The Role of Hepatocyte Hemojuvelin in the Regulation of Bone Morphogenic Protein-6 and Hepcidin Expression in Vivo*

Received for publication, January 31, 2010, and in revised form, March 14, 2010 Published, JBC Papers in Press, April 2, 2010, DOI 10.1074/jbc.M110.109488

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Both hemojuvelin (HJV) and bone morphogenic protein-6 (BMP6) are essential for hepatic expression. Hepcidin is the key peptide hormone in iron homeostasis, and is secreted predominantly by hepatocytes. HJV expression is detected in hepatocytes, as well as in skeletal and heart muscle. HJV binds BMP6 and increases hepatic expression presumably by acting as a BMP co-receptor. We characterized the role of hepatocyte HJV in the regulation of BMP6 and hepatic expression. In HJV-null (Hjv−/−) mice that have severe iron overload and marked suppression of hepatic expression, we detected 4-fold higher hepatic BMP6 mRNA than in wild-type counterparts. These results indicate that Hjv−/− mice do not lack BMP6. Furthermore, iron depletion in Hjv−/− mice decreased hepatic BMP6 mRNA. Expression of HJV in hepatocytes of Hjv−/− mice using an AAV2/8 vector, increased hepatic hepcidin mRNA by 65-fold (Hjv/H11546), demonstrating that normal levels of hepatic BMP6 expression is correlated with a decrease in hepatic iron-loading. Together, our data indicate that the regulation of hepatic BMP6 expression by iron is independent of HJV. Moreover, iron depletion in Hjv−/− mice decreased hepatic BMP6 mRNA.

Hepatic hepcidin expression is mediated via the BMP signaling cascade. BMP signaling is initiated upon the binding of BMP ligands to BMP receptor complexes on the cell surface. This binding triggers the phosphorylation of Smad1, Smad5, and Smad8 (Smad1/5/8) in the cytoplasm. The phosphorylated Smads (pSmad1/5/8) form heteromeric complexes with Smad4 and then translocate to the nucleus where they induce the transcription of target genes. Liver-specific disruption of Smad4 in mice markedly decreases hepcidin expression and causes iron accumulation in particular organs.

Hepatocytes are the only known cell type in the body where both HJV and hepcidin are co-expressed (3, 10, 12, 16). In hematoma cell lines, hepcidin expression can be induced robustly by BMP2, BMP4, and BMP6 independently of HJV (10, 17, 18). Recent studies show that BMP6, rather than other BMP ligands, plays an essential role in iron homeostasis. BMP6 mRNA in the liver and the small intestine is positively regulated by bodily iron-loading (5, 19). Disruption of both BMP6 alleles in mice markedly suppresses hepatic hepcidin expression and causes severe iron overload (6, 7). These phenotypes resemble those reported in Hjv−/− mice (12, 13). Therefore, BMP6 and HJV are both important in the induction of hepcidin expression in vivo.

HJV binds to BMP2, 4, 5, and 6 (7, 17, 20). Studies in hematoma cell lines indicate that HJV acts as a BMP co-receptor to enhance BMP signaling and hepcidin expression (17, 18). Recent studies suggest that HJV increases hepcidin expression by preferentially binding to BMP6 (7). In Hjv−/− mice, suppressed hepcidin expression is correlated with a decrease in phosphorylated Smad1/5/8 in the liver (18). Therefore, HJV induces hepcidin expression through the activation of BMP signaling. However, whether the induction of hepcidin expression by HJV is accomplished via the regulation of BMP6 expression or by acting as a BMP co-receptor in vivo, is not known.

Iron is an indispensable element for life. Iron homeostasis is controlled elegantly by hepcidin (1, 2). Hepcidin is a peptide hormone that is secreted predominantly by the hepatocytes in the liver. Under physiological conditions, its expression is regulated positively by bodily iron-loading (3, 4). Recent studies demonstrate that normal levels of hepatic hepcidin expression require the presence of both hemojuvelin (HJV) and bone morphogenic protein-6 (BMP6) (5–7).

HJV is a GPI-linked membrane protein that is encoded by the gene, Hfe2, in humans (8, 9). Repulsive guidance molecule c (RGMc) is the ortholog of HJV in mice, and it is encoded by the gene, Hfe2. For simplicity, we will use Hjv for the gene and HJV for the protein in human and mice in this report. Hjv is expressed highly in skeletal and heart muscle, and at a relatively low level in hepatocytes (9, 10). Homozygous or compound heterozygous mutations of HJV in humans and disruption of both Hjv alleles (Hjv−/−) in mice, markedly reduce hepatic hepcidin expression and cause severe iron overload (9, 11–13). Thus, HJV is a critical upstream regulator of hepcidin transcription. However, the precise role of HJV expression in different tissues in the regulation of hepcidin expression in vivo has not been addressed.

Hepatic hepcidin expression is mediated via the BMP signaling cascade (14). BMP signaling is initiated upon the binding of BMP ligands to BMP receptor complexes on the cell surface. This binding triggers the phosphorylation of Smad1, Smad5, and Smad8 (Smad1/5/8) in the cytoplasm. The phosphorylated Smads (pSmad1/5/8) form heteromeric complexes with Smad4 and then translocate to the nucleus where they induce the transcription of target genes (15). Liver-specific disruption of Smad4 in mice markedly decreases hepcidin expression and causes iron accumulation in particular organs (14).

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* This work was supported, in whole or in part, by National Institutes of Health Grant DK080765 (to A.-S. Z.).
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† The abbreviations used are: HJV, hemojuvelin; BMP, bone morphogenic protein; PBS, phosphate-buffered saline; IL, interleukin; WT, wild type; AAV, adeno-associated virus; qRT-PCR, quantitative RT-PCR; ANOVA, analysis of variance.
In this study, we characterized the role of hepatocyte HJV in the regulation of BMP6 and hepcidin expression using Hjv−/− mice as a model. Our results showed that the regulation of hepatic BMP6 expression by iron is independent of Hjv expression and that expression of Hjv in hepatocytes plays an essential role in hepcidin expression by potentiating the BMP6-mediated signaling.

**EXPERIMENTAL PROCEDURES**

**Preparation of Adeno-associated Virus (AAV) 2/8-Hjv Vector**—Full-length mouse Hjv open reading frame was amplified from a mouse liver cDNA library by PCR using the Expand High Fidelity PCR System (Roche Applied Science, Indianapolis, IN). The primers used for amplification are 5′-atgggccagtcccctagtcccg-gtccc-3′ (forward) and 5′-ctggcctacttgaaagaacgacgacaccaga-3′ (reverse). Gel-purified amplicons were inserted into the pGEM-T vector (Promega, Madison, WI). After verification by sequencing, Hjv cDNA was subcloned into an AAV2/8 vector containing a strong liver-specific promoter (LSP). The LSP is a combination of two copies of a human α1-microglobulin/bikunin enhancer and the promoter from the human thyroid hormone-binding globulin gene (21–24). Vectors were generated at the Gene Therapy Center Virus Vector Core Facility, University of North Carolina at Chapel Hill by co-transfecting 293 cells with AAV2/8-Hjv vector, the AAV packaging plasmid and pAdHelper. The titer of AAV2/8-Hjv vector was ~4 × 10^{12}. The viral vector stocks were handled according to Biohazard Safety Level 2 guidelines published by the National Institutes of Health.

**Animal Analysis—Hjv−/−** mice on 129/SvEvTac (129/S) background were obtained from Dr. Nancy Andrews (Duke University). Both Hjv−/− and wild-type 129/S mice were bred and maintained in the Department of Comparative Medicine of Oregon Health & Science University. For vector treatment, 8-week-old male mice were injected with ~5 × 10^{11} vector particles per mouse via the tail vein. Two weeks later, mice were anesthetized by intraperitoneal injection of mouse mixture (ketamine 7.5 mg, xylazine 1.5 mg, and acepromazine 0.25 mg per ml). Blood was collected by cardiac puncture, and serum was subsequently collected from clotted samples after centrifugation for 10 min at 10,000 × g at 4°C. Liver, skeletal muscle (gastrocnemius), spleen and small intestine were rapidly removed, snap-frozen in liquid nitrogen and then stored at −80°C for qRT-PCR, Western blot and tissue nonheme iron assays. Each group consisted of 5 animals.

Bodily iron stores in Hjv−/− mice were depleted by feeding them a low iron diet. Five-week-old male animals were randomly assigned to two different categories with free access to a control diet (200 ppm iron, TestDiet, Richmond, IN; control group) or were pair-fed an iron-deficient diet (less than 1 ppm iron, TestDiet, Richmond, IN; low iron group). After 25 days, animals were euthanized to collect blood, liver, spleen, and small intestine tissues as described above for virus-injected mice. Each group consisted of 5 animals. All procedures for animal use met the requirements of the OHSU DCM.

**RT-PCR and Quantitative RT-PCR—**Total RNA from liver, skeletal muscle and small intestine was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). Contaminating genomic DNA was removed by DNase treatment, followed by another cycle of RNA purification using the RNeasy kit (Qiagen, Valencia, CA). cDNA preparation and qRT-PCR analysis of Hjv, hepcidin, BMP6, IL-6, and β-actin transcripts were conducted as previously described (25). The mouse primers used for qRT-PCR are listed in Table 1. All primers were verified for linearity of amplification. The results are expressed as the amount relative to that of β-actin for each sample.

The levels of Hjv transcripts in liver, skeletal muscle, and small intestine were also analyzed by semi-quantitative RT-PCR using the primers listed in Table 1. AAV2/8-Hjv vector DNA was used as positive controls for Hjv. Water was used as a negative control. After 35 cycles of amplification, the amplicons were separated on a 2% agarose gel. Images were taken under UV light after staining with ethidium bromide.

**Immunodetection—**Mouse liver tissue extracts were prepared using NET-Triton (150 mM NaCl, 5 mM EDTA, 10 mM Tris (pH 7.4), and 1% Triton X-100) supplemented with 1X protease inhibitor mixture (Roche), 1 mM sodium fluoride (Sigma) and 1 mM sodium orthovanadate (Sigma). Liver extracts (250 μg of protein per lane) were separated using 11% SDS-PAGE under reducing conditions, followed by transfer to a nitrocellulose membrane. Membranes were probed with rabbit anti-phosphorylated Smad1/5/8 (pSmad, 1:1000; Cell Signaling Technology) or rabbit anti-Smad1/5/8 (Smad, 1:500; Santa Cruz Biotechnology). Bands were detected either by horseradish peroxidase-coupled secondary antibody and enhanced chemiluminescence (SuperSignal WestPico; Pierce) or by fluorescently labeled secondary antibodies as described previously for quantification (26). For the latter, the intensity of each band was recorded as arbitrary units. The amounts of pSmad are expressed relative to total Smad for each sample.

**Nonheme Iron Assay—**Nonheme iron concentrations in the liver and spleen tissues were determined as previously described (13) with the following modifications. Briefly, 50–150 mg of wet liver tissues were digested in 250–750 μl of acid buffer (27) at 65°C for 72 h. The supernatant was collected by centrifugation at 10,000 × g for 5 min, followed by the addition of chromogen (1.86 mM bathophenanthroline sulfonate, 143 mM thioglycolic acid in water) and OD measurement at 535 nm. Each sample was measured twice in triplicate. Iron concen-

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**Table 1**

| Mouse gene | Forward primer | Reverse primer |
|------------|----------------|----------------|
| β-Actin    | 5′-GAAGCAGGAGCTCAGCTCACTATTG-3′ | 5′-CCACAGGAAGCTCACCATACCAAGA-3′ |
| Hjv        | 5′-ATCCTCACATTGTGCAAGTTTT-3′ | 5′-GTCCTCTGAGCCTATGTTTTCTC-3′ |
| Hepcidin   | 5′-GACTGAGCACACACCTTTCTC-3′ | 5′-CCCAAGTGTAGTCTGTCTGTA-3′ |
| BMP6       | 5′-TGTTCTCTTCTTTTTG-3′ | 5′-TTGGGATGATGATACCTTGTGA-3′ |
| IL-6       | 5′-TTCCATTCAGTGGCTTCTTG-3′ | 5′-TTGGGATGATGATACCTTGTGA-3′ |

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Hepatocyte HJV, BMP6, and Hepcidin Expression

This table lists the primers used for RT-PCR and qRT-PCR.
Hepatocyte HJV, BMP6, and Hepcidin Expression

Expression is expressed as micrograms of iron per gram of wet tissue.

Serum Iron Assay—Serum iron concentrations were measured using a serum iron/TIBC Reagent Set (Teco Diagnostics, Anaheim, CA) according to the manufacturer's instructions. Each sample was measured in triplicate. Serum iron concentrations are expressed as micrograms of iron per deciliter of serum.

Statistical Analysis—The standard deviation (S.D.) and the paired two-tailed Student’s t test were used to compare two sets of data. One-way ANOVA and Tukey’s post test were used to compare three or more sets of data.

RESULTS

Hjv−/− Mice Have Increased Hepatic BMP6 mRNA Expression—We sought to explore the role of hepatocyte HJV in the regulation of BMP6 and hepcidin expression. To determine whether HJV is required for BMP6 expression, we first examined the levels of BMP6 mRNA in 10-week-old male Hjv-null (Hjv−/−) mice compared with the age- and gender-matched wild-type control animals. We observed that BMP6 mRNA levels in the liver were about 4-fold higher in Hjv−/− mice than in wild-type mice (Fig. 1A). No significant difference in the BMP6 mRNA was found in the small intestine between the two groups (Fig. 1B). Consistent with the previous reports (12, 13), a markedly reduced hepcidin expression (Fig. 1C), and severe iron overload in the liver (data not shown) were detected in Hjv−/− mice. Hepatic hepcidin mRNA was ∼28-fold lower, and the liver nonheme iron was about 14-fold higher than in the wild-type mice. The liver is the major storage site of excess iron in the body (28), and nonheme iron levels in the liver are widely used as a direct indicator of bodily iron-loading. Our observations indicate that the reduced hepatic hepcidin expression detected in Hjv−/− mice is not caused by the lack of BMP6 expression.

To gain further insight into the role of HJV in the regulation of BMP6 expression, we next examined whether reduction in iron-loading in Hjv−/− mice was able to suppress BMP6 expression.

Depletion of Bodily Iron-loading in Hjv−/− Mice Decreases Hepatic BMP6 mRNA Expression as Well as Hepcidin Expression—5-week-old male Hjv−/− mice were fed an iron-deficient rodent diet (less than 1 ppm iron; low-iron group) for 25 days to deplete bodily iron stores. Gender and age-matched Hjv−/− mice pair-fed a control diet (200 ppm iron; control group) served as controls. The extent of iron depletion was assessed by analysis of liver nonheme iron. Results revealed a ∼62% decrease in liver nonheme iron in the low iron group compared with the control group (Fig. 2A). Interestingly, no significant change in serum iron levels was detected (data not shown). This might be due to the fact that the liver nonheme iron level in the low iron group was still about 7-fold greater than in the wild-type mice (data not shown). Because liver iron stores can be mobilized into the circulation, the depletion of the bodily iron load in the low iron group is limited to the decrease in stored iron.

We next measured the levels of BMP6 mRNA in both the liver and small intestine by qRT-PCR, to determine whether the expression of BMP6 could be regulated by the decrease of stored iron in Hjv−/− mice. In the liver, there was approximately a 52% decrease in BMP6 mRNA levels in the low iron group compared with controls (Fig. 2B). The decrease is statistically significant. However, no change was detected in the small intestine. These results indicate that in the absence of HJV, BMP6 mRNA expression in the liver is suppressed by a decrease in stored iron.

To further examine whether the decrease in hepatic BMP6 expression could alter hepcidin expression in Hjv−/− mice, we compared the levels of hepcidin mRNA in the liver between the control and low-iron groups. Surprisingly, hepcidin levels were ∼84-fold lower in the low-iron group than in the control groups (Fig. 2C). These data indicate that a mild decrease in hepatic BMP6 expression could result in a marked reduction of hepcidin expression in Hjv−/− mice. Whereas these observations indirectly suggest that hepatic BMP6 can induce hepcidin
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expression independently of HJV, it is important to note that the absolute levels of hepcidin mRNA expression in Hjv−/− mice are considerably lower than in wild-type animals.

Together, our results indicate that the reduction of hepcidin expression in Hjv−/− mice does not result from a lack of BMP6 expression and that the regulation of hepatic BMP6 expression by iron is independent of HJV. Because hepatocytes are the only

known site in which HJV and hepcidin are co-expressed (3, 10, 12, 16), we next examined the role of hepatocyte HJV in hepcidin expression.

AAV2/8 Vector Specifically Introduced Hjv into Hepatocytes—To determine the role of hepatocyte HJV in hepcidin expression, we used AAV2/8 vector that has a strong liver-specific enhancer/promoter (21–24), as a tool to specifically introduce exogenous mouse Hjv cDNA into the Hjv−/− murine hepatocytes. Previous studies demonstrate that this vector is able to specifically target the gene of interest into the hepatocytes and that the expression of the introduced gene is evenly distributed in hepatocytes throughout the liver (22, 29).

We injected ~5 × 10^11 particles of AAV2/8-Hjv vector into 8-week-old male wild type or Hjv−/− mice via the tail vein. Control animals were administered with equal volume of PBS. Mice were sacrificed for analysis at 2 weeks after the injection. The expression profiles of introduced Hjv mRNA in liver, skeletal muscle, and small intestine were analyzed by RT-PCR. The primers used for the analysis were designed to amplify the sequence deleted in Hjv−/− mice. Hjv mRNA transcripts were only detectable in the livers of Hjv−/− mice that were injected with AAV2/8-Hjv vector (Fig. 3A). No signal was found in either skeletal muscle or small intestine in these mice (Fig. 3A). These results are consistent with previous reports (22, 29, 30), showing the specificity of AAV2/8 vector to introduce the gene of interest into hepatocytes. Together, our data suggest that AAV2/8 vector targets Hjv into the hepatocytes for expression.

To determine the relative abundance of introduced Hjv mRNA in the liver of Hjv−/− mice, we conducted qRT-PCR analysis. The liver Hjv mRNA levels in Hjv−/− mice injected with AAV2/8-Hjv vector was ~15% of the levels seen in the wild type mice (Fig. 3B, left panel; Hjv/Hjv−/− versus −/WT). A similar increase in Hjv mRNA was also detected in the wild type animals that were administered with AAV2/8-Hjv vector (Fig. 3B, left panel; Hjv/WT versus −/WT). No significant increase of Hjv mRNA was detected in either skeletal muscle or small intestine from the Hjv−/− mice that were injected the AAV2/8-Hjv vector (Fig. 3B, middle and right panels). This is in agreement with the RT–PCR analysis (Fig. 3A). Intriguingly, results revealed a low but detectable level of Hjv mRNA in the small intestine of the wild-type mice by both RT-PCR and qRT-PCR analyses (Fig. 3, A and B). Hjv mRNA was undetectable in the small intestine of Hjv−/− mice (Fig. 3, A and B). Together, the results show that AAV2/8 vector is able to introduce Hjv into hepatocytes at a relatively low level. We next evaluated the effect of the introduced exogenous Hjv on hepcidin expression in hepatocytes.

Specific Expression of Hjv in Hepatocytes Robustly Induces Hepatic Hpcidin Expression and Decreases Bodily Iron-loading—The role of hepatocyte HJV in hepatic hepcidin expression was examined by analysis of hepcidin mRNA levels in the liver by qRT-PCR. We detected ~65-fold higher hepcidin mRNA in Hjv−/− mice injected with AAV2/8-Hjv vector than in Hjv−/− mice injected with PBS (Fig. 4A). Surprisingly, the hepcidin mRNA levels in the Hjv−/− mice injected with AAV2/8-Hjv vector were about 2.5-fold higher than those detected in wild-type animals injected with PBS (Fig. 4A). These results indicate that hepatocyte HJV is a robust inducer of hepcidin expression.

FIGURE 2. Depletion of bodily iron-loading decreases hepatic BMP6 mRNA in Hjv−/− mice. 5-week-old male Hjv−/− mice were pair-fed either a control diet (200 ppm Fe) or an iron-deficient diet (less than 1 ppm Fe) for 25 days. There are 5 animals per group. A, nonheme iron in the liver and spleen. Nonheme iron concentrations in the liver and spleen tissues were measured after digestion with acid buffer. Each sample was measured twice in triplicate. Iron concentrations are expressed as microgram per gram wet tissue. B, BMP6 mRNA. BMP6 mRNA in both the liver and small intestine (intestine) was analyzed by qRT-PCR. Results are expressed as the amount relative to the corresponding β-actin mRNA in each tissue. C, hepcidin mRNA. Hepcidin mRNA in the liver was analyzed by qRT-PCR. Results are expressed as the amount relative to the corresponding β-actin mRNA in each tissue. The mean values and the S.D. for each group are presented. The paired two-tailed Student’s t test was used to evaluate the statistical significance of the results between Hjv−/− mice fed a control (ctrl) or an iron-deficient diet (ID). **, p < 0.01; ***, p < 0.001.

ctrl, control group. ID, iron-depleted group.
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A. positive blank wild type mice Hjv-/- mice

| AAV  | Hjv | - | - | Hjv | - | - | Hjv |
|------|-----|---|---|-----|---|---|-----|
| liver | - | - | Hjv | - | - | Hjv |

![Graph](image_url)

In contrast to the Hjv-/- mice, introduction of a similar amount of excess exogenous Hjv mRNA into the hepatocytes of wild-type animals revealed no significant change in hepcidin mRNA (Figs. 3B and 4A). These data suggest that under physiological conditions, the induction of hepcidin expression is modulated by other factors.

We next characterized iron homeostasis in Hjv-/- mice injected with the AAV2/8-Hjv vector. Because hepatocyte blocks iron absorption from the small intestine and iron efflux from macrophages into circulation, we reasoned that the increased hepcidin expression in Hjv-/- mice injected with AAV2/8-Hjv vector would result in a decrease in serum iron and liver iron concentrations, as well as an increase in iron accumulation in the spleen. As predicted, we detected about 1.7-fold decrease of serum iron in Hjv-/- mice injected with AAV2/8-Hjv vector in comparison with Hjv-/- mice injected with PBS. Serum iron levels were comparable with those in the wild-type mice injected with PBS (Fig. 4B). Liver nonheme iron concentrations displayed a statistically significant decrease of about 15% (Fig. 4C). As mammals do not possess an efficient machinery to excrete excess iron from the body, this mild decrease of liver nonheme iron might represent the amount of iron that was blocked from absorption in the small intestine. Detection of nonheme iron in the spleen revealed a 7-fold increase in Hjv-/- mice injected with AAV2/8-Hjv vector relative to Hjv-/- mice injected with PBS (Fig. 4D). This is in agreement with the spleen being enriched with macrophages and that hepcidin reduces iron export from splenic macrophages (28). These changes are consistent with the profound increase of hepatic hepcidin expression in these animals. Because the induction of hepatic hepcidin expression is mediated via the activation of the BMP signaling pathway under physiological conditions (14, 18), we next examined whether the induced hepcidin expression by hepatocyte HJV occurred through increasing BMP signaling.

Expression of HJV in Hepatocytes in the Hjv-/- Mice Increases BMP Signaling—We used phosphorylated Smad1/5/8 (pSmad) in the liver extracts as an indicator for BMP signaling in hepatocytes, because pSmad levels directly reflect the status of BMP signaling (15). Immunoblot analysis showed a significantly higher pSmad level in Hjv-/- mice injected with AAV2/8-
is positively regulated by iron (5, 19) and that knock-out of the BMP6 gene in mice suppresses hepcidin expression and causes severe iron overload (6, 7). HJV preferentially binds BMP6 to induce hepcidin expression (7). We reasoned that the induction of hepcidin expression by hepatocyte HJV could be mediated either by increasing BMP6 expression or by acting as a BMP co-receptor that is independent of BMP6 expression. To test these hypotheses, BMP6 mRNA levels in both the liver and small intestine were analyzed by qRT-PCR. Interestingly, no significant change in BMP6 mRNA levels was detected in either tissue when exogenous HJV was introduced into either Hjv−/− mice or wild-type mice when compared with their corresponding controls (Fig. 6, A and B). These results suggest that increased hepcidin expression by hepatocyte HJV is not caused by the induction of de novo BMP6 expression. Rather, our data favor the idea that HJV induces hepcidin expression by acting as a BMP co-receptor.

Because hepatic hepcidin expression could also be induced by the inflammatory factor IL-6 (1), we also measured the levels of IL-6 mRNA in the liver by qRT-PCR to determine whether inflammation from viral infection could be a contributing factor to the observed increase in hepcidin expression. No change in IL-6 mRNA was detected in either the wild-type or the Hjv−/− mice that were injected with AAV2/8-Hjv vector (Fig. 6C). These observations, along with previous findings that AAV2/8 vector does not cause inflammation in mice and that administration of control vector does not change hepcidin expression (30), ruled out inflammation as a factor. Together, our data support the model that hepatocyte HJV induces hepcidin expression through potentiating BMP6-mediated signaling, rather than by stimulating BMP6 expression.  

**Hepatic BMP6 mRNA Levels Are Positively Correlated with Liver Nonheme Iron Levels**—Previous studies show that hepatic BMP6 mRNA is positively correlated with bodily iron-loading (5), but whether it is regulated by serum iron or by the stored iron in the liver is not clear. We compared results from three sets of experiments that are presented above. First, Hjv−/− mice had about 4-fold higher hepatic BMP6 mRNA than wild-type mice (Fig. 1A). Hjv−/− mice exhibited both high serum iron and significant increase of liver iron-loading (Fig. 4, B and C). These results suggest that hepatic BMP6 expression could be regulated by either serum iron, by liver iron, or by both. Second,
depletion of liver stored iron, but not serum iron, in Hjv<sup>−/−</sup> mice decreased hepatic BMP6 mRNA (Fig. 2, A and B, data not shown). This observation implies that BMP6 expression is associated with liver iron-loading, not serum iron concentrations. Third, in Hjv<sup>−/−</sup> mice that were injected with AAV2/8-Hjv vector, the marked decline of serum iron (Fig. 4B) and slight decrease in liver iron (Fig. 4C) did not alter the high levels of hepatic BMP6 mRNA (Fig. 6A). This also indicates that hepatic BMP6 expression is determined by the liver iron level. Together, our data suggest that the hepatic BMP6 mRNA level is positively correlated with the degree of liver iron-loading, not the concentration of serum iron.

**DISCUSSION**

In this study we showed that the regulation of BMP6 expression by iron is independent of Hjv expression. Rather, BMP6 mRNA levels positively correlate with hepatic iron-loading in mice. Our data support the idea that HJV potentiates the BMP6-mediated signaling to induce hepcidin expression. Hepatocyte-specific expression of Hjv is sufficient to activate BMP signaling.

The finding that the level of hepatic BMP6 mRNA increases with iron-loading is consistent with other studies (5). In contrast to an earlier report that BMP6 levels in the small intestine increase with iron-loading (19), intestinal BMP6 mRNA levels were not detectably different between wild-type and Hjv<sup>−/−</sup> mice. The basis of the difference is not known. Our results showing that BMP6 and hepcidin mRNA are lowered by a low iron diet independently of Hjv expression, complement a recent study demonstrating that bleeding Hjv<sup>−/−</sup> mice repeatedly to reduce iron levels resulted in lower hepcidin levels (31). In addition, the degree of liver iron overload in Hjv<sup>−/−</sup> mice versus their wild-type counterparts is comparable with those in B6 and D2 mice fed high iron versus iron-deficient diet, and the extent to which BMP6 mRNA increases in these animals is also similar (5). Thus BMP signaling is intact in Hjv<sup>−/−</sup> mice. Hjv appears to be involved in establishing a set-point for BMP signaling. Our data suggest that the degree of liver iron-loading, rather than the serum iron concentration, is the determinant for hepatic BMP6 expression.

The induction of hepcidin expression only requires a small amount of HJV. We showed here that specific expression of Hjv in hepatocytes at 15% of the levels of the wild type animals was able to correct the severely reduced hepcidin expression in Hjv<sup>−/−</sup> mice. Hepatocytes are the only known site where both HJV and hepcidin are co-expressed in the body (1). These results are in agreement with previous reports, which showed that iron homeostasis in humans with heterozygous mutations in HJV as well as in heterozygous Hjv<sup>−/−</sup> mice, is normal (9, 13). Therefore, HJV in hepatocytes is not a limiting factor for hepcidin expression. This finding is also supported by the lack of increase in hepcidin expression in wild-type mice in which excess Hjv is introduced into hepatocytes.

The strong increase in hepcidin expression by hepatocyte Hjv was closely correlated with a significant increase in BMP signaling in the Hjv<sup>−/−</sup> mice, but was not associated with a change in BMP6 mRNA in the liver. These findings have two important implications with respect to the regulation of hepcidin expression by HJV and BMP6. First, they support the previous observations that BMP signaling plays a critical role in hepcidin expression. In Hjv<sup>−/−</sup> mice, inhibition of hepcidin expression is associated with decreased BMP signaling (18). Liver-specific knockdown of Smad4, a critical protein in the BMP signaling pathway, in mice abolishes hepcidin expression and results in severe iron overload (14). Secondly, the lack of association between the induction of hepcidin expression by hepatocyte HJV and the changes in BMP6 expression in the liver, provided another line of evidence to support the hypothesis that HJV is not involved in the regulation of BMP6 expression. Rather, our data strongly indicate that hepatocyte HJV...
induces hepcidin expression by potentiating BMP6-mediated signaling. Therefore, our data support the model that HJV acts as a BMP co-receptor (18).

Our results showed an intriguing unanticipated phenomenon in Hjv−/− mice, that expression of a low level of HJV in the hepatocytes alone was able to increase hepcidin mRNA levels to about 2.3-fold higher than those in the wild-type counterparts. We hypothesize that this might result from the following two possibilities. One possibility could be due to the significantly higher hepatic BMP6 expression in Hjv−/− mice than in the wild-type mice. These results lead us to speculate that when HJV is expressed in hepatocytes, hepcidin expression is determined by the level of BMP6 in the liver. The second possibility might be that the lack of HJV expression in skeletal muscle indirectly affects hepatic hepcidin expression. Skeletal muscle is the major site of HJV expression in the body (9). It constitutes about 42% body mass for an average adult male and 36% body mass for an average adult female. Membrane HJV from skeletal muscle could be released into blood circulation as a soluble form after cleavage by the protease furin (25, 33), but its source is unknown. Soluble HJV binds BMP6 as well as other BMP ligands (17). Therefore, soluble HJV could negatively regulate hepcidin expression by competing with cell surface HJV for limited BMP ligands. The role of skeletal muscle HJV in the regulation of hepcidin expression is not known and will be the subject of our future studies.

On the basis of previous observations and the results obtained in this study, we propose a model for the regulation of BMP6 and hepcidin expression by hepatocyte HJV. Hepatic BMP6 expression is positively modulated by liver iron stores independently of HJV expression. In the absence of HJV expression, BMP6 induces hepcidin expression at a low basal level. When HJV is expressed in hepatocytes, HJV acts as a co-receptor for BMP6 to potentiate the BMP signaling. HJV/BMP6 sets the basal hepcidin expression to a level that is sufficient to regulate iron homeostasis. Therefore, maintenance of iron homeostasis requires the expression of both HJV and BMP6. Importantly, our results also provide a potential therapeutic approach for treatment of juvenile hemochromatosis patients with HIV mutations, using the AAV2/8 vector.

Acknowledgments—We thank Dr. Nancy Andrews at Duke University for Hjv−/− mice and Julia Maxson, Maja Chloupkova, Kristina DeMaster, and Juxing Chen for critical reading of this manuscript and helpful comments.

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