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Hemojuvelin and bone morphogenetic protein (BMP) signaling in iron homeostasis

Amanda B. Core, Susanna Canali and Jodie L. Babitt*

Division of Nephrology, Program in Membrane Biology, Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Program in Anemia

Signaling Research, Boston, MA, USA

Mutations in hemojuvelin (HJV) are the most common cause of the juvenile-onset form of the iron overload disorder hereditary hemochromatosis. The discovery that HJV functions as a co-receptor for the bone morphogenetic protein (BMP) family of signaling molecules helped to identify this signaling pathway as a central regulator of the key iron hormone hepcidin in the control of systemic iron homeostasis. This review highlights recent work uncovering the mechanism of action of HJV and the BMP-SMAD signaling pathway in regulating hepcidin expression in the liver, as well as additional studies investigating possible extra-hepatic functions of HJV. This review also explores the interaction between HJV, the BMP-SMAD signaling pathway and other regulators of hepcidin expression in systemic iron balance.

Keywords: hemojuvelin, bone morphogenetic protein, hepcidin, iron, hemochromatosis, repulsive guidance molecule

**Correspondence:**
Jodie L. Babitt, Massachusetts General Hospital, 185 Cambridge St., CPZN-8208, Boston, MA 02114, USA
e-mail: babitt.jodie@mgh.harvard.edu

**Edited by:**
Paolo Arosio, University of Brescia, Italy

**Reviewed by:**
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**JUVENILE HEMOCROMATOSIS IS CAUSED BY MUTATIONS IN THE GENES ENCODING HEPcidIN OR HEMOJUVELIN**

Juvenile Hemochromatosis (JH) is an autosomal recessive disorder caused by a failure to prevent excess iron entry into the bloodstream, and characterized by progressive tissue iron overload (Pietrangelo, 2010). Although iron’s redox properties are critical for its role in many fundamental biological processes from cellular respiration to oxygen transport, iron excess can lead to toxic free radical generation. If left untreated, JH patients develop multorgan dysfunction as a consequence of iron overload, including cirrhosis, cardiomyopathy, diabetes mellitus, and hypogonadotrophic hypogonadism, before the age of 30 (Pietrangelo, 2010).

The identification of hepcidin as a master regulator of systemic iron balance was a major advance in understanding the pathophysiology of JH (Ganz, 2013). A defensin-like peptide produced predominantly by hepatocytes, hepcidin controls iron entry into the bloodstream from dietary sources, recycled red blood cells, and body storage sites by inducing degradation of the iron exporter ferroportin (Ganz, 2013). Hepcidin expression is stimulated by iron and inflammation to limit iron availability, while hepcidin is inhibited by iron deficiency, anemia, and hypoxia to increase iron availability for erythropoiesis (Babitt and Lin, 2010; Ganz, 2013). Hepcidin deficiency is the common pathogenic mechanism underlying both adult and juvenile-onset hemochromatosis and contributes to the pathogenesis of iron loading anemias such as thalassemia, while its overproduction causes anemia of inflammation and iron refractory iron deficiency anemia (IRIDA) (Ganz, 2013). JH is caused by mutations in the gene encoding hepcidin itself (HAMP) or, more commonly, hemojuvelin (HJV, also known as HFE2 or RGMc) (Roetto et al., 2003; Papanikolaou et al., 2004).

**BMP-SMAD SIGNALING VIA HJV IS A CENTRAL REGULATOR OF HEPcidIN**

A breakthrough in understanding the mechanism of action of HJV in hepcidin regulation came when HJV was discovered to function as a co-receptor for the bone morphogenetic protein (BMP) signaling pathway (Babitt et al., 2006), analogous to its RGM family homologs (Babitt et al., 2005; Samad et al., 2005). Importantly, this BMP signaling function of HJV was demonstrated to be crucial for its role in regulating hepcidin expression (Babitt et al., 2006) (Figure 1).

BMPs belong to the Transforming Growth Factor-beta (TGF-β) superfamily of ligands (Shi and Massagué, 2003). In the canonical signaling pathway, BMP ligands bind to type I and type II serine threonine kinase receptors to induce phosphorylation of cytoplasmic SMAD1, SMAD5, and SMAD8 proteins. These SMAD proteins form a complex with SMAD4 and translocate to
Table 1 | Mutations of the HJV gene linked to JH.

<table>
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<tr>
<th>Residue mutation</th>
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<th>Type of mutation</th>
<th>Nucleotide change</th>
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the nucleus to regulate gene transcription. This signaling pathway is further regulated at multiple levels in order to generate a precise signal in a specific cellular context (Shi and Massagué, 2003).

HJV and other RGM family members function as BMP co-receptors that bind selectively to BMP ligands and receptors to enhance SMAD phosphorylation in response to BMP signals (Babitt et al., 2005, 2006; Samad et al., 2005). All RGMs
FIGURE 1 | Schematic diagram showing the central role of the BMP6-HJV-SMAD signaling pathway in hepcidin regulation and the proposed interaction with other hepcidin regulators. BMP6 binds to the BMP type I and type II receptors (BMPR) and the co-receptor HJV to increase phosphorylation of SMAD1, SMAD5, and SMAD8 proteins (SMAD1/5/8), which translocate to the nucleus to increase hepcidin transcription. Numerous other hepcidin regulators have been identified, many of which are proposed to intersect with the central BMP6/HJV/SMAD pathway at various levels as shown. Proposed iron-mediated hepcidin regulators are shown in yellow, inflammatory mediators in blue, iron deficiency mediators in purple, and anemia mediators in red. Abbreviations: TFR2, transferrin receptor 2; IL6, interleukin 6; sHJV, soluble hemojuvelin, TWSG1, twisted gastrulation 1, GDF15, growth and differentiation factor 15, TMPRSS6, transmembrane serine proteinase 6; EGF, epidermal growth factor; HGF, hepatocyte growth factor, mTOR, mammalian target of rapamycin.

share the ability to bind to the BMP2/BMP4 subfamily and enhance BMP2/BMP4 signaling (Babitt et al., 2005, 2006; Samad et al., 2005; Wu et al., 2012). Moreover, all RGMs utilize BMP type I receptors ALK2, ALK3, and ALK6, and allow preferential signaling through the BMP type II receptor ACTRIIA (Xia et al., 2007, 2008, 2010). However, HJV is unique from other RGMs in that it exhibits preferential ability to bind to the BMP5/BMP6/BMP7 subfamily compared with RGMA and RGMB (Wu et al., 2012).

The BMP-HJV-SMAD signaling pathway activates hepcidin transcription directly through specific BMP-responsive elements (BMP-REs) on the hepcidin promoter (Casanovas et al., 2009; Truksa et al., 2009a). A mutation in the proximal BMP-RE was associated with a more severe iron overload phenotype in a patient with classical HFE hemochromatosis, demonstrating its importance in hepcidin regulation in humans (Island et al., 2009). In mice, liver-specific disruption of Smad4, the BMP receptors type 1 Alk2 or Alk3, or the ligand BMP6 result in hepcidin deficiency and iron overload, supporting the important role of these specific BMP-SMAD pathway components, in conjunction with HJV, in hepcidin regulation in vivo (Wang et al., 2005; Andriopoulos et al., 2009; Meynard et al., 2009; Steinbicker et al., 2011a).

SOLUBLE HJV

In addition to the GPI-anchored membrane form of HJV, endogenous soluble HJV (sHJV) protein is detectable in human and rodent serum. (Lin et al., 2005; Zhang et al., 2007; Chen et al., 2013). Multiple mechanisms have been proposed for endogenous sHJV generation, including cleavage by the pro-protein convertase furin and the type II transmembrane serine protease TMPRSS6 (Kuninger et al., 2008; Lin et al., 2008; Silvestri et al., 2008a,b). Whereas membrane HJV is a co-receptor for the BMP signaling complex (Babitt et al., 2006), sHJV can antagonize BMP signaling, presumably by binding and sequestering BMP ligands from interacting with cell-surface BMP type I and type II receptors (Babitt et al., 2007) (Figure 1). Indeed, the relative binding affinity of HJV for various BMP ligands roughly correlated with the ability of sHJV to inhibit their biological activity (Babitt et al., 2007; Wu et al., 2012).

Although exogenous sHJV inhibits BMP-SMAD signaling, the source, amount, and physiologic role(s) of endogenously produced sHJV in vivo are not well-understood. There is some evidence suggesting that endogenous sHJV is increased by iron deficiency and reduced by iron loading (Lin et al., 2005; Zhang et al., 2007; Silvestri et al., 2008a; Brasse-Lagnel et al., 2010; Chen et al., 2013). Interestingly, the furin cleaved form of sHJV appears to be more potent to inhibit BMP signaling and hepcidin compared with the TMPRSS6-cleaved form (Maxson et al., 2010). Whether HJV cleavage mainly represents a mechanism to remove the activating effects of liver membrane HJV, or whether endogenous sHJV has a direct BMP-SMAD inhibiting effect remains uncertain.

EXTRA-HEPATIC FUNCTIONS OF HJV

In addition to the liver, HJV mRNA is also highly expressed in skeletal muscle and heart (Niederkofler et al., 2004; Papanikolaou et al., 2004), and has been detected in other...
Iron stimulates BMP-SMAD signaling to regulate hepcidin

Iron regulates the activity of the BMP6-SMAD pathway to modulate hepcidin expression. Both circulating and liver iron appear to stimulate this pathway through different mechanisms (Ramos et al., 2011; Corradini et al., 2011a). In mice, liver iron content is positively correlated with liver Bmp6 mRNA levels and overall activity of the Smad signaling pathway (Kautz et al., 2008; Corradini et al., 2011a). Moreover, hepcidin induction by iron is inhibited by a neutralizing BMP6 antibody (Corradini et al., 2011a). These data suggest that liver iron modulates BMP6-SMAD signaling and hepcidin expression at least in part by regulating expression of BMP6 mRNA (Figure 1). It appears that liver iron regulates BMP6 expression mainly in nonparenchymal cells (Enns et al., 2013), and that iron loading in specific liver cell types may be important for this regulation (Daba et al., 2013). However, the mechanism by which hepatic iron levels regulate BMP6 remains unknown. Notably, hepcidin is still increased to a lesser extent by chronic iron loading in Bmp6 and Hjv knockout mice, suggesting that these pathways do not completely account for hepcidin regulation by chronic iron loading (Ramos et al., 2011; Gkouvatsos et al., 2014).

Increases in circulating iron stimulate SMAD1/5/8 phosphorylation and hepcidin expression without affecting Bmp6 mRNA levels (Corradini et al., 2011a). How circulating iron activates SMAD1/5/8 phosphorylation is unknown, but may involve an interaction with other proteins that are mutated in adult-onset hereditary hemochromatosis (see section HFE and TFR2). Hepatic liver membrane protein expression itself does not appear to be regulated by iron (Krijt et al., 2012).

Iron administration and BMP6-SMAD signaling also up-regulate inhibitory SMAD7 and SMAD6, and Tmprss6 (see section Tmprss6), that can act as feedback inhibitors of BMP-SMAD signaling and hepcidin expression (Kautz et al., 2008; Mleczko-Sanecka et al., 2010; Meynard et al., 2011; Corradini et al., 2011a; Vujic Spasic et al., 2013). It has been hypothesized that these pathways may help prevent excessive hepcidin increases by iron to provide tight homeostatic control (Meynard et al., 2011; Corradini et al., 2011a).

Interaction of HJV and the BMP-SMAD signaling pathway with other hepcidin regulators

HFE and TFR2

Adult-onset hereditary hemochromatosis is a less severe iron-overload disorder that manifests later in life compared with JH, and is associated with mutations in HFE or TFR2 (encoding transferrin receptor 2) (Pietrangelo, 2010). Liver expression of HFE and TFR2 are clearly important for iron homeostasis regulation because mice with a hepatocyte-specific knockout of either gene have a similar iron-overload phenotype compared with global Hfe or Tfr2 knockout mice (Wallace et al., 2007; Vujic Sasic et al., 2008). Moreover, liver transplantation corrects much of the HFE hemochromatosis phenotype (Garuti et al., 2010; Bardou-Jacquet et al., 2014). Liver hepcidin expression is inappropriately low in mice and humans with HFE or TFR2 mutations, suggesting that both HFE and TFR2 positively regulate liver hepcidin expression (Ahmad et al., 2002; Fleming et al., 2002; Bridle et al., 2003; Muckenthaler et al., 2003; Kawabata et al., 2005; Nemeth et al., 2005; Piperno et al., 2007). HFE and TFR2 are also postulated to function in iron sensing by the liver. The current working model is that when iron-bound transferrin increases in circulation, it binds to transferrin receptor 1 (TFR1) and displaces HFE, which then signals by some mechanism to stimulate hepcidin expression, possibly through an interaction with TFR2 (Schmidt et al., 2008; Gao et al., 2009).

It has been proposed that HFE and TFR2 may form a “supercomplex” with HJV to stimulate hepcidin expression via the BMP-SMAD pathway. Studies supporting this model have demonstrated that liver BMP-SMAD signaling is impaired in mice and humans with HFE and/or TFR2 mutations, suggesting an interaction at some level between HFE, TFR2 and the BMP-SMAD pathway (Corradini et al., 2009, 2011b; Kautz et al., 2009; Wallace et al., 2009; Bolondi et al., 2010; Ryan et al., 2010). Recently, it was published in an overexpression tissue culture system using tagged proteins that HFE and TFR2 can form a complex with HJV (D’Alessio et al., 2012). However, it is not been shown whether these proteins endogenously interact in vivo. Moreover, the more severe iron overload phenotype of HJV mutations and combined HFE/TFR2 mutations compared with either HFE or TFR2 mutations alone suggest that the function of these proteins is not entirely overlapping (Pietrangelo et al., 2005; Wallace et al., 2009). Thus, while it appears that HFE and TFR2 interact at some level with the BMP-HJV-SMAD pathway to regulate liver hepcidin expression (Figure 1), the precise molecular mechanisms of how HFE and TFR2 contribute to hepcidin regulation remain an active area of investigation.

The inflammatory pathway

In addition to iron, inflammatory stimuli also induce hepcidin expression (Ganz, 2013). The most well-characterized pathway is through IL6 activating the Janus kinase JAK2 to phosphorylate STAT3, which then activates the hepcidin promoter directly via a
Although inflammation downregulates liver Hjv mRNA expression (Krijt et al., 2004; Niederkofler et al., 2005; Constanze et al., 2007), liver SMAD1/5/8 signaling is often activated in the context of inflammation (Theurl et al., 2011) and is essential for hepcidin regulation by inflammation. Indeed, blocking BMP signaling with a small molecule BMP type I receptor inhibitor or a shHjv recombinant protein inhibits IL6-induced hepcidin expression in cell culture (Babitt et al., 2007; Yu et al., 2008). Moreover, mice with a hepatocyte-specific knockout of Smad4 exhibit blunted hepcidin response to IL6 treatment (Wang et al., 2005). Importantly, BMP pathway inhibitors lower hepcidin, increase iron availability for erythropoiesis, and ameliorate anemia in animal models of anemia of inflammation (Theurl et al., 2011; Steinbicker et al., 2011b; Sun et al., 2013).

At least two mechanisms are proposed to account for the crosstalk between the BMP-SMAD and IL6-STAT3 pathways in hepcidin regulation. First, there may be an interaction at the level of the hepcidin promoter, where the proximal BMP-RE and the STAT3 binding site are in close proximity (Figure 1). In support of this hypothesis, mutation of the proximal BMP-RE impairs hepcidin promoter activation not only by BMPs, but also by IL6 (Casanovas et al., 2009). Second, inflammation induces hepatic expression of another TGF-β superfamily member, Activin B, which can stimulate hepcidin expression by activating SMAD1/5/8 signaling in hepatoma-derived cell cultures (Besson-Fournier et al., 2012) (Figure 1). Whether Activin B contributes to hepcidin regulation by inflammation in vivo remains to be determined.

**TMPRSS6**

The serine protease TMPRSS6 has been implicated in hepcidin inhibition by iron deficiency. Mutations in TMPRSS6 are linked to IRIDA associated with inappropriately high hepcidin levels (Du et al., 2008; Finberg et al., 2008; Folgueras et al., 2008). Moreover, genome-wide association studies have linked common single nucleotide polymorphisms in TMPRSS6 to iron status and hemoglobin level, supporting an important role for TMPRSS6 in regulating systemic iron homeostasis and normal erythropoiesis (Benyamin et al., 2009; Chambers et al., 2009; Tanaka et al., 2010). TMPRSS6 is proposed to regulate hepcidin expression through an interaction with BMP and the BMP-SMAD pathway in the liver. Specifically, when both proteins are overexpressed in cell culture, TMPRSS6 binds and cleaves HJV to generate shJv, thereby inhibiting BMP-SMAD signaling (Silvestri et al., 2008b) (Figure 1). In mouse models, the combined deficiency of Hjf or Bmp6 and Tmprss6 causes iron overload, suggesting that there is a genetic interaction between Tmprss6 and the Bmp6-HJV-SMAD pathway (Truksa et al., 2009b; Finberg et al., 2010; Lenoir et al., 2011). Interestingly, liver membrane expression of Hjf is decreased (Krijt et al., 2011), and serum shJv levels are unchanged (Chen et al., 2013), in Tmprss6 knockout mice compared with wild-type mice, which seem contrary to the proposed hypothesis that Tmprss6 acts to cleave HJV from the liver membrane surface. Future work is needed to fully understand the mechanism of action of Tmprss6 in hepcidin regulation and iron homeostasis in vivo.

**NEOGENIN**

In addition to Tmprss6, the deleted in colorectal cancer (DCC) family member neogenin is also proposed to function as an HJV interacting protein that modifies BMP-SMAD signaling and iron homeostasis (Figure 1). In particular, neogenin binds to HJV, like other RGM family members (Matsunaga et al., 2004; Zhang et al., 2005; Conrad et al., 2010). Moreover, neogenin mutant mice exhibit reduced hepcidin levels and iron overload consistent with a role for neogenin in regulating hepcidin and systemic iron balance in vivo (Lee et al., 2010). However, the mechanism of action of neogenin in hepatic and iron homeostasis regulation is still not fully understood. In some studies, neogenin increased HJV cleavage (Enns et al., 2012), while in other studies, neogenin reduced HJV secretion (Lee et al., 2010). Moreover, neogenin was variably shown to inhibit (Hagihara et al., 2011), have no effect (Xia et al., 2008), or stimulate BMP signaling (Lee et al., 2010). Whether neogenin and HJV interact in a cell autonomous or cell non-autonomous manner in vivo remains unclear, and how this interaction occurs may be important for downstream functional effects.

**OTHER PATHWAYS**

Hepcidin suppression by erythropoietic drive appears to be mediated by secreted factor(s) released by proliferating red blood cell precursors in the bone marrow (Pak et al., 2006; Vokurka et al., 2006). Two proposed erythroid hepcidin regulators are the TGF-β/BMP superfamily modulators growth and differentiation factor 15 (GDF15) and twisted gastrulation 1 (TWSG1), at least in the context of ineffective erythropoiesis in iron loading anemias (Tanno et al., 2007, 2009) (Figure 1). The role of GDF15 and TWSG1 in hepcidin suppression by erythropoietic drive in other contexts has been questioned (Ashby et al., 2010; Casanovas et al., 2013). Recently, erythropherrone has been proposed as a novel erythroid regulator (Kautz et al., 2013), but its mechanism of action is not yet reported.

A number of other hormones, growth factors and signaling pathways have recently been implicated in hepcidin regulation including testosterone, estrogen, hepatocyte growth factor (HGF), epidermal growth factor (EGF), endoplasmic reticulum stress, gluconeogenic signals and the Ras/RAF and mTOR signaling pathways (Oliveira et al., 2009; Vecchi et al., 2009, 2014; Goodnough et al., 2012; Hou et al., 2012; Yang et al., 2012; Guo et al., 2013; Latour et al., 2014; Mleczko-Sanecka et al., 2014). Notably, the majority of these pathways appear to regulate hepcidin through an intersection with the BMP-SMAD pathway at some level (Goodnough et al., 2012; Guo et al., 2013; Latour et al., 2014; Mleczko-Sanecka et al., 2014) (Figure 1).

**CONCLUSION**

Understanding the genetic basis for JH has yielded important insights into the molecular mechanisms of systemic iron homeostasis. Hepcidin and its receptor ferroportin are key regulators of body iron balance, and the BMP-SMAD pathway via the co-receptor HJV is a central regulator of hepcidin production.
Figure 1. Knowledge of these pathways has already led to the development of novel therapeutic strategies that target the molecular mechanisms underlying iron homeostasis disorders, with several new treatments currently being evaluated in human clinical trials (Fung and Nemeth, 2013). Future work will be needed to fully understand the mechanisms by which iron levels are sensed by the liver and integrated with other pathways to regulate BMP-SMAD signaling, hepcidin expression, and systemic iron homeostasis.

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