**Bmp6 Expression in Murine Liver Non Parenchymal Cells: A Mechanism to Control their High Iron Exporter Activity and Protect Hepatocytes from Iron Overload?**

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**Abstract**

*Bmp6* is the main activator of hepcidin, the liver hormone that negatively regulates plasma iron influx by degrading the sole iron exporter ferroportin in enterocytes and macrophages. *Bmp6* expression is modulated by iron but the molecular mechanisms are unknown. Although hepcidin is expressed almost exclusively by hepatocytes (HCs), *Bmp6* is produced also by non-parenchymal cells (NPCs), mainly sinusoidal endothelial cells (LSECs). To investigate the regulation of *Bmp6* in HCs and NPCs, liver cells were isolated from adult wild-type mice whose diet was modified in iron content in acute or chronic manner and in disease models of iron deficiency (*Tmprss6* KO mouse) and overload (*Hjv* KO mouse). With manipulation of dietary iron in wild-type mice, *Bmp6* and *Tfr1* expression in both HCs and NPCs was inversely related, as expected. When hepcidin expression is abnormal in murine models of iron overload (*Hjv* KO mice) and deficiency (*Tmprss6* KO mice), *Bmp6* expression in NPCs was not related to *Tfr1*. Despite the low *Bmp6* in NPCs from *Tmprss6* KO mice, *Tfr1* mRNA was also low. Conversely, despite body iron overload and high expression of *Bmp6* in NPCs from *Hjv* KO mice, *Tfr1* mRNA and protein were increased. However, in the same cells ferritin L was only slightly increased, but the iron content was not, suggesting that *Bmp6* in these cells reflects the high intracellular iron import and export. We propose that NPCs, sensing the iron flux, not only increase hepcidin through *Bmp6* with a paracrine mechanism to control systemic iron homeostasis but, controlling hepcidin, they regulate their own ferroportin, inducing iron retention or release and further modulating *Bmp6* production in an autocrine manner. This mechanism, that contributes to protect HC from iron loading or deficiency, is lost in disease models of hepcidin production.
Introduction

Hepcidin, the master regulator of iron metabolism, is a liver peptide hormone that negatively regulates dietary iron absorption and iron release from macrophages by binding and degrading the sole cellular iron exporter ferroportin [1]. Although hepcidin activation is mediated by both circulating and liver iron content, how these two signals govern hepcidin changes is not fully clarified. The characterization of hemojuvelin (HJV), the protein mutated in type 2A hemochromatosis [2], as Bone Morphogenetic Protein (BMP)-coreceptor, functionally linked the BMP-Sons of Mothers Against Decapentaplegic (SMAD) pathway to hepcidin and iron regulation [3]. HJV selectively uses the BMP type II receptor ActRIIA [4], highly expressed in the liver, and the type I receptors ALK2 and ALK3 [5]. In the presence of the ligand, constitutively active type II receptor phosphorylates type I receptor, which phosphorylates SMAD1/5/8 proteins (R-SMADs) that in turn interact with SMAD4. The resulting multiprotein complex translocates to the nucleus to activate target genes [6]. Liver conditional inactivation of Smad4 [7] or Alk3 [5] causes severe iron overload due to downregulation of hepcidin, similar to the phenotype of Hjv−/− [8,9] and Hamp−/− [10] mice, whereas liver specific deletion of Alk2 blunts the response of hepcidin to increased iron levels [5].

BMPs are members of the transforming growth factor beta (TGF-beta) superfamily [11]. In vitro several BMPs as BMP2, BMP4 and BMP6 activate hepcidin in the presence of HJV [12]. In vivo, Bmp6 is the sole BMP that regulates hepcidin expression in mice. Bmp6 inactivation causes severe iron overload due to strong hepcidin downregulation and ferroportin stabilization, a phenotype comparable to that of Hjv−/− mice [9], suggesting that Bmp2 and Bmp4 do not compensate for the lack of Bmp6. Transcription of Bmp6 is suppressed in iron deficiency and upregulated in iron overload [13]; this regulation is liver specific [14] and no other tissue modulates Bmp6 in response to iron, in agreement with the central role of the liver in iron homeostasis.

The liver is composed by several cell types: parenchymal cells, essentially hepatocytes (HCs), and non-parenchymal cells (NPCs). Among the latter Kupffer cells are resident macrophages (constituting 80–90% of body tissue macrophages), sinusoidal endothelial cells (LSECs) have filter functions between blood and hepatocytes and high endocytic capacity for many ligands, hepatic stellate cells (HSc), or Ito cells, localize between the sinusoids and HCs, and are involved in liver fibrosis when activated [15]. Recently, NPCs (KCs, LSECs and HSc) were reported to express high levels of Bmp6 compared to HCs [16,17], suggesting that they play a role in hepcidin regulation. Here we extend this observation analyzing Bmp6 expression in isolated liver cell populations of wild type mice after changing the iron status by acute and chronic diets, and in disease models with opposite and pathological hepcidin levels: the iron loaded Hjv−/− mice which have low hepcidin, and the iron deficient Tmprss6−/− mice characterized by high hepcidin levels. We demonstrate that Bmp6 is highly expressed in NPCs and that chronic changes in iron status induced by diet modulate Bmp6 in all cell types according to their intracellular iron content that is influenced by the hepcidin effect on their iron export capacity. However, in NPCs cells, characterized by high ferroportin activity, Bmp6 expression is independent on cell iron content and more related to their iron uptake and release. We also show that Bmp6 expression in LSECs is independent from HCs iron. In addition, in our in vivo models Bmp6 increase both in parenchymal and NPCs does not induce Tmprss6 transcription in HCs.

Experimental Procedures

Animal and diets

Wild type C57BL/6 male mice, obtained from Charles River, Tmprss6 KO mice on a mixed 129/Ola X C57BL/6 background [18], and Hjv KO mice on an inbred 129S6/SvEvTac background
were housed under a standard 12-hour light/dark cycle with water and chow ad libitum in a pathogen-free animal facility of the San Raffaele Scientific Institute in accordance with the European Union guidelines. The study was approved by the Institutional Animal Care and Use Committee of the San Raffaele Scientific Institute (IACUC number: 514). Only male mice were analyzed when 7–8 week old. To induce stable changes of the iron status, 4-week-old C57BL/6 male mice were fed an iron-balanced (IB; carbonile iron 200 mg/kg; SAFE), an iron-loaded (IL; carbonile iron 8.3 g/kg; SAFE), or an iron-deficient diet (ID), with virtually no iron (< 3 mg iron/kg; SAFE) for 3 weeks. To induce acute iron changes, C57BL/6 animals pretreated by 2 weeks ID diet to induce iron depletion, were administered 1 day an IB, ID or IL diet [19]. The animals were anesthetized by isoflurane and were sacrificed by cervical dislocation. All efforts were made to minimize suffering, 3 Livers and spleens were snap-frozen for isolation of RNA or dried for tissue iron content analysis. Liver cell populations were separated as described below.

Analysis of hematological and iron parameters
Hemoglobin (Hb) levels were determined using a Sysmex KX-21 automated blood cell analyser (Sysmex America) from 0.2 mL of blood extracted by caudal puncture from anesthetized mice. Iron parameters were analyzed as previously described [20]. Briefly, transferrin saturation was calculated as the ratio of serum iron and total iron binding capacity levels, using The Total Iron Binding Capacity Kit (Randox Laboratories Ltd.), according to the manufacturer’s instructions.

To measure liver (LIC) and spleen iron content (SIC) tissue samples were dried at 110°C overnight, weighed, and digested in 1 mL of 3M HCl, 0.6M trichloroacetic acid for 20 hours at 65°C. The cleared acid extract was added to 1 mL of working chromogen reagent (1 volume of 0.1% bathophenanthroline sulfate and 1% thioglycolic acid solution, 5 volumes of water, and 5 volumes of saturated sodium acetate). The solutions were then incubated for 30 minutes at room temperature until color development and the absorbance was measured at 535 nm. A standard curve was plotted using the acid solution containing increasing amounts of iron diluted from a stock solution of Titrisol iron standard (Merck, Darmstadt, Germany) [20].

Liver cells separation
Liver cells were isolated according to Liu et al. [21] with some modifications. Briefly, mice were anesthetized and sacrificed by cervical dislocation. All efforts were made to minimize suffering, 3 Livers were perfused in situ through the inferior vena cava with 0.16 mg/mL collagenase IV (Sigma-Aldrich) in an isosmotic saline solution after transection of the portal vein. After perfusion, livers were removed, teased with scalpels and incubated for 10 min at 37°C in a shaking water bath in a collagenase-solution containing DNase I. Cell suspensions were filtered through a 100-μm and a 70-μm cell strainer. Single cell suspension was then centrifuged at 50g for 3 min to pellet HCs. Cell pellet was washed three times with PBS containing 0.1% BSA and supernatants (containing NPCs) were collected. HCs were further purified by centrifugation on 10% Optiprep (1.06 g/mL) at 50 g for 10 min. NPCs were pelleted at 400 g for 10 min, resuspended in Optiprep 17.6% and stratified onto a 8.2% Optiprep. The cell fraction between the interface of the 8.2 and 17.6% Optiprep was enriched in KCs and LSECs. Middle layers were collected, cells were separated by centrifugation, and LSECs were isolated using MACS CD146 MicroBeads (Miltenyi Biotec). KCs were recovered from the flowthrough. Cell purity was validated by mRNA expression of specific genes: Tmprss6 for HCs, Cd146 for LSECs, and Cd45 for KCs.
Spleen macrophages separation
Mice were anesthetized and sacrificed by cervical dislocation. All efforts were made to minimize suffering.3 Spleen was isolated and spleen capsule was punctured to send the spleen content out. Cells were resuspended in HBSS medium (Gibco Cell Culture, Portland, OR) and centrifuged at 370g for 10 min. Cell pellet was resuspended in HBSS and incubated on ice with 8 volumes of NH₄Cl for 10 min to lyse erythrocyte. After centrifugation at 370g for 10 min cell pellet was resuspended in HBSS, stratified onto FBS (Gibco Cell Culture, Portland, OR) and centrifuge at 370g for 10 min. Cell pellet was washed two times with PBS containing 0.5% BSA and 2mM EDTA. To isolate spleen macrophage population, cells were incubated with MACS anti-F4/80 biotin conjugated and pulled down with Streptavidin MicroBeads (Miltenyi Biotec). F4/80 negative cells were further incubated with MACS CD11 MicroBeads (Miltenyi Biotec).

qRT-PCR
RNAs from isolated HCs were extracted using the guanidinium thiocyanate–phenol–chloroform method (Trizol Reagent, Invitrogen), following the manufacturer’s (Invitrogen) recommendations. RNAs from isolated KCs, LSECs and spleen-derived cells were extracted using the RNeasy mini kit (Qiagen), following manufacturer’s instructions. Total RNA (200 ng for KCs and LSECs and 2 μg for HCs) was retro-transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem), according to the manufacturer’s instructions. Gene expression levels were measured by quantitative real-time PCR using TaqMan Gene Expression Master Mix (Applied Biosystem). Primers used for qRT-PCR are listed in the S1 Table.

Western Blot analysis on liver cells
Liver cells were lysed in lysis buffer (200mM Tris-HCl [pH 8]; 1mM EDTA; 100mM NaCl; 10% glycerol; 0.5% NP-40) and proteins extracts were quantified using the Bio-Rad Protein Assay (Bio-Rad) according to the manufacturer’s instructions. Fifty g of protein extracts were re-suspended in Laemmli buffer, incubated 5 minutes at 95°C, loaded onto a 12% SDS-PAGE and then transferred to Hybond C membrane (Amersham Bioscience Europe GmbH) by standard Western blot technique. Membrane were stained with Ponceau staining for protein quantification and then blocked with 2% nonfat milk in TBS (0.5M Tris-HCl [pH 7.4] and 0.15M NaCl) containing 0.1% Tween 20 (TBST). Blocked membranes were then incubated overnight with anti-Tfr1 (1:2000; Zymed Laboratories. Inc., San Francisco) or with anti-FtL (1:1000; Sigma-Aldrich). After washing with TBST, blots were incubated 1 hour with relevant HRP-conjugated antisera in TBST with 2% nonfat milk and developed using a chemoluminescence detection kit (ECL; Amersham Biosciences).

Electro mobility shift assay (EMSA)
EMSA was performed as already described [22]. Briefly, the 32P-labeled IRE probe was generated by in vitro transcription of the plasmid pSPT-Fer. Cell extracts (2 μg of total proteins) were incubated with a molar excess of 32P-labeled IRE probe (100.000 cpm) in the presence or absence of 2% β-mercaptoethanol. Proteins were separated by non-denaturing polyacrylamide gel electrophoresis and dried gels were exposed to autoradiography. Band intensity of the exposed films was quantified by densitometry.

Quantification of cellular iron
The concentration of Iron in the different biological matrices examined was measured by inductively coupled plasma mass spectrometry (ICP-MS) using a Perkin Elmer ELAN DRC II
instrument (Perkin Elmer Sciex, Woodbridge, ON, Canada). The total quant technique analytical method, with external calibration using a dynamic reaction cell, was adopted. The coefficients of variation ranged from 4.5% to 7.6% among analytical series and from 5% to 10.5% between the series. The instrument was calibrated using standard solution at a concentration of 10 μg/L (Multielement ICP-MS Calibration Standard 3, Matrix per Volume: 5% HNO₃ per 100 mL, Perkin Elmer Plus).

Each sample underwent two-fold determination. The accuracy of the method was calculated in ultrapure water. Bovine liver standard reference materials (NIST 1640 and MS1577b, respectively, National Institute of Standard and Technology, Gaithersburg, MD, USA) were used to better approximate the results from biological matrices. It ranged between 86.5 and 88.5%. The detection limit, determined on the basis of three standard deviations of the background signal, was determined at 0.005 μg.

Statistical analysis
Data are presented as mean ± SE. Student’s t-test was used to calculate significance (P<0.05).

Results
*Bmp6* is expressed mainly in NPCs and correlates with intracellular iron content

To investigate *Bmp6* expression under basal conditions, HCs, KCs and liver sinusoidal LSECs were isolated from adult male mice as described in Material and Methods. HSCs were not included in the analysis because of the low amount of cells we were able to purify from a single animal. The purity of the preparations was assessed by specific markers: *Tmprss6* for HCs, *Cd45* for KCs and *Cd146* for LSECs. HCs and LSECs expressed high levels of *Tmprss6* ([S1A Fig](#)), whereas *Cd45* ([S1C Fig](#)) was predominantly expressed in KCs. *Hamp* was exclusively expressed in HCs ([Fig 1A](#)), whereas *Bmp6* was highly expressed in NPCs, mainly in LSECs ([Fig 1B](#)). To investigate whether *Bmp6* expression depends on cell iron content, we measured the Iron Regulatory Proteins (IRPs)/Iron Responsive Elements (IRE) binding activity that directly correlates to cell iron [23]. Alternatively we quantified *Tfr1* mRNA that is stabilized by IRPs in iron deficiency and degraded in iron overload and thus indirectly estimates intracellular iron content [23]. In all cell types, *Bmp6* levels inversely correlated with both IRPs binding activity ([Fig 1C](#)) and *Tfr1* expression ([Fig 1D](#)). Since *Tfr1* changes are easier to assess and more pronounced than changes of IRPs activity, we used *Tfr1* expression to investigate cells iron content in subsequent experiments. Notably, ferroportin (*Fpn*) expression was low in HCs and high in NPCs, especially in KCs ([Fig 1E](#)). The latter finding, considering the lower levels of IRPs binding activity in NPCs as compared to HCs ([Fig 1C](#)), indicates that NPCs have a prevalent iron exporter functions.

Chronic dietary iron changes modulate *Bmp6* in all cell types

Liver and spleen are iron storage organs able to accumulate or release iron according to body needs. To investigate whether and how dietary iron changes modulate *Bmp6* expression in liver cells and in spleen macrophages, different cohorts of mice were fed an iron balanced (IB), iron deficient (ID) or iron loaded (IL) diet for 3 weeks. Iron parameters changed as expected: LIC ([S2A Fig](#)), SIC ([S2B Fig](#)) and transferrin saturation (TS, [S2C Fig](#)) were significantly increased and decreased in IL- and ID-treated mice, respectively. Also Hb was significantly increased in animals maintained an IL diet and decreased following an ID diet ([S2D Fig](#)). In HCs, *Hamp* ([S2E Fig](#)), Inhibitor of differentiation 1 (*Id1*, a target of the Bmp-Smad pathway) ([S2F Fig](#)) and *Bmp6* ([Fig 2A](#) and [S8 Fig](#)) were transcriptionally modulated in the same direction of iron
changes. Both KCs (Fig 2C) and LSECs (Fig 2E) upregulated Bmp6 expression in dietary iron overload, while the opposite occurred in iron deficiency. In all cells (Fig 2B, 2D and 2F) Tfr1 was stabilized in iron deficiency and downregulated in iron overload. We concluded that in wild type animals Bmp6 is regulated in all liver cell types, according to their intracellular iron content, which reflects the difference between iron uptake and release. A similar regulation does not occur in the spleen: total spleen (S3A Fig), spleen-derived macrophages, as F4/80+ (S3B Fig), Cd11b+ (S3C Fig) and F4/80− Cd11b− cells (S3D Fig), did not change Bmp6 expression following systemic iron variations.

Changes in hepcidin expression do not require a change in LSECs Bmp6 expression in response to acute mild iron changes

To investigate the kinetic of Bmp6 response after acute dietary iron variation, mice were iron-depleted by a low-iron diet for 2 weeks and maintained for 1 day the same diet (< 3 mg/kg iron, defined as ID in Figs 3 and 4), or switched to a standard diet (200 mg/kg iron, defined as IB in Figs 3 and 4) or to a rich iron diet (8.3 mg/kg iron, defined as IL in Figs 3 and 4).
**Fig 2. Bmp6 and Tfr1 expression in chronic dietary iron changes.** Bmp6 (A, C, E) and Tfr1 (B, D, F) mRNA expression was evaluated by qRT-PCR in isolated HCs (A, B), KCs (C, D) and LSECs (E, F) from 4–12 mice/group. mRNA expression ratio was normalized relative to housekeeping Hprt1. Mean control value of IB-treated mice was set to 1. Error bars indicate SE. *: \( P < .05 \); **: \( P < .01 \); ***: \( P < .001 \); ns: not significant.

**Fig 3. Iron parameters in acute dietary iron changes.** Transferrin saturation (TS, A) and non-heme liver (LIC, B) iron content were measured in mice (6–8/group) treated by an iron deficient diet for two weeks and then challenged with 1 day ID (ID-1 day), iron balanced (IB-1 day) and iron loaded (IL-1 day) diets. Hamp (C) expression was evaluated in HCs isolated from 4–7 mice/group. Hprt1 was used as housekeeping gene. mRNA expression ratio was normalized to control (ID-1 day) mean value set to 1. Error bars indicate SE. *: \( P < .05 \); **: \( P < .01 \); ***: \( P < .001 \); ns: not significant.
1 day ID, standard (IB) or IL diet, according to described protocols [19]. Results after IL diet were as expected: TS (Fig 3A), LIC (Fig 3B) and Hamp (Fig 3C) were all increased, Bmp6 was increased and Tfr1 mRNA decreased in all cell types (Fig 4). In animals switched to a standard (IB) diet, the response of circulating and intracellular iron was uncoupled: TS was increased (Fig 3A), whereas LIC (Fig 3B) was unchanged and Hamp was only moderately increased compared with animals maintained an ID diet (Fig 3C). HCs and KCs Bmp6 was concordant with Hamp and moderately increased in comparison with ID (Fig 4A and 4C). Remarkably, LSECs Bmp6 remained low as in ID (Fig 4E).

Considering intracellular iron we observed a discrepancy between LIC and Tfr1 mRNA: although LIC was similar in IB and ID (Fig 3B), HCs Tfr1 was strongly induced only in ID (Fig 4B) and downregulated in IB as well as in IL mice, which had the highest LIC (Fig 3B). This observation suggests that a threshold of intracellular iron should likely be achieved to induce reduction of Tfr1 expression and that Tfr1 mRNA is a better sensor of iron decrease than increase.
KCs behaved as HCs: in IB *Bmp6* was slightly upregulated (Fig 4C) and *Tfr1* mRNA mildly decreased (Fig 4D) compared to ID mice. However, in LSECs both *Bmp6* (Fig 4E) and *Tfr1* expression (Fig 4F) were comparable between ID and IB mice, suggesting that, although iron absorption increases significantly when animals previously iron depleted are switched to 1 day IB diet [19], this is not sufficient to activate *Bmp6* in LSECs, the activation requiring higher iron burden, as occurs in IL diet (Fig 4E).

**Bmp6 regulation in the iron loaded *Hjv* KO mice**

To investigate the regulation of *Bmp6* in pathological iron overload, we analyzed *Hjv* KO mice, characterized by severe iron overload with increased TS (S4A Fig) and LIC (S4B Fig and [8,9]), due to inactivation of the Bmp6-coreceptor *Hjv* that results in strong *Hamp* reduction (S4C Fig). Severe hepcidin insufficiency causes ferroportin stabilization and, although iron is increased in the circulation and in the liver, the spleen is iron poor (S4D Fig) [8,9].

In *Hjv* KO HCs *Bmp6* mRNA was increased (Fig 5A and S8 Fig) and *Tfr1* expression was suppressed (Fig 5B), compatible with high iron content, as demonstrated also by degradation of *Tfr1* protein (S5A Fig). *Bmp6* was increased also in KCs and LSECs (Fig 5C and 5E). However, unlike HCs, *Tfr1* was up-regulated in NPCs both at mRNA (Fig 5D and 5F) and protein levels (S5B and S5C Fig).

**Fig 5. Bmp6 and Tfr1 expression in *Hjv* KO mice.** *Bmp6* (A, C, E) and *Tfr1* (B, D, F) mRNA expression was evaluated in liver cells isolated from 6–10 mice by qRT-PCR, using *Hprt1* as the referred housekeeping gene. mRNA expression ratio was normalized to control wild type (wt) mean value set to 1. Error bars indicate SE. **: P < 0.01; ***: P < 0.001; ****: P < 0.0001.

doi:10.1371/journal.pone.0122696.g005
Table 1. Total iron quantification in liver cells from wild type and Hjv KO mice.

|            | HCs          | KCs          | LSECs        |
|------------|--------------|--------------|--------------|
| wild type  | 2.127±0.442  | 0.115±0.096  | 0.248±0.045  |
| Hjv KO     | 125.639±87.937* | 0.405±0.252ns | 0.481±0.267ns |

HCs: hepatocytes. KCs: kupffer cells. LSECs: liver sinusoidal endothelial cells.

*: Hjv KO vs wild type P = 0.036.

ns: Hjv KO vs wild type non significant.

doi:10.1371/journal.pone.0122696.t001

To determine the iron content in Hjv KO liver cells populations, HCs, KCs and LSECs were processed by inductively coupled plasma mass spectrometry (ICP-MS). HCs from Hjv KO mice are severely iron loaded, as expected (Table 1). In contrast, iron levels in KCs and LSECs are comparable to the iron replete wild type littermates (Table 1), likely because of their elevated iron export ability due to low hepcidin and ferroportin stabilization. To further investigate the regulation of iron-related proteins in this model, we analyzed ferritin L (FtL) levels by Western Blot analysis. FtL is strongly increased in Hjv KO HCs (S5A Fig) as expected due to high iron levels. However FtL is increased also in Hjv KO KCs and LSECs (S5B and S5C Fig), which had intracellular iron comparable to wild type animals (Table 1).

Regulation of Bmp6 in the iron deficient Tmprss6 KO mice

The Tmprss6 KO mouse model is characterized by iron deficient anemia, as assessed by low hemoglobin (data not shown), low LIC (S6A Fig), low TS (S6B Fig), due to hyperactivation of the BMP-SMAD pathway [24] and inappropriately high hepcidin levels (S6C Fig and [18]). Spleen iron of Tmprss6 KO mice is comparable with that of wild type littermates (S6D Fig), thus inappropriate for its condition of iron deficiency.

In isolated HCs Bmp6 expression was low (Fig 6A) and TfR1 elevated (Fig 6B). However, Tfr1 expression in KCs (Fig 6D) and LSECs (Fig 6F) was similar to wild type littermates, reflecting spleen iron content (S6D Fig) and compatible with iron retention due to high hepcidin. Bmp6 in KCs (Fig 6C) and LSECs (Fig 6E) was significantly lower than in wild type cells, as in HCs. In analogy with the Hjv KO model but in opposite direction, these results suggest that NPCs modulate Bmp6 in a way independent on their iron content, in this case reflecting low iron uptake.

Tmprss6 expression is independent of Bmp6 expression

Recently Bmp6 was shown to be a positive regulator of Tmprss6 expression in mice treated with prolonged iron rich diet [25]. Tmprss6 up-regulation was interpreted as a negative feedback mechanism to avoid excessive hepcidin production in response to iron increase [25]. We took advantage of the analyzed models to investigate whether Bmp6 produced by HCs and/or NPCs could play a role in the regulation of Tmprss6. As shown in S7 Fig, in our models we did not observe a transcriptional activation of Tmprss6 in all high Bmp6 conditions, including dietary both chronic (S7A Fig) and acute (S7B Fig) and disease-associated (S7C Fig) iron overload. Only a trend toward a slight increase was observed in chronic IL diet.

Discussion

The liver hormone hepcidin controls systemic iron homeostasis in response to different stimuli, such as variations of total body iron, erythropoiesis expansion, hypoxia and inflammation. In response to iron increase, hepcidin is up-regulated by the BMP-SMAD signaling...
pathway, activated by BMP6 and inhibited by the serine protease TMPRSS6 [26]. At the same time iron regulates BMP6 expression in a homeostatic manner [13]. The orchestration of iron metabolism by Bmp6 is a liver-specific function, since systemic iron changes do not modify Bmp6 expression in other organs [14]. However, how Bmp6 expression is upregulated by iron remains elusive and the specific contribution of the different liver cell types in Bmp6 regulation in physiological and pathological conditions remains poorly understood.

In the present study we investigated the Bmp6 expression in isolated liver cells, in response to physiological iron changes and in murine models of iron disorders. We also attempted to compare cell iron content and Bmp6 expression in the different cell types. As a measure of iron content we used Tfr1 mRNA, since in basal conditions its transcriptional regulation corresponds to the IRP1 activity (Fig 1C) in the different cell types.

We confirm that Hamp expression in basal conditions is high although HCs Bmp6 is low, compatible with a paracrine hepcidin stimulation by the elevated NPCs Bmp6, as previously observed [27]. Interestingly, KCs and LSECs have lower IRP binding activity than HCs and express higher levels of ferroportin, findings compatible with an important function of iron exporters. At variance with published data [27] we observed that Bmp6 changes are consensual to systemic and intracellular iron in all liver cells, including HCs, when variations of the iron status are stably induced by the chronic (3-weeks) diet. We propose that in steady state the

![Fig 6. Bmp6 and Tfr1 expression in Tmprss6 KO mice. Bmp6 (A, C, E) and Tfr1 (B, D, F) expression was measured in liver cells isolated from wild type (wt) and Tmprss6 KO mice (4–6 mice/group) by qRT-PCR, using Hprt1 as housekeeping gene. mRNA expression ratio was normalized to wt mean value set to 1. Error bars indicate SE. *: P < .05; **: P < .01; ns: not significant.](http://journal.pone.0122696.g006)
equilibrium is reached between iron uptake and hepcidin-mediated iron export and that Bmp6 expression reflects this equilibrium in all liver cells, including NPCs. We excluded that in the same chronic conditions spleen macrophages participate to Bmp6 regulation, confirming the central role of liver Bmp6 in hepcidin control.

Acute dietary iron changes in iron-depleted mice caused marked variations of Hamp and Bmp6 in all liver cells only when animals were exposed to the iron-enriched (IL) diet. In IB-treated ones TS increased but Bmp6 increase was mild in HCs and KCs and absent in LSECs, that remain relatively iron free likely because of their rapid iron release. In the interpretation of our results, the dynamics of iron uptake and release should be considered, the latter being more important in NPCs than in HCs, because of their higher ferroportin expression. It should also be noted that in the acute model of iron variation the effect of hepcidin on ferroportin is not as evident as in chronic models.

In the 1-day diet, despite HCs iron and Bmp6 are both increased in IB-treated mice, LSECs do not up-regulate Bmp6. Thus LSECs do not respond to acute and likely modest and transient dietary iron changes.

A signal from HCs that drives Bmp6 activation in NPCs has been previously described [27]. Our results suggest that Bmp6 expression in LSECs increases only when circulating iron is stably high as in IL diet.

Disease models of iron overload (Hjv KO) and deficiency (Tmprss6 KO) are especially informative to define Bmp6 regulation. Since characterized by abnormal hepcidin response, opposite to the physiological one, these models dissociate Bmp6 expression from hepcidin response. Hjv KO mice have appropriately high Bmp6, as observed [19] but low hepcidin, and Tmprss6 KO have the opposite phenotype of high hepcidin and low Bmp6. In our hands the direct correlation between iron (Tfr1 mRNA) and Bmp6 is maintained in HCs, but not in NPCs whose Tfr1 expression was similar to that of spleen macrophages. Although HCs have high (in case of Hjv KO) or low (in case of Tmprss6 KO) iron content, KCs and LSECs show high Tfr1 in Hjv KO mice, and low Tfr1 in Tmprss6 KO animals, while Bmp6 is upregulated in the former, and downregulated in the latter in all liver cell examined. To clarify the discrepancy observed between Tfr1 and Bmp6 we measured Tfr1 and ferritin L protein and intracellular iron content in isolated liver cells from Hjv KO mice, which showed the more striking discrepancy between Tfr1 and Bmp6. We found that both Tfr1 and ferritin L proteins were increased in NPCs although the iron content was not significantly different from wild type cells but strikingly lower than HCs iron (Table 1). To reconcile these data with the high levels of Tfr1 we reasoned that the iron taken up from the circulation is sequestered into ferritin and rapidly exported by Fpn (not degraded by hepcidin), leaving low the labile iron pool. The effect of abnormal hepcidin levels is quite evident in NPCs also in Tmprss6 KO mice. Since NPCs opposite to HCs, have high ferroportin expression and considering the iron status of the models studied, is plausible to conclude that Bmp6 expression in NPCs reflects their iron flux (high in Hjv KO and low in Tmprss6 KO).

It has been suggested that ferritin from HCs may act as a paracrine molecule to regulate Bmp6 expression in LSECs [28]. Both HCs and NPCs regulate Bmp6 expression apparently in response to changes of HCs iron, in chronic conditions. This has been proposed to require a signal/cytokine to transmit information on the iron state from HCs to NPCs [27]. We favor a different interpretation based on the following observations. First, a mild HCs iron increase in acute dietary iron treatment does not activate Bmp6 in LSECs. Second, Fpn inactivation in HCs, macrophages and duodenum, that leads to liver iron overload and decreases serum iron and TS, does not increase, but even down-regulate, liver Bmp6 expression [29]. In this mouse model, KCs are highly iron loaded, whereas HCs show only mild iron accumulation. However, Bmp6 is downregulated in the total liver of these mice. These results agree with our model. In
wild type

Hjv KO

Tmprss6 KO
mice with conditional deletion of Fpn HC s iron does not regulate Bmp6 expression that is low despite the mild iron increase. Moreover this mice model is compatible with the concept that iron flux (the uptake by Tfr1 and the export by ferroportin) regulates Bmp6 expression. With conditional deletion of Fpn the uptake of iron is low because of low TS, and the export is blocked because of Fpn inactivation.

We observe that NPCs Bmp6 is discrepant with iron content measured as Tfr1 mRNA (or protein) in several conditions. In the interpretation of the results, the higher ferroportin expression of NPCs in comparison with HCs should be considered. Our results are compatible with the possibility that NPCs regulate Bmp6 expression sensing the iron flux, while Tfr1 reflects the labile iron pool resulting from the uptake/release balance, the latter being highly dependent on hepcidin itself and more evident when levels of hepcidin are stable in chronic conditions. This would explain why Bmp6 is low in Tmprss6 KO (where iron uptake is minimal, but the release is reduced by ferroportin degradation) and high in Hjv KO, where the uptake is massive but massive is also the export, due to the hepcidin deficiency. These disease models highlight the physiological crosstalk of liver cells, because of their altered hepcidin response.

In conclusion we have strengthened the crosstalk among the different liver cell types and showed a crucial role of NPCs in the modulation of Bmp6 both in physiological and pathological conditions. Although HCs are able to increase Bmp6 in iron overload, the major contribution is provided by the high Bmp6-expressing NPCs, especially LSECs, likely because they are in direct contact with the circulation and may behave as a liver iron sensor. Increasing hepcidin through Bmp6, they contribute not only to systemic, but also to local iron regulation, withholding iron, as a possible protective mechanism of HCs (Fig 7). In Hjv KO model Bmp6 production persists, but lack of hepcidin response abolishes the protective effect of LSECs and KCs on HCs iron loading. In Tmprss6 KO mice Bmp6 expression is low, irrespective of the relatively high iron content of NPCs that is determined by the high hepcidin levels. This iron sequestration contributes to worsen systemic and HCs iron deficiency, as illustrated in Fig 7.

We have uncovered a previously unrecognized mechanism of controlled storage and release of iron by NPCs: the positive feedback between Bmp6 expression in these cells and ferroportin degradation by the induced hepcidin would allow these cells to accumulate iron for later needs and at the same time to protect HCs from iron overload.

**Supporting Information**

**S1 Fig. Isolated liver cells characterization.** Liver cells were isolated from 4 mice. Tmprss6 (A), Cd146 (B) and Cd45 (C) mRNA expression was quantified by qRT-PCR relative to housekeeping Hprt1 mRNA to evaluate the purity of HCs, KCs and LSECs, respectively. Error bars indicate SE. *: P<.05; **: P<.01; ***: P<.001.

**S2 Fig. Iron parameters and hepatocyte iron-related genes in chronic dietary iron changes.** Mice were fed an iron balanced (IB), iron deficient (ID) and iron loading (IL) diet for 3 weeks (6–8 mice/group). Non-heme liver (LIC, A), spleen (SIC, B) iron content, transferrin saturation (TS, C) and hemoglobin levels (Hb, D) are shown. In isolated hepatocytes hepcidin (Hamp) and Id1 mRNA expression was quantified by qRT-PCR relative to housekeeping
Hprt1 gene. mRNA expression ratio was normalized setting control (IB) mean value to 1. Error bars indicate SE. *: P < .05; **: P < .01; ***: P < .001.

(S3 Fig. Bmp6 expression in total spleen and spleen-derived cells. Spleen and spleen-derived cells were isolated from mice maintained an iron balanced (IB), iron deficient (ID) and iron loading (IL) diet for 3 weeks. Bmp6 expression from total spleen (A, 3–4 mice), from F4/80+ cells (B, 6 mice), from Cd11b+ cells (C, 6 mice) and from negative fractions (D, 6 mice) was quantified by qRT-PCR relative to Hprt1 as the housekeeping gene. Error bars indicate SE. ns: not significant.

(S4 Fig. Iron parameters and hepcidin levels in Hjv KO mice. Transferrin saturation (TS, A), non-heme sliver iron content (LIC, B) and non-heme spleen iron content (SIC, D) were measured in wild type (wt) and Hjv KO animals (4 mice/group). In isolated HCs, hepcidin (Hamp, C) expression was measured by qRT-PCR, using Hprt1 as housekeeping gene and mRNA expression ratio was normalized to control (wt) mean values set to 1. Error bars indicate SE. *: P < .05; **: P < .01; ***: P < .001.

(S5 Fig. Tfr1 and Ftl protein levels in cells isolated from Hjv KO mice. HCs (A), Kcs (B) and LSECs (C) were isolated from wild type (wt) and Hjv KO mice. Cells were lysed in Lysis Buffer as described in Material and Methods and protein extracts were loaded onto a 12% SDS PAGE and processed for Western Blot analysis. Anti-Tfr1 and anti-FtL Ab were used to detect endogenous Tfr1 and FtL respectively. Equal protein transfer were verified by Ponceau staining.

(S6 Fig. Iron parameters and hepcidin levels in Tmprss6 KO mice. Non-heme liver (LIC, A) and spleen (SIC, D) iron content and transferrin saturation (TS, B) in wild type (wt) and Tmprss6 KO mice (4–6 mice/group). Hepcidin mRNA expression (Hamp, C) was evaluated in isolated HCs by qRT-PCR relative to the housekeeping Hprt1 gene. mRNA expression was normalized to control (wt) mean values set to 1. Error bars indicate SE. *: P < .05; **: P < .01; ns: not significant.

(S7 Fig. Hepatocytes Tmprss6 expression in iron-loading and iron deficient conditions. Tmprss6 expression was measured by qRT-PCR in HCs isolated from: A) mice maintained 3 weeks an iron balanced (IB), iron deficient (ID) and iron loading (IL) diet (6 mice/group). mRNA expression ratio was normalized to a control (IB) mean values set to 1. B) mice maintained 2 weeks an ID diet and then treated with 1 day ID (ID-1 day), IB (IB-1 day), IL (IL-1 day) diet (4 mice/group). C) wild type (wt) and Hjv KO mice (4 mice/group). Hprt1 was used as housekeeping gene for mRNA quantification. Error bars indicate SE. ns: not significant.

(S8 Fig. Bmp6 and Tfr1 expression. Bmp6 (A, C, E) and Tfr1 (B, D, F) expression were assessed by qRT-PCR in liver cells population from C57BL/6, maintained a 3 weeks iron balanced (IB), iron deficient (ID) and iron loading (IL) diets (A, B), Hjv KO mice (C, D) and Tmprss6 KO animals (E, F) male mice. Hprt1 was used as housekeeping gene for mRNA quantification. Error bars indicate SE.
S1 Table. Oligonucleotides used for qRT-PCR.

Acknowledgments

We wish to thank Carlos Lopez-Otin (University of Oviedo, Oviedo, Spain) and Nancy Andrews (Duke University, Durham, NS) for the gift of Tmprss6 KO and Hjv KO mice, respectively. This work was partially supported by “Telethon Fondazione Onlus” Grant GGP12025, Ministero Sanità, MIUR-PRIN (Progetto di Rilevante Interesse Nazionale) 2010–2011 and Ricerca Finalizzata RF-2010-2312048 to CC. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Contributions

Conceived and designed the experiments: LS CC. Performed the experiments: LS MR AP AN AC MEG. Analyzed the data: LS MR. Contributed reagents/materials/analysis tools: PA. Wrote the paper: LS CC.

References

1. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, et al. (2004) Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 306: 2090–2093. PMID: 15514116
2. Papanikolaou G, Samuels ME, Ludwig EH, MacDonald ML, Franchini PL, Dube MP, et al. (2004) Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. Nature genetics 36: 77–82. PMID: 14647275
3. Babitt JL, Huang FW, Wrighting DM, Xia Y, Sidis Y, Samad TA, et al. (2006) Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. Nature genetics 36: 531–539. PMID: 16604073
4. Xia Y, Yu PB, Sidis Y, Beppu H, Bloch KD, Schneyer AL, et al. (2007) Repulsive guidance molecule RGMa alters utilization of bone morphogenetic protein (BMP) type II receptors by BMP2 and BMP4. The Journal of biological chemistry 282: 18129–18140. PMID: 17472960
5. Steinbicker AU, Bartnikas TB, Lohmeyer LK, Leyton P, Mayeur C, Kao SM, et al. (2011) Perturbation of hepcidin expression by BMP type I receptor deletion induces iron overload in mice. Blood 118: 4224–4230. doi:10.1182/blood-2011-03-339952 PMID: 21841161
6. Nohe A, Keating E, Knaus P, Petersen NO (2004) Signal transduction of bone morphogenetic protein receptors. Cellular signalling 16: 291–299. PMID: 14687659
7. Wang RH, Li C, Xu X, Zheng Y, Xiao C, Zerfas P, et al. (2005) A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression. Cell metabolism 2: 399–409. PMID: 16330325
8. Niederkofler V, Salie R, Arber S (2005) Hemojuvelin is essential for dietary iron sensing, and its mutation leads to severe iron overload. The Journal of clinical investigation 115: 2180–2186. PMID: 15721058
9. Huang FW, Pinkus JL, Pinkus GS, Fleming MD, Andrews NC (2005) A mouse model of juvenile hemochromatosis. The Journal of clinical investigation 115: 2187–2191. PMID: 16075059
10. Nicolas G, Bennoun M, Devaux I, Beaumont C, Grandchamp B, Kahn A, et al. (2001) Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. Proceedings of the National Academy of Sciences of the United States of America 98: 8780–8785. PMID: 11447267
11. Bragdon B, Moseychuk O, Saldanha S, King D, Julian J, Nohe A (2011) Bone morphogenetic proteins: a critical review. Cellular signalling 23: 609–620. doi: 10.1016/j.cellsig.2010.10.003 PMID: 20959140
12. Xia Y, Babitt JL, Sidis Y, Chung RT, Lin HY (2008) Hemojuvelin regulates hepcidin expression via a selective subset of BMP ligands and receptors independently of neogenin. Blood 111: 5195–5204. doi: 10.1182/blood-2007-09-111567 PMID: 18326817
13. Kautz L, Meynard D, Monnier A, Damaud V, Bouvet R, Wang RH, et al. (2008) Iron regulates phosphorylation of Smad1/5/8 and gene expression of Bmp6, Smad7, Id1, and Atoh8 in the mouse liver. Blood 112: 1509–1509. doi: 10.1182/blood-2008-03-143954 PMID: 18539898
14. Kautz L, Besson-Fournier C, Meynard D, Latour C, Roth MP, Coppin H (2011) Iron overload induces BMP6 expression in the liver but not in the duodenum. Haematologica 96: 199–203. doi: 10.3324/haematol.2010.031963 PMID: 20952515

15. Yin C, Evasion KJ, Asahina K, Stainier DY (2013) Hepatic stellate cells in liver development, regeneration, and cancer. The Journal of clinical investigation 123: 1902–1910. doi: 10.1172/JCI66369 PMID: 23635788

16. Knittel T, Fellmer P, Muller L, Ramadori G (1997) Bone morphogenetic protein-6 is expressed in non-parenchymal liver cells and upregulated by transforming growth factor-beta 1. Experimental cell research 232: 263–269. PMID: 9168801

17. Zhang AS, Anderson SA, Wang J, Yang F, DeMaster K, Ahmed R, et al. (2011) Suppression of hepatic hepcidin expression in response to acute iron deprivation is associated with an increase of matriptase-2 protein. Blood 117: 1687–1699. doi: 10.1182/blood-2010-06-287292 PMID: 21115976

18. Folgueras AR, de Lara FM, Pendas AM, Garabaya C, Rodriguez F, Astudillo A, et al. (2008) Membrane-bound serine protease matriptase-2 (Tmprss6) is an essential regulator of iron homeostasis. Blood 112: 2539–2545. doi: 10.1182/blood-2008-04-149773 PMID: 18523150

19. Ramos E, Kautz L, Rodriguez R, Hansen M, Gabayan V, Ginzburg Y, et al. (2011) Evidence for distinct pathways of hepcidin regulation by acute and chronic iron loading in mice. Hepatology 53: 1333–1341. doi: 10.1002/hep.24178 PMID: 21480335

20. Pagani A, Nai A, Corna G, Bosurgi L, Rovere-Querini P, Camaschella C, et al. (2011) Low hepcidin accounts for the proinflammatory status associated with iron deficiency. Blood 118: 736–746. doi: 10.1182/blood-2011-02-337212 PMID: 21628413

21. Liu W, Hou Y, Chen H, Wei H, Lin W, Li J, et al. (2011) Sample preparation method for isolation of single-cell types from mouse liver for proteomic studies. Proteomics 11: 3556–3564. doi: 10.1002/pmic.201100157 PMID: 21751380

22. Campanella A, Levi S, Cairo G, Biasiotto G, Arosio P (2004) Blotting analysis of native IRP1: a novel approach to distinguish the different forms of IRP1 in cells and tissues. Biochemistry 43: 195–204. PMID: 14705945

23. Rouault TA (2006) The role of iron regulatory proteins in mammalian iron homeostasis and disease. Nature chemical biology 2: 406–414. PMID: 16850017

24. Riba M, Rausa M, Sorosina M, Cittaro D, Garcia Manteiga JM, Nai A, et al. (2013) A strong anti-inflammatory signature revealed by liver transcription profiling of Tmprss6-/- mice. PloS one 8: e66694. doi: 10.1371/journal.pone.0066694 PMID: 23922777

25. Meynard D, Vaja V, Sun CC, Corradini E, Chen S, Lopez-Otin C, et al. (2011) Regulation of TMPRSS6 by BMP6 and iron in human cells and mice. Blood 118: 747–756. doi: 10.1182/blood-2011-04-348698 PMID: 21622652

26. Hentze MW, Muckenthaler MU, Galy B, Camaschella C (2010) Two to Tango: regulation of Mammalian iron metabolism. Cell 142: 24–38. doi: 10.1016/j.cell.2010.06.028 PMID: 20603012

27. Enns CA, Ahmed R, Wang J, Ueno A, Worthen C, Tsukamoto H, et al. (2013) Increased iron loading induces Bmp6 expression in the non-parenchymal cells of the liver independent of the BMP-signaling pathway. PloS one 8: e60534. doi: 10.1371/journal.pone.0060534 PMID: 23562526

28. Feng Q, Migas MC, Waheed A, Britton RS, Fleming RE (2012) Ferritin upregulates hepatic expression of bone morphogenetic protein 6 and hepcidin in mice. American journal of physiology Gastrointestinal and liver physiology 302: G1397–1404. doi: 10.1152/ajpgi.00200.2012 PMID: 22517766

29. Zhang Z, Guo X, Herrera C, Tao Y, Wu Q, Wu A, et al. (2014) Bmp6 expression can be regulated independently of liver iron in mice. PloS one 9: e84906. doi: 10.1371/journal.pone.0084906 PMID: 24454764