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Chloé Latour[1], Céline Besson-Fournier[1], Ophélie Gourbeyre[1], Delphine Meynard[1], Marie-Paule Roth[$1,] Hélène Coppin[$1]

[1] IRSD, Université de Toulouse, INSERM, INRA, ENVT, UPS, Toulouse, France

[$]HC and MPR contributed equally to this manuscript

Correspondence to: Hélène COPPIN & Marie-Paule ROTH
Institut de Recherche en Santé Digestive (IRSD)
Inserm U1220 Bat B
CHU Purpan
Place du Docteur Baylac
CS 60039
F-31024 Toulouse Cedex 3

e-mail: helene.coppin@inserm.fr & marie-paule.roth@inserm.fr
tel: (33) 5 62 74 45 08
fax: (33) 5 62 74 45 58

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Key points

- Loss of Bmp6 further represses hepcidin expression in the liver of *Hjv* knockout mice and markedly worsens the iron phenotype of females
- Induction of hepcidin by LPS is not prevented by lack of Bmp6 and/or Hjv but its level post-stimulation is blunted compared with controls

Abstract

Lack of either BMP6 or the BMP co-receptor hemojuvelin (HJV) in mice leads to a similar phenotype with hepcidin insufficiency, hepatic iron loading, and extrahepatic iron accumulation in males. This is consistent with the current views that HJV is a co-receptor for BMP6 in hepatocytes. To determine whether BMP6 and HJV may also signal to hepcidin independently of each other, we intercrossed *Hjv*$^{-/-}$ and *Bmp6*$^{-/-}$ mice and compared the phenotype of animals of the F2 progeny. Loss of Bmp6 further repressed Smad signaling and hepcidin expression in the liver of *Hjv*$^{-/-}$ mice of both genders, and led to iron accumulation in the pancreas and the heart of females. These data suggest that, in *Hjv*$^{-/-}$ females, Bmp6 can provide a signal adequate to maintain hepcidin to a level sufficient to avoid extrahepatic iron loading. We also examined the impact of Bmp6 and/or Hjv deletion on the regulation of hepcidin by inflammation. Our data show that lack of one or both molecules does not prevent induction of hepcidin by LPS. However, BMP/Smad signaling in unchallenged animals is determinant for the level of hepcidin reached after stimulation, which is consistent with a synergy between IL6/STAT3 and BMP/SMAD signaling in regulating hepcidin during inflammation.

Introduction

Liver sinusoidal endothelial cells (LSEC) produce bone morphogenetic protein BMP6 which acts on the hepatocyte co-receptor hemojuvelin (HJV) to regulate hepcidin production in a paracrine fashion[1,2]. Both are required to ensure optimal maintenance of iron homeostasis. In humans, mutations in the HJV gene are responsible for juvenile hemochromatosis, an early onset form of hereditary hemochromatosis caused by profound hepcidin insufficiency[3]. Whereas loss of function mutations in BMP6 have not been described so far, heterozygous mutations in the pro-peptide have been identified in patients with iron overload and lead to inappropriate hepcidin synthesis[4,5]. In the mouse, deletion of either *Bmp6* or *Hjv* leads to a similar phenotype characterized by hepcidin insufficiency, severe iron loading and extrahepatic iron accumulation in males[6-11], which is consistent with the current views that HJV is a coreceptor for BMP6.

Whether BMP6 and HJV may also signal to hepcidin independently of each other is still a topic of discussion[11]. To provide direct evidence that BMP6 and HJV can separately stimulate hepcidin, we intercrossed *Hjv* and *Bmp6* knockout mice and looked whether deletion of both *Bmp6* and *Hjv* in mice of the F2 progeny was aggravating the phenotype of single knockout animals. Whether BMP6 and HJV are both required for the upregulation of hepcidin by inflammatory stimuli is another unresolved issue. We took the opportunity of having genetically comparable single and double knockout animals to challenge them with LPS and examine the impact of Bmp6 and/or Hjv deletion on Smad signaling and hepcidin expression after stimulation.

Methods

Mouse crosses. *Hjv*$^{-/-}$ mice on a 129S6/SvEvTac background[9] were bred to *Bmp6*^{tm1Rob} mice (*Bmp6*$^{-/-}$) on a CD1 background[12]. Experiments were done on 12 w.o. wild-type, *Bmp6*$^{-/-}$, *Hjv*$^{-/-}$, and *Bmp6*$^{-/-}$ *Hjv*$^{-/-}$ littermates of the F2 progeny. Experimental protocols were approved by the Midi-Pyrénées Animal Ethics Committee. Animals were given free access to tap water and standard laboratory mouse chow diet (250 mg iron/kg; SAFE, Augy, France). Littermates carrying the different genotypes were also challenged with an intraperitoneal injection of LPS (1µg/g body weight) and livers were harvested 4 hours later.

Quantitation of liver mRNA levels. Quantitative PCR reactions were run on a LightCycler® 480 System (Roche Diagnostics), using primers previously referenced[13]. ΔCt values were obtained by subtracting the *Hprt* reference gene Ct to the target gene Ct.

Serum hepcidin. Serum hepcidin levels were quantified using the Intrinsic LifeSciences (La Jolla, CA) Hepcidin-Murine Compete™ competitive ELISA.

Quantitative iron measurement and tissue iron staining. Transferrin saturation was obtained through the determination of serum iron and latent iron-binding capacity. Quantitative measurement of non-heme iron in the liver, heart, and pancreas was performed according to the method of Torrance & Bothwell[14]. Non-transferrin bound iron (NTBI) was measured using the FeROS™ eLPI kit (Aferrix).

Protein extraction and western-blot analysis were performed as previously described[13]***.***

Statistical analyses**.** Means of quantitative variables (log-transformed for serum hepcidin) were compared with two-way ANOVA followed by Sidak's multiple comparison tests of planned contrasts between pairs of means.

Results and discussion

We first measured by quantitative PCR the amount of hepcidin mRNA in the liver of the genetically comparable F2 littermates (Fig. 1A). As previously reported in mice of other genetic backgrounds[11], hepcidin expression in the liver of *Bmp6*$^{-/-}$ and *Hjv*$^{-/-}$ mice is much lower than in wild-type mice, particularly in males. However, deletion of Bmp6 more dramatically repressed the already reduced hepcidin expression of *Hjv*$^{-/-}$ mice, regardless of sex. These observations were confirmed at the protein level (Fig. 1B). Gene expression of *Id1* (Fig. 1C) and *Smad7* (Fig. 1D), two targets of Bmp/Smad signaling, was also further repressed in double knockout mice, compared with single knockouts, providing indirect evidence that deletion of Bmp6 further alters Smad1/5/8 signaling in the liver of *Hjv* knockout mice. This was confirmed by western-blot (Fig. 2C, lanes 11-13).

In line with the repression of hepcidin, targeted disruption of the *Bmp6* and/or the *Hjv* gene leads to a strong increase in transferrin saturation (Fig. S1A) and liver iron accumulation (Fig. S1B) in mice of both genders. However**,** whereas *Bmp6*$^{-/-}$ or *Hjv*$^{-/-}$ males have iron deposits in acinar cells of the exocrine pancreas (Fig. 1E and S2A) and in the heart (Fig. 1F and S2B), females do not accumulate iron in these extrahepatic tissues, which reflects the fact that, in the absence of testosterone, their hepcidin is less strongly repressed[10] and their NTBI lower (Fig. 1G) than in males. Nevertheless, the concomitant loss of Bmp6 suppresses this hepcidin advantage over males. As a consequence, and in

contrast to single *Bmp6*$^{-/-}$ or *Hjv*$^{-/-}$ females, double knockout females have substantial NTBI amounts and massive iron loading in all extrahepatic tissues examined (Fig. S2A&B).

Interestingly, while LPS significantly induces hepcidin mRNA (Fig. 2A) and protein (Fig. 2B) not only in wild-type males and females, but also in single and double knockout animals, the level reached after stimulation depends on basal expression of hepcidin. It is highest in wild-type mice, intermediate in *Bmp6*$^{-/-}$ and in *Hjv*$^{-/-}$ mice, and lowest in double knockout animals. This is compatible with the proposed synergy between IL6/STAT3 and BMP/SMAD signaling in regulating hepcidin during inflammation[15]. Smad5 signaling was previously shown to be activated by activin B as a consequence of LPS stimulation[16] but to have no impact on hepcidin induction[13]. Here, although activation of Smad5 by LPS was similar in wild-type and in *Bmp6*$^{-/-}$ mice (Fig. 2C&D, lanes 3-6), the level of hepcidin reached after stimulation was much lower in *Bmp6*$^{-/-}$ mice. In contrast, *Hjv*$^{-/-}$ mice present with reduced Smad5 activation compared with *Bmp6*$^{-/-}$ mice (Fig. 2C&D, lanes 5-8) but have similar induction of hepcidin. These data confirm recent *in vitro* observations showing that HJV augments Smad5 signaling by activin B[17]. They also definitely indicate the lack of relationship between activation of Smad1/5/8 signaling by inflammatory stimuli, which is facilitated by HJV, and elevation of hepcidin expression.

In conclusion, analysis of this *Bmp6*$^{-/-}$ x *Hjv*$^{-/-}$ intercross clearly shows that deletion of both *Bmp6* and *Hjv* further represses hepcidin and aggravates the phenotype of single knockout animals. This indicates that, when one actor of the major hepcidin signaling pathway is lacking, alternative pathways, although less efficient to activate Smad1/5/8, succeed in maintaining hepcidin to a level avoiding extrahepatic iron accumulation in females. These can be initiated by interaction of HJV with LSEC-produced BMP2 whose deletion in mice also leads to iron overload[18,19], or by direct binding of BMP6 to preformed BMP type I/type II receptor complexes that exist at the membrane in the absence of ligands and co-receptors[20-22]. The suppression of these alternative pathways, as here in *Bmp6/Hjv* double knockout animals, leads to a greater repression of hepcidin in mice of both genders and a substantial exacerbation of the extrahepatic iron overload phenotype in females. Our data also show that induction of hepcidin by LPS *in vivo* is linked to BMP/Smad signaling before but not after stimulation. Notably, the less severely affected *Bmp6*$^{-/-}$ or *Hjv*$^{-/-}$ females produce, when challenged with LPS, more hepcidin than unchallenged wild-type mice. Thus, in females, treatments targeting BMP type I receptors, previously shown to attenuate induction of hepcidin gene expression by various inflammatory stimuli[23-25], will be more effective against anemia of inflammation than treatments that would target only hemojuvelin, as the latter would not prevent BMP6 to signal to hepcidin independently of HJV.

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Authorship Contributions. C.L. genotyped the mice. C.L. and O.G. killed the mice and sampled the different tissues. C.L. performed RT-PCR experiments and hepcidin ELISAs. C.B.F. did quantitative iron measurements and western-blots. O.G. performed Perls' Prussian blue staining of deparaffinized tissue sections. D.M. helped with data analysis and interpretation. M.P.R. and H.C. led and supervised the project through all stages, helped in data analyses and wrote the manuscript with suggestions and comments from all authors.

Disclosure of conflicts of interest. The authors have no conflict of interest to declare.

References

1. Canali S, Zumbrennen-Bullough KB, Core AB, et al. Endothelial cells produce bone morphogenetic protein 6 required for iron homeostasis in mice. *Blood*. 2017;129(4):405-414.
2. Parrow NL, Fleming RE. Liver sinusoidal endothelial cells as iron sensors. *Blood*. 2017;129(4):397-398.
3. Papanikolaou G, Samuels ME, Ludwig EH, et al. Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. *Nat Genet*. 2004;36(1):77-82.
4. Daher R, Kannengiesser C, Houamel D, et al. Heterozygous Mutations in BMP6 Pro-peptide Lead to Inappropriate Hepcidin Synthesis and Moderate Iron Overload in Humans. *Gastroenterology*. 2016;150(3):672-683.
5. Piubelli C, Castagna A, Marchi G, et al. Identification of new BMP6 pro-peptide mutations in patients with iron overload. *Am J Hematol*. 2017;92(6):562-568.
6. Andriopoulos B, Jr., Corradini E, Xia Y, et al. BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. *Nat Genet*. 2009;41(4):482-487.
7. Meynard D, Kautz L, Darnaud V, Canonne-Hergaux F, Coppin H, Roth MP. Lack of the bone morphogenetic protein BMP6 induces massive iron overload. *Nat Genet*. 2009;41(4):478-481.
8. Niederkofler V, Salie R, Arber S. Hemojuvelin is essential for dietary iron sensing, and its mutation leads to severe iron overload. *J Clin Invest*. 2005;115(8):2180-2186.
9. Huang FW, Pinkus JL, Pinkus GS, Fleming MD, Andrews NC. A mouse model of juvenile hemochromatosis. *J Clin Invest*. 2005;115(8):2187-2191.
10. Latour C, Kautz L, Besson-Fournier C, et al. Testosterone perturbs systemic iron balance through activation of epidermal growth factor receptor signaling in the liver and repression of hepcidin. *Hepatology*. 2014;59(2):683-694.
11. Latour C, Besson-Fournier C, Meynard D, et al. Differing impact of the deletion of hemochromatosis-associated molecules HFE and transferrin receptor-2 on the iron phenotype of mice lacking bone morphogenetic protein 6 or hemojuvelin. *Hepatology*. 2016;63(1):126-137.
12. Solloway MJ, Dudley AT, Bikoff EK, Lyons KM, Hogan BL, Robertson EJ. Mice lacking Bmp6 function. *Dev Genet*. 1998;22(4):321-339.
13. Besson-Fournier C, Gineste A, Latour C, et al. Hepcidin upregulation by inflammation is independent of Smad1/5/8 signaling by activin B. *Blood*. 2017;129(4):533-536.
14. Torrance JD, Bothwell, T.H. Tissue iron stores. In: Cook JD, ed. Iron Methods in Hematology. Vol. 1. New York: Churchill Livingstone; 1980:90-115.
15. Verga Falzacappa MV, Vujic Spasic M, Kessler R, Stolte J, Hentze MW, Muckenthaler MU. STAT3 mediates hepatic hepcidin expression and its inflammatory stimulation. *Blood*. 2007;109(1):353-358.
16. Besson-Fournier C, Latour C, Kautz L, et al. Induction of activin B by inflammatory stimuli up-regulates expression of the iron-regulatory peptide hepcidin through Smad1/5/8 signaling. *Blood*. 2012;120(2):431-439.
17. Canali S, Core AB, Zumbrennen-Bullough KB, et al. Activin B Induces Noncanonical SMAD1/5/8 Signaling via BMP Type I Receptors in Hepatocytes: Evidence for a Role in Hepcidin Induction by Inflammation in Male Mice. *Endocrinology*. 2016;157(3):1146-1162.
18. Koch PS, Olsavszky V, Ulbrich F, et al. Angiocrine Bmp2 signaling in murine liver controls normal iron homeostasis. *Blood*. 2017;129(4):415-419.
19. Canali S, Wang CY, Zumbrennen-Bullough KB, Bayer A, Babitt JL. Bmp2 controls iron homeostasis in mice independent of Bmp6. *Am J Hematol*. 2017.
20. Ehrlich M, Horbelt D, Marom B, Knaus P, Henis YI. Homomeric and heteromeric complexes among TGF-beta and BMP receptors and their roles in signaling. *Cell Signal*. 2011;23(9):1424-1432.
21. Nohe A, Hassel S, Ehrlich M, et al. The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. *J Biol Chem*. 2002;277(7):5330-5338.

22. Hartung A, Bitton-Worms K, Rechtman MM, et al. Different routes of bone morphogenic protein (BMP) receptor endocytosis influence BMP signaling. *Mol Cell Biol*. 2006;26(20):7791-7805.
23. Mayeur C, Kolodziej SA, Wang A, et al. Oral administration of a bone morphogenetic protein type I receptor inhibitor prevents the development of anemia of inflammation. *Haematologica*. 2015;100(2):e68-71.
24. Steinbicker AU, Sachidanandan C, Vonner AJ, et al. Inhibition of bone morphogenetic protein signaling attenuates anemia associated with inflammation. *Blood*. 2011;117(18):4915-4923.
25. Theurl I, Schroll A, Sonnweber T, et al. Pharmacologic inhibition of hepcidin expression reverses anemia of chronic inflammation in rats. *Blood*. 2011;118(18):4977-4984.

Figure legends

Fig. 1. Deletion of Bmp6 further represses hepcidin expression in *Hjv* knockout mice and worsens the iron overload phenotype of females. (A) Hepcidin (*Hamp*), **(C)** *Id1*, and **(D)** *Smad7* mRNA expression were measured by quantitative PCR in the liver of male (M) and female (F) littermates that had the wild-type (WT), *Bmp6*$^{-/-}$, *Hjv*$^{-/-}$ or double knockout genotype. **(B)** Serum hepcidin levels were measured by competitive ELISA on the same mice. Quantitative measurement of non-heme iron in the pancreas **(E)**, and the heart **(F)** was performed according to the method recommended by Torrance & Bothwell[14]. Non-transferrin-bound iron **(G)** was measured using the FeROS™ eLPI kit (Aferrix LTD). Box and whiskers plots are shown on the graphs. Data were obtained on 10 WT, 10 *Bmp6*$^{-/-}$, 10 *Hjv*$^{-/-}$, and 18 double knockout males, and on 11 WT, 10 *Bmp6*$^{-/-}$, 8 *Hjv*$^{-/-}$, and 11 double knockout females. Means of quantitative PCR ΔCt values, log-transformed serum hepcidin, tissue iron content, and NTBI arbitrary units in mice of the different genotypes were compared with two-way ANOVA followed by Sidak's multiple comparison tests of planned contrasts between pairs of means. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

Fig. 2. The lack of Bmp6 and/or Hjv does not prevent the induction of hepcidin by LPS but the level of hepcidin reached after stimulation is linked to baseline BMP/Smad signaling in unchallenged animals. (A) Hepcidin (*Hamp*) mRNA expression was measured by quantitative PCR before and after LPS stimulation in the liver of male (M) and female (F) littermates that had the wild-type (WT), *Bmp6*$^{-/-}$, *Hjv*$^{-/-}$ or double knockout genotype. **(B)** Serum hepcidin levels were measured by competitive ELISA on the same mice. Means ± SD are shown on the graphs. Data on LPS-challenged mice were obtained on 8 WT, 4 *Bmp6*$^{-/-}$, 9 *Hjv*$^{-/-}$, and 5 double knockout males, and on 6 WT, 4 *Bmp6*$^{-/-}$, 7 *Hjv*$^{-/-}$, and 4 double knockout females. Means of quantitative PCR ΔCt values and log-transformed serum hepcidin before and after LPS were compared with Student's t-tests. **(C)** Representative western blot of 7 independent experiments for phospho-Smad5, total Smad5, phospho-Stat3, and total Stat3 expression in the liver of WT (Bmp6+/Hjv+), *Bmp6*$^{-/-}$(Bmp6-/Hjv+), *Hjv*$^{-/-}$ (Bmp6+/Hjv-) and double knockout (Bmp6-/Hjv-) male littermates before (LPS-) and after (LPS+) LPS stimulation. Western blots were analyzed on a Chemidoc MP Imaging System (Bio-Rad). **(D)** Quantification was obtained with the Image Lab Software. Means of PSmad5/Smad5 ratios were compared with two-way ANOVA. Results of comparisons of unchallenged knockout with unchallenged wild-type mice as well as of LPS-challenged with unchallenged mice of the same genotype are shown above the bars. Results of comparisons between knockout mice are shown by connecting lines. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.