was confirmed by immunohistochemistry using an antibody raised against the mature domain of BMP6 (Fig. 1c, i-ii) and against hepcidin (Fig. 1c, iii-iv). BMP6 was previously shown to be restricted to non-parenchymal liver cells (stellate and Kupffer cells) [33]. However, we found that the BMP6 localization in iron loaded livers was dominantly in hepatocytes of periportal zone of liver acini which was not observed in *Bmp6*<sup>-/-</sup> mice (Fig. 1d). To further explore whether iron affects the biodistribution of BMP6 and to confirm hepatic source of BMP6, we developed a procedure of labeling BMP6 with  $^{99m}\text{Tc}$  using the Isolink labeling kit. BMP6 structure and function were well maintained following labeling as evidenced by gel electrophoresis and Western blot of  $^{99m}\text{Tc}$ -BMP6, as well as the activity of a BMP-responsive luciferase reporter (BRE-Luc) in C2C12 cells (data not shown).  $^{99m}\text{Tc}$ -BMP6 concomitantly administered with iron to rats, predominantly accumulated in the liver at 3 hours following an iron injection, while its uptake by the duodenum, jejunum, kidney and blood was similar to control animals (Fig. 1e). To determine whether accumulation in the liver was specific for BMP6 we used  $^{99m}\text{Tc}$  labeled BMP7 and found similar results in iron loaded and control animals (data not shown).

#### BMP6 circulated in serum and correlated with induced iron overload in WT mice

Recently published data suggested that in WT mice, iron significantly increased BMP6 in serum, represented as a 23kDa band by Western blot analysis using the Santa Cruz S-20 anti-BMP6 antibody [23]. However, this antibody was shown to be nonspecific for mouse BMP6 in Western blot experiments as the 23kDa band was also observed in *Bmp6*<sup>-/-</sup> mice [24]. Under similar experimental conditions, using LC-MS, we did not detect BMP6 in the total serum sample of iron loaded mice. Moreover, the 23kDa band detected by a Coomassie staining did not contain BMPs but several unrelated proteins predominantly retinol binding protein 4 (Rbp4) and Apolipoprotein M (apoM) (Fig. 2a).

In order to determine whether BMP6 circulates, we analyzed biological fluids of mouse and human using the human BMP6 DuoSet ELISA (R&D Systems), but failed to detect BMP6 in the serum (data not shown). To enhance the assay sensitivity and simplify the BMP6 measurement procedure, we developed an alternative assay,



the Proximity Extension Assay (PEA) which is reported to measure protein levels from 0.01-10 000 pM (Olink Bioscience). Our results indicated that the lower detection limit or sensitivity of the assay was 10 pg/mL, as this is the concentration of two standard deviations above the background. For BMP6 detection we used mouse BMP6 antibodies (R&D Systems) and determined that BMP6 circulates at  $55.46 \pm 9.8$  pg/mL in only 1  $\mu$ L of mouse serum. Iron challenge, as measured by PEA, increased circulating levels of BMP6 in WT mice at 12 hours to  $109.96 \pm 25.4$  and at 24 hours to  $128.7 \pm 21$  pg/mL (Fig. 2b). The background level in *Bmp6*<sup>-/-</sup> mice was in the range 5 - 10% of control values in WT mice (data not shown).

Iron injection resulted in delayed Smad 1/5/8 signaling and hepcidin response in *Bmp6*<sup>-/-</sup> mice

We next tested the effects of an iron overload on the temporal dynamics of hepcidin expression in *Bmp6*<sup>-/-</sup> mice relative to WT control mice. In *Bmp6*<sup>-/-</sup> mice, liver *Hamp* mRNA induction by iron was delayed and dampened compared to WT mice. There was a trend toward increased *Hamp* mRNA expression by 2-fold at 8 hours and 4.3-fold at 24 hours after an iron injection, as demonstrated by qPCR (Fig. 3a) and immunohistochemical staining (Fig. 3b).

As BMP signaling has been shown to induce hepcidin expression, we tested whether phosphorylation of Smad 1/5/8 was modulated in the liver extract of iron challenged *Bmp6*<sup>-/-</sup> mice. At 24 hours iron increased 1.9-fold the Smad 1/5/8 phosphorylation in liver lysates of *Bmp6*<sup>-/-</sup> mice (Fig. 3c), suggesting that other BMPs have been activated to stimulate liver Smad signaling, however, insufficient for an appropriate *Hamp* response.

Iron administration affected BMPs in liver, duodenum and circulation of *Bmp6*<sup>-/-</sup> mice

Next, we investigated the expression of other BMPs and their potential role in the hepcidin regulation. *Bmp2*, *4*, *5*, *7* and *9* transcripts were not changed in the liver and duodenum of WT mice during 8 hours following iron injection, except for decreased *Bmp2* in the duodenum (Fig. 4a-e).

On the contrary, in *Bmp6*<sup>-/-</sup> mice, iron time-dependently increased *Bmp2*, *4*, *5*, and *9* expression in the duodenum (Fig. 5a-d). In the liver, *Bmp5* mRNA was slightly increased at 24 hours after iron administration in *Bmp6*<sup>-/-</sup> mice (Fig. 5c). As BMP9 has been previously claimed to circulate [26], we measured its serum level, and found in *Bmp6*<sup>-/-</sup> mice an increased serum BMP9 concentration at 24 hours following iron administration (Fig. 5e). Furthermore, immunoblot analyses confirmed that at 12 hours BMP9 protein was increased in the duodenum of *Bmp6*<sup>-/-</sup> mice and decreased at 24 hours (Fig. 5f). This suggested that BMP9 from the gut might have been released into the blood, as an attempt by the duodenum to contribute in the prevention of iron overload caused by the lack of BMP6.

Liver *Bmp7* expression was, however, increased by 3.8-fold at 4 hours (Fig. 6a) and 3.27-fold at 8 hours (not shown), which was also verified by BMP7 immunostaining of liver sections (Fig. 6b). Together, these data suggested that in the liver of *Bmp6*<sup>-/-</sup> mice, *Bmp7* was upregulated at an early time point and *Bmp5* at a later time point after iron injection, while *Bmp2*, *4*, *5* and *9* were time-dependently increased in the duodenum. However, these compensatory mechanisms in *Bmp6*<sup>-/-</sup> mice were not sufficient to invoke an adequate BMP-dependent defense against iron overload to prevent hemochromatosis.

Exogenous BMP7 increased hepcidin expression, reduced iron accumulation and corrected for bone loss in WT and *Bmp6*<sup>-/-</sup> mice

We have previously shown that BMP6 administration increases hepatic *Hamp* mRNA expression and reduces serum iron in a dose-dependent manner [14]. To examine a potential of exogenous BMP7 administration on hepcidin expression and plasma iron distribution, WT and *Bmp6*<sup>-/-</sup> mice were treated i.v. with BMP7. Indeed, BMP7 significantly increased the liver *Hamp* mRNA by 20-fold and reduced the serum iron level in *Bmp6*<sup>-/-</sup> mice at 6 hours (Fig. 7a and b), which was verified by hepcidin immunohistochemistry of liver sections (Fig. 7c). Interestingly, in WT mice, the induction of hepcidin expression and the reduction of serum iron following BMP7 injection were modest as compared to *Bmp6*<sup>-/-</sup> mice. However, as *Bmp6*<sup>-/-</sup> mice contain much less hepcidin, the immunohistochemical analyses of the liver showed only moderate increase in hepcidin staining in *Bmp6*<sup>-/-</sup> mice than in WT mice. The induction of liver Smad1/5/8 phosphorylation by BMP7 in *Bmp6*<sup>-/-</sup> mice was also significant, mirroring the hepcidin response (Fig. 7d). Thus, exogenous BMP7 ameliorated the hepcidin deficiency in *Bmp6*<sup>-/-</sup> mice, suggesting that these animals were primed to respond to compensatory regulation by endogenous BMPs which were however too low to adequately upregulate hepcidin and prevent hemochromatosis. In addition, i.v. therapy with BMP7 (10 µg/kg) for 4 months significantly increased the bone volume in ovariectomized *Bmp6*<sup>-/-</sup> mice (Fig. 8).

## Discussion

Endogenous BMP6 increases hepcidin expression and reduces the serum iron in mice, and has a key role in the iron metabolism [14,15]. Recently, it has been suggested that epithelial cells of the small intestine are the main source of BMP6 upon “sensing” iron *in vivo* and that enterocytes produce and release BMP6 into the circulation to reach the liver and regulate hepcidin expression [23]. In contrast, we and others [24,25] have shown that iron increased the hepatic *Bmp6* expression in WT mice without affecting its expression in the duodenum or jejunum. We were, however, not able to detect BMP6 in serum (5  $\mu$ l) by LC-MS analyses as have been previously suggested [23]. Indeed, we demonstrated that the previously reported ~23kDa band detected by Western blot [23] did not contain BMP6. Furthermore, using the DuoSet ELISA (R&D Systems) we could not detect BMP6 in biological fluids of mouse and human. Therefore, we developed a BMP6 Proximity Extension Assay which enabled us to analyze BMP6 in 1  $\mu$ l serum samples before and following iron exposure. We showed that BMP6 circulates in the serum, and this is the first demonstration of a physiological range of circulating BMP6. Furthermore, we found that iron loading was followed by a BMP6 increase in mouse serum indicating that circulating levels of BMP6 may reflect body iron status. Interestingly, in untreated mice BMP6 concentrations displayed a diurnal variation, with concentrations being lowest in the morning and increasing throughout the day before declining during evening hours. Given the already known hepcidin diurnal rhythm [34], our results suggest that hepcidin variations might reflect the BMP6 circulating pattern. Our further goal is to determine the range of circulating BMP6 in healthy individuals and patients with osteoporosis, and search for any discrepancies. Iron loading was also followed by a pronounced increase of hepatic *Bmp6* mRNA and protein as well as hepcidin mRNA, indicating a major role for hepatic BMP6 in protecting the organism against iron. Consistent with this, the biodistribution of i.v. injected  $^{99m}\text{Tc}$ -BMP6 showed a higher liver uptake of BMP6 in the presence of iron as compared to a lesser uptake in the liver of  $^{99m}\text{Tc}$ -BMP7.

To assess other potential mediators in iron homeostasis, we analyzed the hepcidin response to iron in *Bmp6*<sup>-/-</sup> mice. Twenty four hours following iron injection, hepcidin expression was increased in the liver, but failed to reach the hepcidin level seen in WT mice. Also, iron injection caused hepatic Smad1/5/8 activation, which led to the hypothesis that BMPs, other than BMP6, have been activated to mediate hepcidin expression in response to iron, although at an insufficient level. Similar modulation of hepcidin mRNA has been previously reported in *Bmp6*<sup>-/-</sup> mice at the 21st day of chronic iron loading [19]. These results suggest that in response to iron, increased hepcidin in *Bmp6*<sup>-/-</sup> mice could be regulated via other pathways.

Indeed, in *Bmp6*<sup>-/-</sup> mice, we showed a time-dependent increase of *Bmp2*, *4*, *5* and *9* mRNA in the duodenum following iron injection. In addition, increased duodenal *Bmp9* expression was accompanied by increased serum BMP9, suggesting that BMP9 might have been released from the gut into the circulation. Previous studies have demonstrated that BMP2, 4 and 5 can all bind to hemojuvelin, the BMP co-receptor that plays an essential role in hepcidin regulation and iron homeostasis [9] with high affinity ( $K_D$ 's 4.5 – 17 nM) [35]. Moreover, BMP2, 4, 5 and 9 can all stimulate hepcidin expression *in vitro* and BMP2 can stimulate hepcidin expression *in vivo* [10,18]. Notably, duodenal expression of BMPs was not upregulated by a single injection of iron in WT mice, suggesting that a functional role, if any, for these duodenal BMPs in hepcidin regulation and systemic iron homeostasis may be limited to pathologic conditions or more prolonged iron exposure. Future studies will be needed to further

explore the role of endogenous circulating BMP9 and other duodenal upregulated BMPs in hepcidin regulation and iron homeostasis in *Bmp6*<sup>-/-</sup> mice and other conditions.

In the liver of *Bmp6*<sup>-/-</sup> mice, we found a moderately increased *Bmp5* at 24 hours and more significant increase of *Bmp7* at 4 and 8 hours after an iron injection. Interestingly, iron injection also caused a trend toward increased liver *Bmp5* expression in WT mice, but did not affect liver *Bmp7* expression. Although BMP7 is predominantly produced in the kidney and bone [36], hepatocytes have also been recognized as a source of BMP7 [31,37], albeit with low basal levels. BMP7 has also been demonstrated to bind to hemojuvelin with high affinity ( $K_D$  20nM) [35] and to stimulate hepcidin expression *in vitro* [10]. To determine the BMP7 effect on hepcidin expression *in vivo*, we treated WT and *Bmp6*<sup>-/-</sup> mice with exogenous BMP7, which induced hepcidin expression and Smad 1/5/8 phosphorylation. The more robust response of *Bmp6*<sup>-/-</sup> mice compared to WT animals to exogenous BMP7 suggests an adaptation to BMP6 loss by increasing sensitivity to BMP7, but the exact mechanism should be further explored. In addition, systemically applied BMP7 to ovariectomized *Bmp6*<sup>-/-</sup> mice increased the bone volume and restored the bone microarchitecture and quality of the skeleton.

These results support an alternative mechanism for hepcidin regulation in *Bmp6*<sup>-/-</sup> mice, which involves BMPs other than BMP6. However, although an exogenous dose of BMP7 in *Bmp6*<sup>-/-</sup> mice resulted in an increased bone volume and hepcidin expression which reached levels of those from WT mice, the lack of an early hepcidin response after iron injection in *Bmp6*<sup>-/-</sup> mice was presumably due to an insufficient endogenous amount of BMP required to substitute for BMP6. BMP6 is an essential, but not exclusive endogenous regulator of hepcidin in prevention of hemochromatosis in *Bmp6*<sup>-/-</sup> mice.

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FOOTNOTES:

Conflict of Interest Disclosures: JLB and HYL have ownership interest in a start-up company Ferrumax Pharmaceuticals, which has licensed technology from the Massachusetts General Hospital based on their work.

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The abbreviations used are: BMP, bone morphogenetic protein; Hamp, hepcidin; HJV, hemojuvelin; pSmad 1/5/8, phosphorylated Smad1, Smad5 and Smad8 protein

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## Figure legends

**Table 1.** Sequences of primers used for gene expression analysis

**Fig. 1** Iron increased hepatic *Bmp6* and hepcidin expression. Three months old WT mice received an i.v. injection of iron-dextran at 200 mg/kg body weight (n=5/group). Four and 8 hours after the injection, tissues were analyzed for (a) *Bmp6* relative to *Gapdh* mRNA by qRT-PCR, (b) hepatic *Hamp* relative to *Gapdh* mRNA by qRT-PCR, (c) immunohistochemical staining of liver using BMP6 monoclonal antibody (i-ii) and hepcidin antibody (iv-v) including negative control (iii/vi) at original magnification x20 and (d) immunohistochemical staining of iron loaded liver using BMP6 monoclonal antibody (original magnification x40) in WT and *Bmp6*<sup>-/-</sup> mice. (e) Rats (n=4/group) treated with 200 mg/kg iron received 24 hours later a single injection of <sup>99m</sup>Tc-BMP6 at a dose level of 10 µg/kg, while control animals received only <sup>99m</sup>Tc-BMP6. Animals were sacrificed 3 hours following a <sup>99m</sup>Tc-BMP6 injection. The organ uptake is expressed as a percent of injected dose per gram of tissue wet weight. Results are reported as the mean ± SEM. Exact *P* values are shown.

**Fig. 2** Iron increased BMP6 circulating levels in WT mice. Serum samples of mice loaded with iron were submitted to SDS gel electrophoresis after which each protein band was analyzed by (a) liquid chromatography-mass spectrometry (LC-MS) including the 23 kDa band previously identified as a mature BMP6 [23]. For BMP6 quantification, serum was collected at zero, 12 and 24 hours following iron injection and BMP6 was measured in (b) Proximity Extension Assay (\**P* = 0.01 compared with time zero and 0.038 compared with 24 hours control). Results are reported as the mean ± SEM. Exact *P* values are shown.

**Fig. 3** *Bmp6*<sup>-/-</sup> mice showed delayed hepcidin response and Smad 1/5/8 signaling to iron loading. Three months old *Bmp6*<sup>-/-</sup> mice were treated with iron-dextran and were killed at 0, 4, 8 and 24 hours following injection (n=5/group). Liver tissues were analyzed for hepatic (a) *Hamp* relative to *Gapdh* mRNA by qRT-PCR, (b) immunohistochemical staining with hepcidin antibodies (original magnification x20) and (c) hepatic phosphorylated Smad 1/5/8 relative to Smad 1/5/8 protein level by Western blot at 4, 8 and 24 hours following iron injection. Results are reported as the mean ± SEM. Exact *P* values are shown.

**Fig. 4** Iron decreased *Bmp2* expression in duodenum, while other *Bmps* remained unchanged in WT mice. Three months old WT mice received an i.v. injection of iron-dextran at 200 mg/kg body weight (n=5/group). Four and 8 hours after the injection, tissues were analyzed for (a) *Bmp2*, (b) *Bmp4*, (c) *Bmp5*, (d) *Bmp7* and (e) *Bmp9* relative to *Gapdh* mRNA by qRT-PCR. Results are reported as the mean ± SEM. Exact *P* values are shown.

**Fig. 5** Iron increased expression of *Bmp2*, 4, 5 and 9 in duodenum of *Bmp6*<sup>-/-</sup> mice. Three months old *Bmp6*<sup>-/-</sup> mice were treated i.v. with iron-dextran at 200 mg/kg and were killed at 0, 4 and 24 hours after injection (n=5/group). Tissues were analyzed for (a) *Bmp2*, (b) *Bmp4*, (c) *Bmp5* and (d) *Bmp9* relative to *Gapdh* mRNA by qRT-PCR. (e) BMP9 serum levels were measured with commercial BMP9 ELISA at several time points between 0 and 24 hours after iron injection (\**P* = 0.04 compared with zero point and 0.007 compared with 24

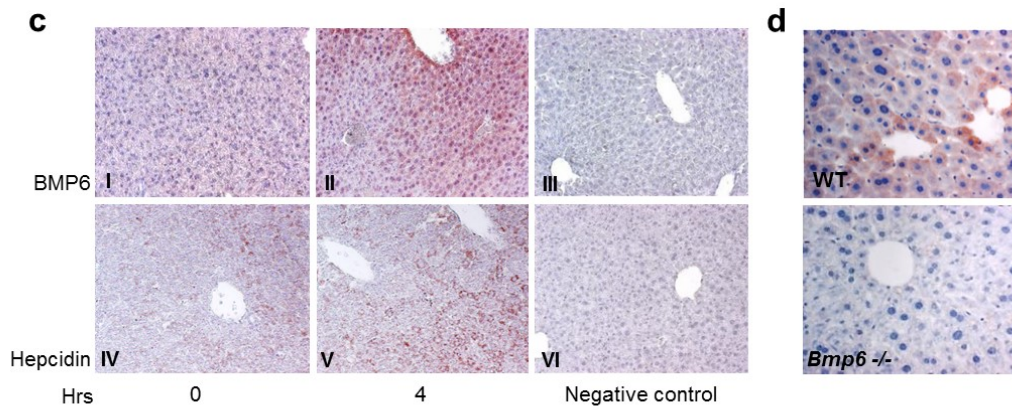
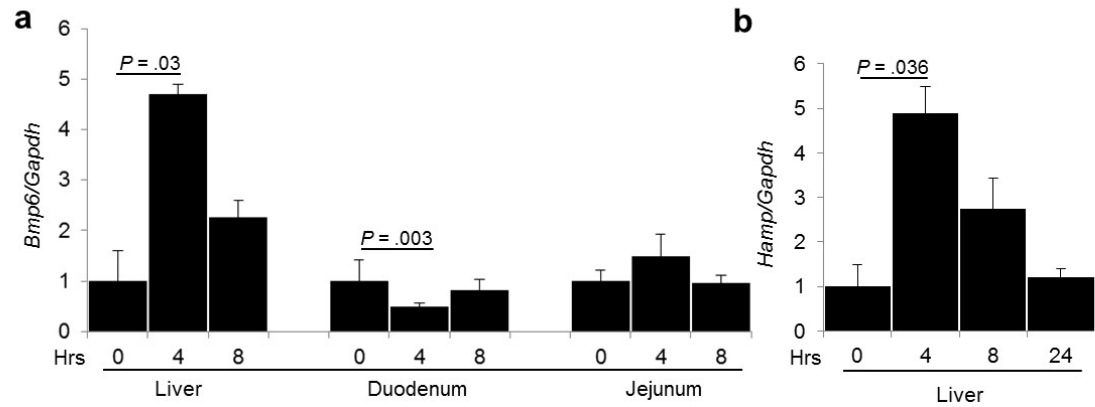
hours control) and (f) BMP9 relative to  $\beta$ -actin expression by Western blot at 12 and 24 hours following iron overload in the duodenum. Results are reported as the mean  $\pm$  SEM. Exact *P* values are shown.

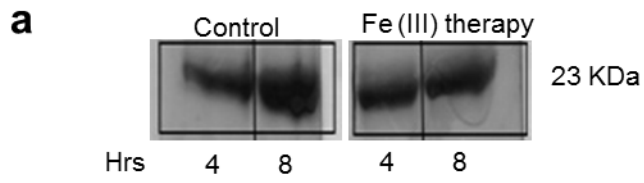
**Fig. 6** Iron induced *Bmp7* expression in the liver of *Bmp6*<sup>-/-</sup> mice. Three months old *Bmp6*<sup>-/-</sup> mice were treated i.v. with iron-dextran at 200 mg/kg and were killed at 0, 4 and 24 hours after injection (n=5/group). Tissues were analyzed for (a) *Bmp7* relative to *Gapdh* mRNA by qRT-PCR, (b) hepatic BMP7 staining (original magnification x20). Results are reported as the mean  $\pm$  SEM. Exact *P* values are shown.

**Fig. 7** Exogenous BMP7 increases hepcidin expression and reduces serum iron in WT and *Bmp6*<sup>-/-</sup> mice. Three months old WT and *Bmp6*<sup>-/-</sup> mice received an i.v. injection of BMP7 at 500  $\mu$ g/kg and 6 hours after blood and liver were harvested (n=5/group). Tissues were analyzed for (a) hepatic *Hamp* relative to *Gapdh* mRNA by qRT-PCR, (b) serum iron, (c) hepatic immunohistochemical staining with hepcidin antibodies (original magnification x20) and (d) hepatic phosphorylated Smad 1/5/8 relative to Smad 1/5/8 protein level by Western blot at 6 hours following BMP7 injection in WT and *Bmp6*<sup>-/-</sup> mice. Results are reported as the mean  $\pm$  SEM. Exact *P* values are shown.

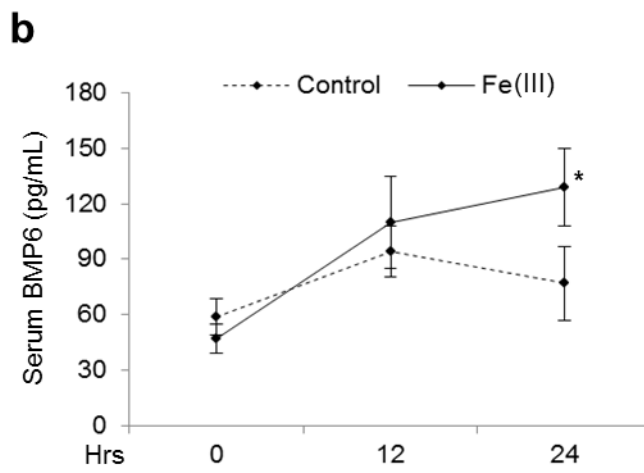
**Fig. 8** Treatment of *Bmp6*<sup>-/-</sup> mice with BMP7 (10  $\mu$ g/kg) restored the bone loss following ovariectomy. *Bmp6*<sup>-/-</sup> mice were ovariectomized (n=14) at 10 weeks of age and were treated 15 days later with BMP7 (10  $\mu$ g/kg, 3x/week) for 4 months (n=7). Femur BMD values were compared to ovariectomized (n=7) and sham *Bmp6*<sup>-/-</sup> mice (n = 7). Results are reported as the mean  $\pm$  SEM. Exact *P* values are shown.

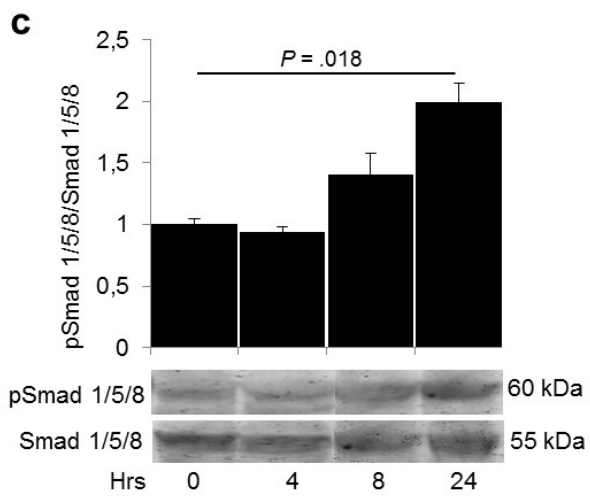
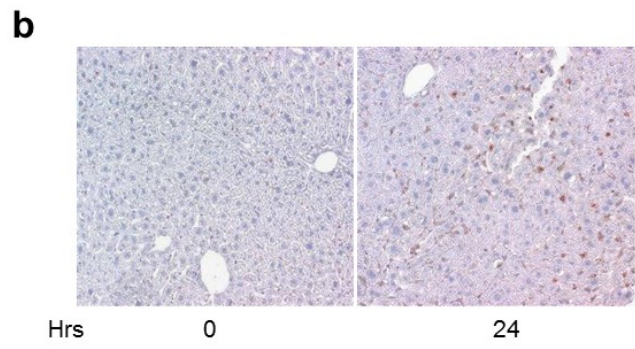
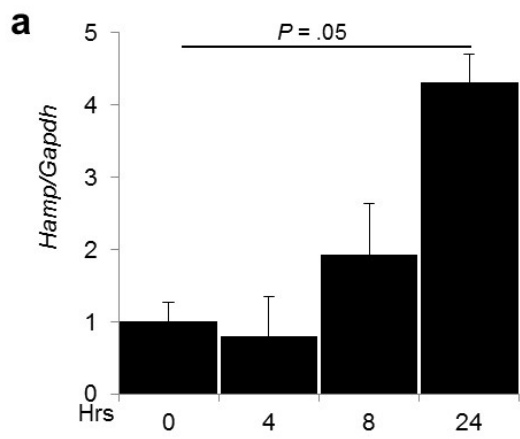
Gene	5'→3' Forward 5'→3' Reverse
Mouse <i>Bmp2</i>	F: GGGACCCGCTGTCTTCTAGT R: TCAACTCAAATTCGCTGAGGAC
Mouse <i>Bmp4</i>	F: GACTTCGAGGCGACACTTCTA R: GCCGGTAAAGATCCCTCATGTAA
Mouse <i>Bmp5</i>	F: TTAGTTAGGGGTATTGTGGGCT R: CCGTCTCTCATGGTTCCGTAG
Mouse <i>Bmp6</i>	F: TCCCCACATCAACGACACCA R: TCCCCACCACACAGTCCTTG
Mouse <i>Bmp7</i>	F: ACGGACAGGGCTTCTCCTAC R: ATGGTGGTATCGAGGGTGGAA
Mouse <i>Bmp9</i>	F: CAGAAGCCGCTGCAGAACTG R: AAGCTCCGCACGATGTTGG
Mouse <i>Hamp</i>	F: TTGCGATACCAATGCAGAAG R: GGGGAGGGCAGGAATAAATA
Mouse <i>Gapdh</i>	F: AGGTCGGTGTGAACGGATTTG R: TGTAGACCATGTAGTTGAGGTCA

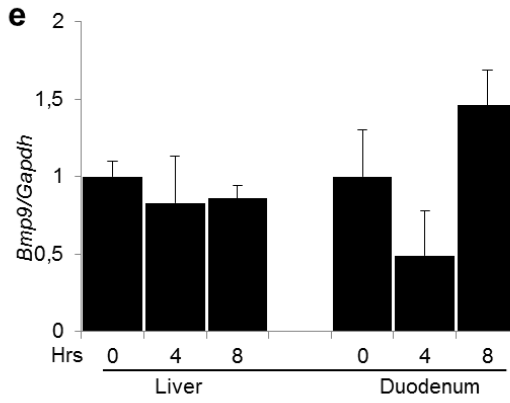
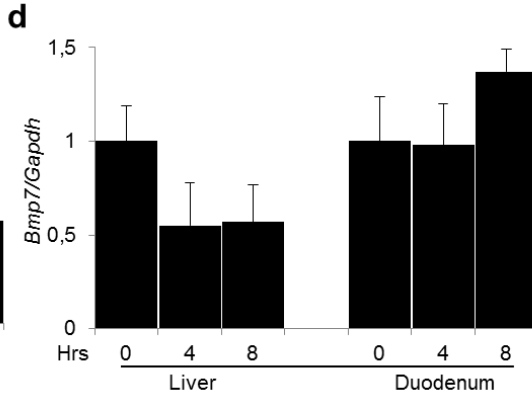
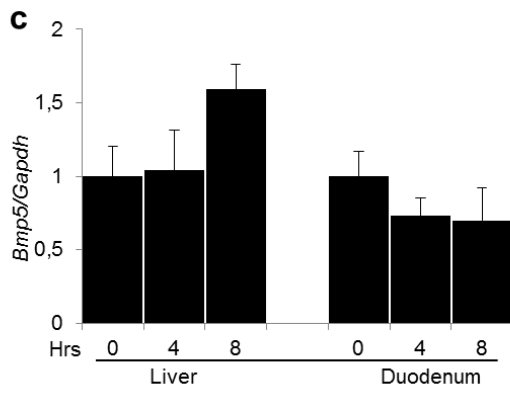
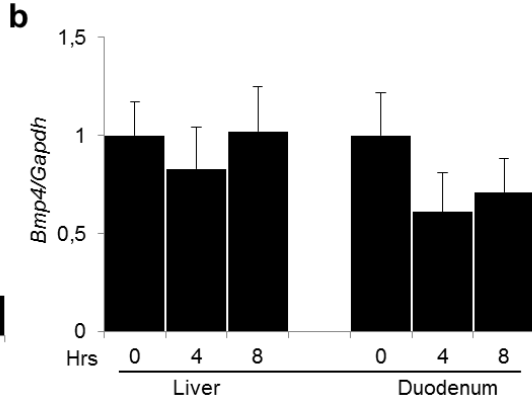
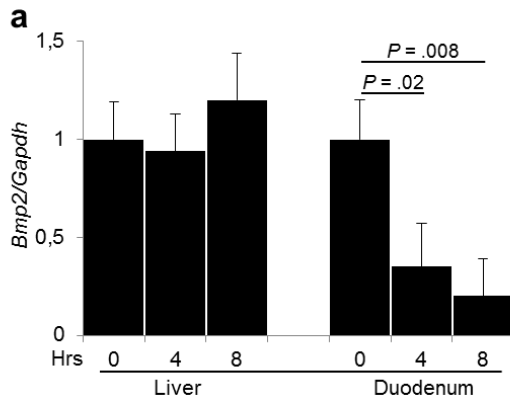


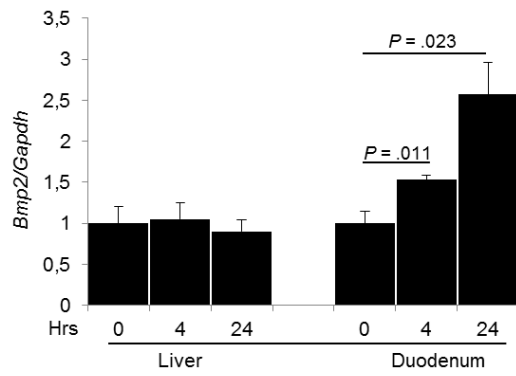
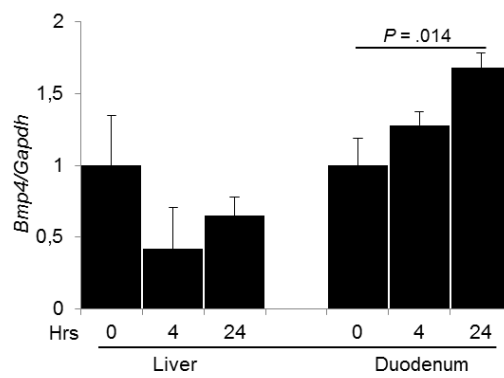
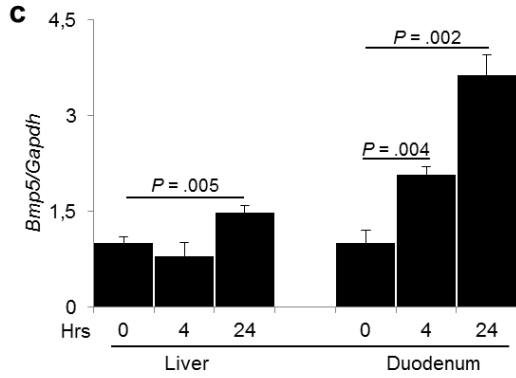
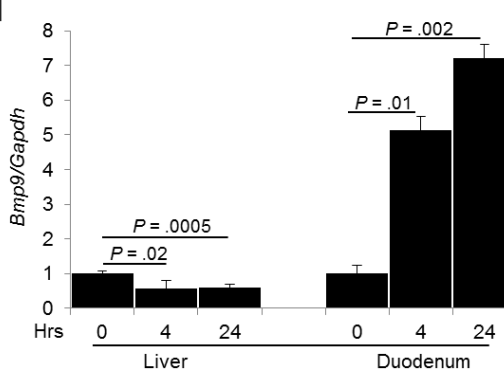
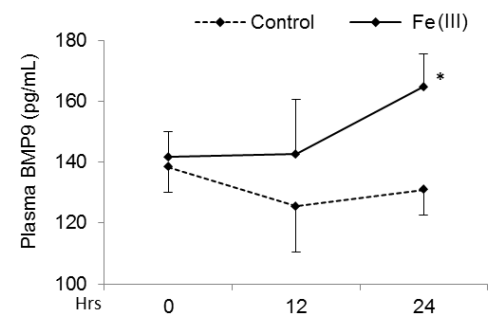


ID	UniProt Accession	Protein Description	Unique Peptides		Posterior error probability (PEP)
			Control	Iron Therapy	
123	Q00724	Retinol-binding protein 4	12	9	5,82E-096
159	Q9Z1R3	Apolipoprotein M	6	5	1,51E-035







**a****b****c****d****e****f**