A novel validated enzyme-linked immunosorbent assay to quantify soluble hemojuvelin in mouse serum

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ABSTRACT

Hemojuvelin is a critical regulator of hepcidin expression and can be cleaved by proteases to form soluble hemojuvelin. Soluble hemojuvelin has been recently identified in human serum but the presence and quantity of soluble hemojuvelin in mouse serum is unknown. We developed a two-site enzyme-linked immunosorbent assay using a monoclonal anti-hemojuvelin as the capture antibody and a biotinylated polyclonal anti-hemojuvelin as the detection antibody to quantify the levels of soluble hemojuvelin in mouse serum. We validated this assay using cell-conditioned media and serum from Hemojuvelin-null and Bone morphogenetic protein 6-null mice. We also used this validated assay to measure serum soluble hemojuvelin concentrations in mice receiving an acute low iron or high iron treatment. This two-site enzyme-linked immunosorbent assay was highly specific for mouse hemojuvelin, with a lower limit of detection at 13.2-26.8 ng/mL of soluble hemojuvelin in mouse serum. The median serum soluble hemojuvelin concentration in wild-type C57BL/6J mice was 57.9±22 ng/mL, which is 4- to 20-fold less than that reported in healthy human volunteers. After acute low iron diet treatment in these mice, serum soluble hemojuvelin levels were increased and correlated with lowered serum iron levels and decreased hepatic hepcidin expression. An acute high iron diet in wild-type mice or chronically iron-overloaded Bone morphogenetic protein 6-null mice did not significantly lower serum soluble hemojuvelin concentrations. Here we report reliable quantitation of mouse serum soluble hemojuvelin using a novel and validated enzyme-linked immunosorbent assay. This assay may provide a useful tool to elucidate the source and physiological role of serum soluble hemojuvelin in hepcidin regulation and iron metabolism using well-established mouse models of iron-related disorders.

Introduction

Hepcidin is a peptide hormone secreted by the liver that plays a central role in iron homeostasis. It is the key regulatory protein that negatively regulates iron influx by inducing the internalization and degradation of ferroportin (the only known mammalian iron exporter protein)[1,2] on the surfaces of duodenal enterocytes and reticuloendothelial macrophages, thereby limiting intestinal iron absorption and mobilization of iron from tissue stores.[3] Hepcidin expression is modulated by circulating iron levels,[4] inflammation,[5,6] the rate of erythropoiesis,[7] and hypoxia.[8]

Hemojuvelin (HJV), a member of the repulsive guidance molecule family and also known as RGMc, is encoded by the gene *HFE2*.[9] It is mainly expressed in the liver, heart and skeletal muscle.[10] HJV is a glycosylphosphatidylinositol-linked membrane bound protein that plays a crucial role in hepatic hepcidin regulation and systemic iron homeostasis. Patients with mutations in HJV have a severe form of hemochromatosis known as juvenile hemochromatosis characterized by iron overload and extremely low hepcidin levels.[9] Membrane HJV functions as a co-receptor that binds to bone morphogenetic protein 6 (BMP6), consequently activating the BMP-SMAD signaling pathway and hepcidin expression.[11-13] Loss-of-function mutations of HJV result in decreased BMP-SMAD signaling and hepcidin expression, leading to iron overload.[11] Indeed, *Hjv*-null mice have decreased hepcidin expression and increased iron deposition in liver, pancreas, and heart but decreased iron levels in tissue macrophages, similar to the findings in patients with juvenile hemochromatosis.[14,15]

Hemojuvelin can be released from cells as soluble HJV (sHJV) when examined *in vitro*, and endogenous sHJV has been detected in the sera of several species including humans.[16-19] Although exogenously added soluble recombinant HJV has been shown to inhibit the BMP-SMAD signaling pathway and hepcidin expression,[20] the source and function of endogenous sHJV are still not clear. Full-length sHJV can be released into the cell culture media by endogenous phosphatidyinositol-specific phospholipase C (PI-PLC) activity.[21] Furin, a pro-protein convertase, cleaves HJV to generate a 40-42 kDa sHJV fragment.[22,23] Recently, the serine protease TMPRSS6, known to be mutated in patients with iron-refractory iron deficiency anemia,[24] was demonstrated to cleave HJV on hepatoma cell surfaces and release sHJV into the cell culture media.[25,26] Neogenin expression has been reported to enhance the secretion of sHJV *in vitro* by an unknown mechanism.[19] In contrast, neogenin-null mice appear to have increased sHJV.[27]

Several studies found that elevated sHJV levels correlated

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with lowered iron status and lowered hepatic hepcidin expression.[19,22] During conditions of iron deficiency and hypoxia, stabilization of transcription factor HIF-1α leads to increased furin expression and furin-mediated release of sHJV.[22] Rats fed with an iron-deficient diet for 3 days have increased serum sHJV as measured by western blot analysis.[19] Studies have also shown that iron loading with ferric ammonium citrate or holo-transferrin in cell culture was associated with increased expression of hepcidin in the cells and lowered sHJV levels released into the cell-conditioned media.[18,19,22,23] These results suggest that serum sHJV levels may affect hepcidin regulation under these conditions.

Recently, a competitive one-site enzyme-linked immunosorbent assay (ELISA) and a two-site ELISA have been used to quantify sHJV concentrations in human serum. Using the competitive ELISA, three studies found sHJV levels in human serum ranged from 780 to 1140 ng/mL in healthy individuals.[16,28,29] Using the two-site ELISA, two studies demonstrated a range of 210 to 1100 ng/mL sHJV in the serum of healthy individuals.[30,31]

None of the reported sHJV ELISA has been applied to study serum sHJV in mice. A specific and reliable ELISA to quantify sHJV in mouse serum would be a valuable tool to study the functional role of serum sHJV in iron metabolism in many available mouse models of iron-related diseases, including hemochromatosis, anemia of chronic disease, thalassemia and chronic kidney disease.

In this study, we developed and validated a novel two-site ELISA to measure sHJV levels in cell culture media and in mouse serum. We also assessed the association between serum sHJV concentration, hepatic hepcidin levels and iron status in mice during acute iron deficiency and iron loading conditions.

Design and Methods

Cell culture

The human hepatocarcinoma cell line Hep3B (HB-8064, ATCC) was cultured in Eagle's Minimum Essential Medium (ATCC) supplemented with 10% fetal bovine serum (ATCC) without antibiotics and maintained at 37°C under 5% CO_2.

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at the Massachusetts General Hospital and the Institutional Animal Care Committee and the Ministry of Science and Technology at the University of Zagreb School of Medicine.

Hjv-null mice on a C57BL/6J background[14] were kindly provided by Dr. Paul Schmidt at the Children's Hospital Boston and bred at the Massachusetts General Hospital. The mice were fed on a standard rodent diet (Prolab 5P75 Isopro RMH 3000) containing 380 ppm iron. Six-week old female *Hjv*-null mice and female littermates were sacrificed without fasting for quantitation of serum sHJV and liver iron content.

All the mice used in following experiments were wild-type C57BL/6J female mice at 9 weeks of age. Prior to low iron diet treatment, wild-type mice were fed on a standard rodent diet (380 ppm iron). These mice were sacrificed at time zero (baseline) or received a low iron diet (2-6 ppm iron, TD.80396, Harlan Teklad) for 24, 48 and 72 h. Prior to high iron diet experiments, wild-type mice were fed on NIH-31 diet (206 ppm iron), then sacrificed at time zero (baseline) or received a high iron diet (2% carbonyl iron, TD.08496, Harlan Teklad) for 24, 48 and 72 h. All the mice were sacrificed under non-fasting conditions.

Bmp6-null and *Tmprss6*-null mice are described in the *Online Supplementary Design and Methods* section.

Iron analyses

Serum iron and liver non-heme iron concentrations were determined as described previously.[20,32]

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was isolated from mouse livers and *Hamp1*, *Bmp6* or *Hjv* mRNA levels relative to *Rpl19* mRNA levels were measured using two-step quantitative real-time reverse transcriptase polymerase chain reactions as described elsewhere.[33]

Antibodies

Mouse monoclonal anti-HJV 18H8 was generated against the extracellular domain of the human hemojuvelin fused to the Fc portion of the human immunoglobulin chain (HJV.Fc, Ferrumax Pharmaceuticals).[34] Goat polyclonal anti-human HJV was generated against Gln36-Asp400 of recombinant human HJV (AF3720, R&D Systems). Biotinylated goat polyclonal anti-human HJV was the biotin-conjugated version of the same antibody (BAF3720, R&D Systems).

Western blot analysis

Ten nanograms of recombinant mouse HJV.His (mRGMc.His, R&D Systems), RGMa.Fc and RGMb.Fc protein (generated in this laboratory as described previously[35,36]) were subjected to reducing gel electrophoresis using NuPAGE® 4-12% Bis-Tris Gel (Invitrogen), transferred to Immun-Blot PVDF Membrane (Biorad), blocked and incubated overnight at 4°C with (i) mouse monoclonal anti-HJV 18H8 (0.4 μg/mL, Ferrumax Pharmaceuticals), (ii) goat polyclonal anti-human HJV (anti-RGMc, 0.1 μg/mL, AF3720, R&D Systems) or (iii) goat anti-human Fc antibody (1.3 μg/mL, Jackson ImmunoResearch Laboratories) in 5% milk in 0.2% Tween 20 and TBS (TTBS). Blots were washed and incubated with horseradish peroxidase-conjugated secondary antibody and detected by chemiluminescence as previously described.[33]

Hep3B cells ($1.5x10^6$ per plate) seeded in 6 cm dishes were transfected with either empty vector pcDNA3 (2 μg/mL) or pcDNA3.mHjv containing the cDNA sequence of full-length mouse *Hjv* (2 μg/mL or 1 μg/mL) using Lipofectamine 2000 (Invitrogen) in opti-MEM medium (Gibco). The culture medium was switched to opti-MEM medium 24 h after transfection. Forty-eight hours after transfection, cell-conditioned medium was collected and concentrated using a 3 kDa molecular weight cut-off ultrafiltration system (Amicon Ultra, Millipore). Western blot analysis of secreted sHJV in cell-conditioned medium (20 μg) using (i) mouse monoclonal anti-HJV 18H8 (0.4 μg/mL) or (ii) goat polyclonal anti-HJV (0.1 μg/mL) was performed and developed as described above.

Mouse liver membrane proteins were extracted using a proteoJET Membrane Protein Extraction Kit (Fermentas) according to the manufacturer's instructions. Western blot of liver membrane protein (25 μg) for RGMc/HJV (goat anti-mouse RGMc, 0.2 μg/mL, AF3634, R&D Systems) relative to pan-cadherin protein (rabbit anti-pan-cadherin, 1 μg/mL, Abcam) was performed as described above.

For detecting HJV protein in mouse serum, albumin and immunoglobulin (IgG) were removed using an Aurum™ Serum Protein Mini Kit (Bio-Rad) and equal amounts of serum protein (20 μg) were analyzed by western blot using (i) mouse monoclonal anti-HJV 18H8 (0.4 μg/mL) or (ii) goat polyclonal anti-HJV (anti-RGMc, 0.1 μg/mL, AF3720, R&D Systems) antibodies.

The enzyme-linked immunosorbent assay protocol

We developed a two-site ELISA using mouse monoclonal anti-HJV 18H8 as the capture antibody and biotinylated goat polyclonal anti-human HJV (anti-RGMc, BAF3720, R&D systems) as the detection antibody. ELISA plates (NUNC-Immuno™ 96-well MaxiSorp plate, Thermo/Fisher) were coated at 100 μL/well with 4 μg/mL of anti-HJV 18H8 and incubated overnight at 4°C with agitation. The next day, non-specific binding sites were blocked with 1% bovine serum albumin in TBS (pH7.4) at 200 rpm with agitation for 2 h at room temperature. Plates were washed three times in ELISA wash buffer (0.05% Tween20, PBS pH7.4) at 150 μL/well prior to addition of the calibrator and test samples.

Recombinant His-tagged mouse HJV/RGMc (mouse HJV.his) (R&D Systems) was used as a calibrator and recombinant mouse RGMa.Fc and RGMb.Fc were used as negative controls in the ELISA. The calibrator and test samples were run in duplicate on the plate. To quantify sHJV in cell-conditioned media, mouse HJV.His was diluted in serial 2-fold dilutions from 64 ng/mL to 0.5 ng/mL in TBS-Casein buffer pH7.5 (BioFx Laboratories) to generate a standard curve. To quantify sHJV in mouse sera, mouse HJV.His was diluted in serial 2-fold dilutions from 16 ng/mL to 0.5 ng/mL in diluted *Hjv*-null mouse serum (5 μL serum + 95 μL TBS-Casein). Cell-conditioned media and mouse serum samples were diluted at 1:50 and 1:20 in TBS-Casein buffer, respectively, and loaded at 100 μL/well. The calibrator and samples were incubated for 2 h at room temperature, washed four times with ELISA wash buffer at 150 μL/well and then incubated with a biotinylated anti-HJV goat polyclonal detection antibody (R&D Systems) at 0.2 μg/mL for 1.5 h at room temperature. After washing four times with ELISA wash buffer, a streptavidin-conjugated horseradish-peroxidase (Jackson Immunoresearch) was applied at 0.2 μg/mL to the plate and incubated for 1 h at room temperature. The plate was washed four more times with ELISA wash buffer prior to development with TMB substrate (100 μL/well) for 10 min followed by 1 N HCl (100 μL/well) to terminate the reaction. Plates were then read on a spectrophotometer at 450 nm. Plates were sealed and gently shaken during all incubations.

Validation of the enzyme-linked immunosorbent assay

The assay was validated by determining the lower limit of detection (LLOD), working range, linearity, dilution linearity, spiking recovery, intra-assay and inter-assay variability. The mean of OD 450 nm readings from wells with 0 ng/mL of the calibrator mouse HJV.His was defined as the background (blank) and subtracted from OD 450 nm readings of the calibrator and tested samples. The in-well sensitivity limit was defined as 2 times the standard deviation of the blank. The lower limit of detection of samples for the assay was defined as 2 times the standard deviation of the blank times the sample dilution factor. To determine intra-assay reproducibility, the coefficient of variance from the means of duplicate samples on the same plate was measured. To determine inter-assay reproducibility, the coefficient of variance from the means of the same sample was analyzed in a multi-plate, multi-day study with three separate experiments. Coefficients of variance of the assay were calculated from the standard deviation of the samples divided by the mean times 100. To determine dilution linearity, samples from two cell-conditioned media from Hep3B cells transfected with pcDNA3.mHjv (2 μg/mL and 1 μg/mL, respectively) were assayed at 20-fold and 50-fold dilutions. The concentrations were corrected for dilution factor (concentration = measured concentration x dilution factor). Percent recovery from the 50-fold dilution was calculated as % recovery = concentration for 50-fold dilution / concentration for 20-fold dilution x 100. To test the spike recovery rate of our assay, mouse HJV.His was spiked into TBS-Casein buffer at 64, 16, 4 and 1 ng/mL, or into diluted wild-type mouse serum at 16, 4 and 1 ng/mL, and assessed for sHJV concentration by ELISA in three independent experiments. The percent recovery of the spiked mouse HJV.His in TBS-Casein Buffer was calculated using the following formula: % Recovery = measured sHJV / expected x 100. The percent recovery of the spiked mouse HJV.His in mouse serum was calculated with the formula: % Recovery = (measured sHJV – measured sHJV in wild-type mouse serum) / expected x 100.

Statistical analyses

Statistical analyses were carried out using GraphPad Prism version 5.0 software (GraphPad Software Inc.) and SPSS v. 20.0 (IBM). An unpaired two-tailed Student's *t* test was used to analyze significant changes in the described parameters between two groups. One-way analysis of variance (ANOVA) with the Dunnett's post-hoc test was used to determine statistical significance for multiple comparisons. Correlations were analyzed using the Pearson correlation test. *P*-values less than 0.05 were considered statistically significant.

Results

Specificity and characteristics of the soluble hemojuvelin enzyme-linked immunosorbent assay

In order to develop a two-site ELISA, we used a mouse monoclonal anti-HJV (18H8) as the capture antibody and a biotinylated goat polyclonal anti-HJV as the detection antibody. We determined the optimal concentration of capture and detection antibodies according to Crowther.[37] Using western blot analysis, we showed that the mouse monoclonal anti-HJV 18H8 and goat polyclonal anti-HJV can both detect mouse HJV specifically without cross-reactivity against mouse RGMa.Fc or RGMb.Fc (comprising the extracellular domains of RGMa and RGMb, which share 50-60% homology to HJV, fused to the Fc portion of the human immunoglobulin chain) (Figure 1A-C). We confirmed the loading of mouse RGMa.Fc and RMGb.Fc proteins using anti-human Fc antibody (Figure 1D).

To generate a standard curve, we used recombinant mouse HJV.His as the calibrator in serial 2-fold dilutions from 64 to 0.5 ng/mL in TBS-Casein buffer. This resulted in OD 450 nm readings from 1.04 to 0.01 (Figure 1E). Standard curves were also generated with mouse RGMa.Fc and RGMb.Fc using the same dilution conditions. These curves resulted in OD 450 nm readings close to zero, indicating that our ELISA specifically detects mouse HJV and not mouse RGMa or RGMb (Figure 1E).

The LLOD or sensitivity of the assay was 0.33±0.25 ng/mL (Table 1). The intra-assay coefficients of variance, based on duplicates from each experiment, ranged from 0.5 to 4.2%. The inter-assay coefficients of variance, based on three separate experiments, ranged from 2.7 to 8.4%. The correlation between OD 450 nm readings and calibrator concentrations was linear with the square of the Pearson correlation coefficient (r^2) being 0.989. The spike recovery rates of 64, 16, 4 and 1 ng/mL of mouse HJV detected in our assay were between 92.3% and 116%.

In order to ensure that our assay can reliably detect and derive at the same final concentration of sHJV regardless of sample dilution, we tested the dilution linearity and recovery percentage of two different samples. Samples from conditioned media at 20-fold dilution from Hep3B cells transfected with 2 μg/mL (Sample 1) and 1 μg/mL (Sample 2) of mouse *Hjv* vector, gave sHJV concentrations of 363.6 and

125.5 ng/mL respectively. The samples were then tested at 50-fold dilution and we obtained recovery rates of 86.0% and 97.9% which are well within the accepted assay linearity of 80-120%[38] (Table 1).

Validation of the soluble hemojuvelin enzyme-linked immunosorbent assay in cell-conditioned media

We measured the concentration of sHJV in Hep3B cell-conditioned media using western blot analysis and ELISA. We were unable to detect endogenous sHJV in conditioned media from empty-vector transfected Hep3B cells by western blot analysis using either monoclonal anti-HJV 18H8 or polyclonal goat anti-HJV. In cells transfected with mHjv cDNA (2 μg/mL) we detected, as expected, a 42-kDa band corresponding to sHJV in the cell-conditioned media using both antibodies (Figure 1F and 1G). The size of this sHJV band was consistent with the size of the furin-cleaved fragment reported in the literature.[18,22]

Hep3B cells transfected with mHjv cDNA secreted 384±17 ng/mL of sHJV into the cell-conditioned media (Figure 1H). Untreated media alone (i.e. without exposure to cells) and cell-conditioned media from Hep3B cells transfected with empty vector gave a background sHJV concentration of 20-25 ng/mL, which was below the LLOD (4-29 ng/mL) of our assay under these conditions.

Quantitation of soluble hemojuvelin in wild-type mouse sera

The detection of sHJV in mouse serum can be influenced by interference from other proteins in the serum, a phe-

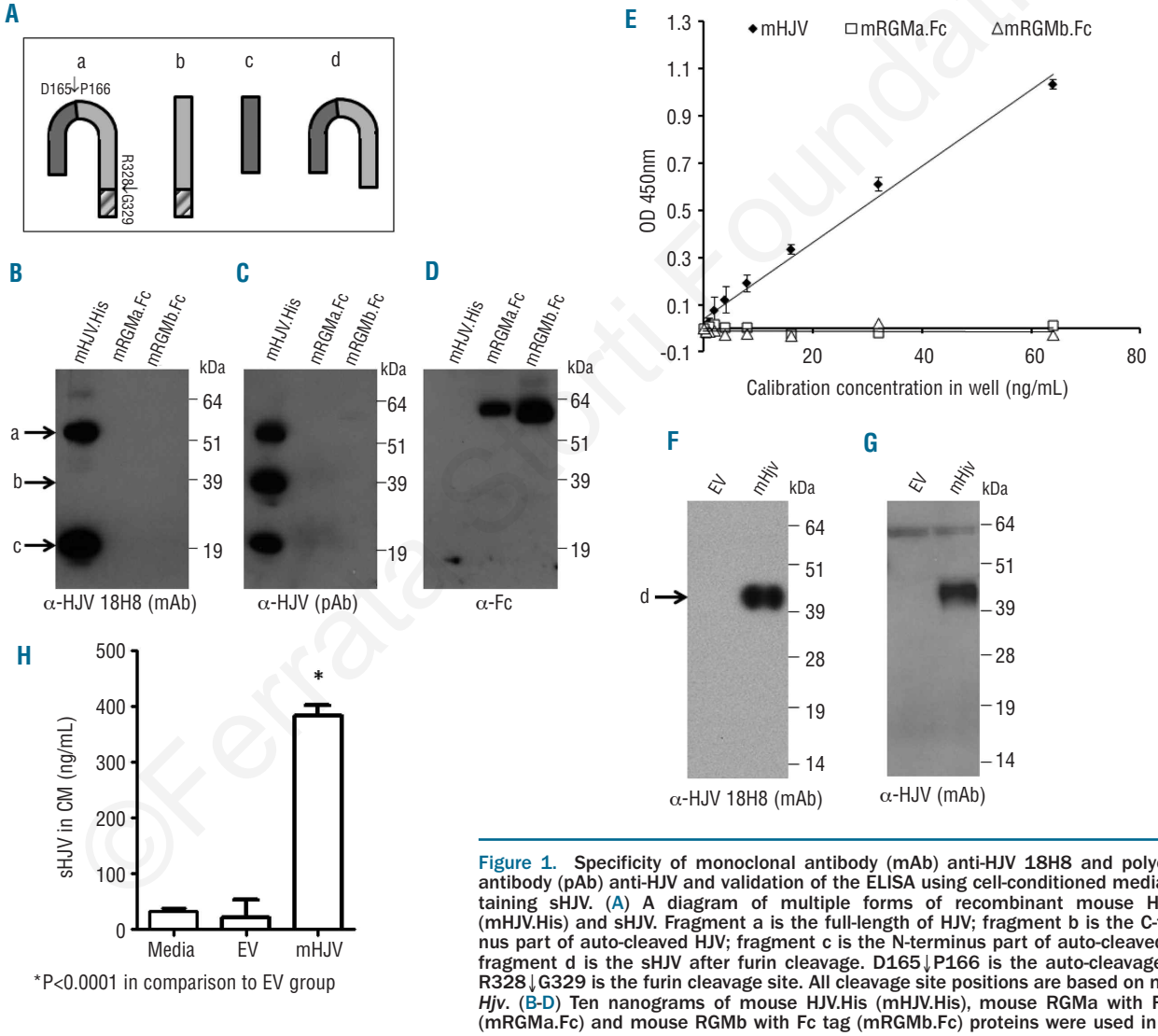


Figure 1. Specificity of monoclonal antibody (mAb) anti-HJV 18H8 and polyclonal antibody (pAb) anti-HJV and validation of the ELISA using cell-conditioned media containing sHJV. (A) A diagram of multiple forms of recombinant mouse HJV.His (mHJV.His) and sHJV. Fragment a is the full-length of HJV; fragment b is the C-terminus part of auto-cleaved HJV; fragment c is the N-terminus part of auto-cleaved HJV; fragment d is the sHJV after furin cleavage. D165↓P166 is the auto-cleavage site; R328↓G329 is the furin cleavage site. All cleavage site positions are based on mouse *Hjv*. (B-D) Ten nanograms of mouse HJV.His (mHJV.His), mouse RGMa with Fc tag (mRGMa.Fc) and mouse RGMb with Fc tag (mRGMb.Fc) proteins were used in western blot analysis. Both mAb anti-HJV 18H8 (capture antibody) and pAb anti-HJV (detection antibody) specifically detect mHJV.His, but not mRGMa.Fc or mRGMb.Fc (B, C). Anti-human Fc antibody confirmed the loading of mRGMa.Fc and mRGMb.Fc (D). (E) The standard curve for the ELISA was generated using 2-fold serial dilutions from 64 to 0.5 ng/mL of mHJV.His as the calibrator diluted in TBS-Casein buffer and plotted in a linear curve with measured OD 450 nm readings. The standard curve of mHJV.His was generated from three independent experiments performed in duplicate (n=6). Error bars represent standard deviations. Standard curves for mRGMa.Fc and mRGMb.Fc were also generated using the same concentrations and plotted with measured OD 450 nm readings. (F-G) A specific sHJV band at 42-kDa can be detected by immunoblot with mAb anti-HJV 18H8 (F) and pAb anti-HJV (G) in cell-conditioned media from Hep3B cells transfected with pcDNA3.*mHjv* (2 μg/mL) but not in cell-conditioned media from Hep3B cells transfected with the same amount of empty vector (EV). (H) The same samples of cell-conditioned media and media alone were quantified by ELISA. Samples were from two independent transfections and analyzed in duplicate (n=4). Error bars represent standard deviations. The *P* value was calculated using the Student's t test.

nomenon known as the matrix effect.[39] To minimize any matrix effects when quantifying sHJV in the mouse sera, we formulated the diluent buffer for the standard curve with serum from *Hjv*-null female mice (5 μL serum + 95 μL TBS-Casein, a 20-fold dilution of serum). The calibrator (mouse HJV.His protein) was diluted in serial 2-fold dilutions from 16 to 0.5 ng/mL. This resulted in OD 450 nm readings from 0.25 to 0.005 (Figure 2A). In contrast, standard curves generated using mouse RGMa.Fc and RGMb.Fc proteins under the same conditions had OD 450 nm readings of less than zero, indicating that our ELISA was specific for mouse HJV but not for soluble mouse RGMa or RGMb in mouse serum (Figure 2A).

We further determined the sensitivity, precision and recovery in our assay for sHJV in mouse serum. The in-well assay sensitivity limit under these conditions was 1±0.34 ng/mL (Table 2). This is equivalent to a LLOD of 13.2-26.8 ng/mL of sHJV in mouse serum, after adjusting for the 20-fold dilution of mouse serum into TBS-Casein buffer. The intra-assay coefficients of variation, based on duplicates from each experiment, were 0.2-18.6% and the inter-assay coefficients of variation, based on three separate experiments, were 1.2-16.7%. Pearson linear regression analysis resulted in an r^2 value of 0.999, indicating excellent linearity. The spike recovery rates using 16, 4 and 1 ng/mL of mouse HJV.His were 95.6-108.8% (Table 2).

We next quantified serum sHJV from wild-type female mice compared to Hjv null mice. First, 6-week old $Hjv^{-/-}$, $Hjv^{+/-}$ and wild-type $Hjv^{+/+}$ littermate female mice were genotypically confirmed by polymerase chain reaction analysis as described previously.[14] We also confirmed that $Hjv^{-/-}$ mice have significantly higher iron load in the liver compared to $Hjv^{+/+}$ and $Hjv^{+/-}$ mice at 6 weeks of age, as previously reported[14,15] (Figure 2B). Using our ELISA, we showed that the median serum sHJV concentration in 6-week old $Hjv^{-/-}$ mice was 6.4±4.9 ng/mL (Figure 2C). This concentration was below the LLOD of our ELISA (13.2-26.8 ng/mL) as reported above. In contrast, the median concentrations of sHJV in 6-week old $Hjv^{+/-}$ and $Hjv^{+/+}$ mouse sera were 56.2 and 57.9 ng/mL, respectively, which were significantly higher than the LLOD and the measured sHJV concentrations in $Hjv^{-/-}$ mouse sera (Figure 2C).

To ensure that the low sHJV concentrations in $Hjv^{-/-}$ mouse sera were due to the lack of sHJV and not due to an effect induced by the iron overload condition in these mice, we tested the serum sHJV concentration in $Bmp6^{-/-}$ mice which had similarly elevated iron levels as seen in $Hjv^{-/-}$ mice[12,13] (*Online Supplementary Figure S1A*). No significant difference was found in serum sHJV levels between $Bmp6^{-/-}$ and $Bmp6^{+/+}$ mice (*Online Supplementary Figure S1B*). In addition, no significant difference was found in serum sHJV levels between $Bmp6^{+/+}$ and $Hjv^{+/+}$ mice of different sex and genetic background (Figure 2C and *Online Supplementary Figure S1B*). These data confirmed that the low serum sHJV signal in $Hjv^{-/-}$ mice was indeed due to loss of *Hjv* and not due to long-term iron overload effects on serum sHJV levels.

TMPRSS6 overexpression has been shown to cleave HJV and generate TMPRSS6 cleaved fragments of sHJV; loss of function of TMPRSS6 did not generate TMPRSS6 cleaved fragments of sHJV *in vitro*.[25,26] We measured serum sHJV levels in $Tmprss6^{-/-}$ mice to determine whether serum sHJV levels are decreased due to loss of TMPRSS6 activity. Intriguingly, serum sHJV levels in $Tmprss6^{-/-}$ mice were not decreased compared to those in $Tmprss6^{+/-}$ and $Tmprss6^{+/+}$ mice (*Online Supplementary Figure S1C*).

Quantitation of soluble hemojuvelin in sera from mice following acute iron diet treatment

To address whether acute changes in iron levels regulate serum sHJV levels, we quantified serum sHJV in mice receiving low iron and high iron diets using our ELISA. Nine-week old C57BL/6J female mice were fed with either a low iron diet (2-6 ppm iron) or a high iron diet (2% carbonyl iron) for 0, 24, 48 and 72 h and were analyzed for iron status and serum sHJV levels.

During acute iron deficiency conditions (low iron diet), serum iron levels remained the same as baseline at 24 h, but were significantly decreased after 48 and 72 h of treatment (Figure 3A). However, there was no change in liver iron content after low iron diet treatment over this time course (Figure 3B). Liver hepcidin mRNA expression was not significantly different at 24 h compared to baseline but was decreased after 48 and 72 h of treatment (Figure 3C). Serum sHJV levels tended upwards at 24 and 48 h, and were significantly increased after 72 h compared to baseline levels (Figure 3D). We also performed western blot analysis on the sera of wild-type mice and mice fed a low iron diet for 72 h to visualize the type of sHJV fragments in the serum. However, due to the minute amounts of sHJV protein in the midst of serum proteins, western blot analysis was not sensitive enough to detect sHJV fragments in the serum (*Online Supplementary Figure S2*).

In order to explore potential associations between hepcidin, serum iron and serum sHJV levels, we performed Pearson linear regression analyses in mice receiving the low iron diet at all time points (n=25). Correlation analyses showed that serum sHJV was inversely associated with serum iron (r=-0.4346, $P<0.05$) (Figure 3E) and liver hepcidin mRNA expression levels (r=-0.4068, $P<0.05$) (Figure 3F). This result is consistent with the inverse correlation of elevated serum sHJV levels to lowered serum iron levels and decreased hepatic hepcidin mRNA expression as published in other reports.[16,22,28]

Interestingly, HJV mRNA expression levels in liver, heart

Table 1. Summary of the sHJV ELISA characteristics using TBS-Casein as the diluent buffer.

Standard curve	Calibrator range	0-64 ng/mL
	In-well sensitivity limit	0.33±0.25 ng/mL
	Reproducibility (intra-assay CV)	2.4% (0.5-4.2%)
	Reproducibility (inter-assay CV)	5.5% (2.7-8.4%)
	Linearity (r^2)	0.989
Spike recovery		
	64 ng/mL	92.3±5.5%
	16 ng/mL	116.0±3.0%
	4 ng/mL	110.6±4.9%
	1 ng/mL	102.5±17.0%

Dilution linearity	Dilution factor	sHJV (ng/mL)	Percent recovery
Sample 1	1:20	363.6	-
	1:50	355.9	97.9%
Sample 2	1:20	125.5	-
	1:50	107.9	86.0%

In-well sensitivity limit and spike recovery rates are presented as mean±SD. The intra- and inter-assay coefficients of variation (CV) are presented as mean (range).

and skeletal muscle remained unchanged in mice receiving the low iron diet (*Online Supplementary Figure S3A-C*). Liver membrane HJV was also not significantly changed after low iron diet treatment (*Online Supplementary Figure S4A,B*). These data suggest that the increase of serum sHJV during acute iron deficiency was not associated with an increase of HJV mRNA expression in the tissues expressing HJV or with a measurable decrease of liver membrane HJV protein levels.

During acute iron loading conditions (high iron diet), serum iron and liver hepcidin mRNA expression were increased, as expected, after 24 h and reached a plateau at 48 to 72 h (Figure 4A and 4C). Liver iron content was also increased after 24 h and continued to increase after 48 and 72 h (Figure 4B). Interestingly, there was a trend towards a decrease in serum sHJV levels after high iron diet treatment over time. However, these changes did not reach significance based on the one-way ANOVA test (Figure 4D). These results indicate that increased serum iron and liver iron content induce liver hepcidin mRNA expression as early as 24 h after starting high iron diet treatment. However, serum HJV levels were not significantly lowered by increases in serum iron and liver iron content.

Different iron concentrations in the rodent diet affected the baseline liver hepcidin mRNA expression in our mice. The baseline group in the low iron diet experiment were originally fed a 380 ppm iron diet prior to the dietary intervention which resulted in a higher baseline liver hepcidin level compared to that in the baseline group (fed on 206 ppm iron) in the high iron diet experiment (Figure 3C).

Discussion

We have established and validated a novel ELISA to specifically measure the concentrations of sHJV in mouse serum. This assay is, to our knowledge, the first to target mouse sHJV. Having a tool that reliably detects and quantifies sHJV levels in mice will be useful to investigate the role of sHJV in the regulation of systemic iron balance.

We developed a two-site ELISA, using a monoclonal anti-hemojuvelin as the capture antibody and a polyclonal anti-hemojuvelin as the detection antibody, to reliably detect and quantify sHJV levels in cell-conditioned media and in mouse sera. We validated our ELISA using the following criteria: specificity, sensitivity, range, recovery, linearity, precision and reproducibility. We achieved excellent results for each of these criteria, as described in the Results section.

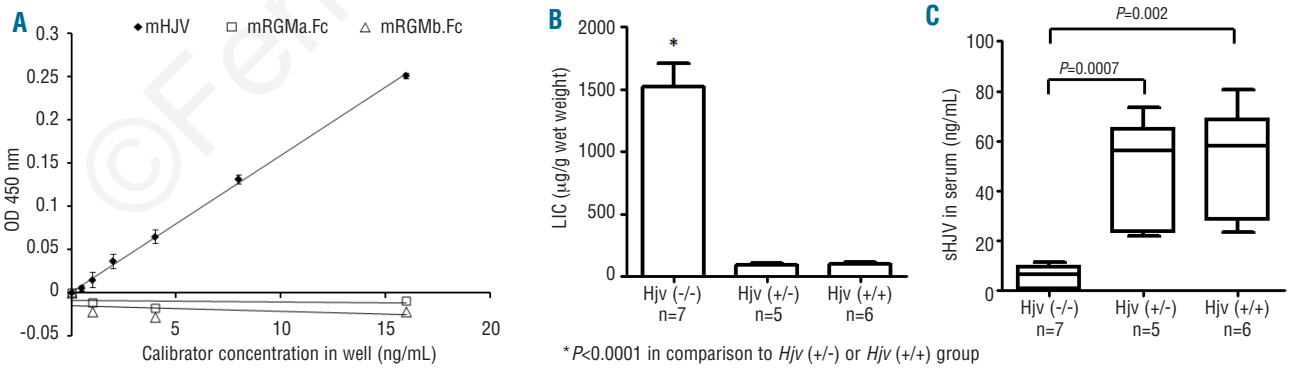
We determined that healthy mice have median concentrations of serum sHJV of 56.2 ng/mL for *Hjv*$^{+/-}$ mice and 57.9 ng/mL for *Hjv*$^{+/+}$ mice. The median concentration of serum sHJV in *Hjv*$^{-/-}$ mice was 6.4 ng/mL, which was below the LLOD of our assay. These serum sHJV concentrations in healthy wild-type mice were 4- to 20-fold lower than those reported in healthy adult human volunteers (210-1140 ng/mL) using both a competitive one-site ELISA[16,29] and a commercially available two-site ELISA.[30,31]

There are several possible explanations for the large differences seen in the concentration of serum sHJV in mice reported here and in humans in previously reported studies. One possibility is that mice have much lower serum sHJV than humans due to species difference. Our assay measures

Table 2. Summary of sHJV ELISA characteristics using *Hjv*-null mouse serum as diluent buffer.

Standard curve	
Calibrator range	0-16 ng/mL
In-well sensitivity limit	1±0.34 ng/mL
Reproducibility (intra-assay CV)	5.6% (0.2-18.6%)
Reproducibility (inter-assay CV)	8.2% (1.2-16.7%)
Linearity (r^2)	0.999
Spike recovery	
16 ng/mL spike	104.9±3.7%
4 ng/mL spike	108.8±10.1%
1 ng/mL spike	95.6±9.2%

In-well sensitivity limit and spike recovery rates are presented as mean±SD. The intra- and inter-assay coefficients of variation (CV) are presented as mean (range).



*P<0.0001 in comparison to *Hjv* (+/-) or *Hjv* (+/+) group

Figure 2. Quantitation of sHJV in mouse serum by ELISA. (A) The standard curve for the sHJV ELISA was generated using 2-fold dilutions from 16 to 0.5 ng/mL of mHJV.His protein as a calibrator diluted in *Hjv*-null mouse serum and plotted in a linear curve with measured OD 450 nm readings. The standard curve of mHJV.His was generated from three independent experiments performed in duplicate (n=6). Error bars represent standard deviations. Standard curves were also generated using 16, 4 and 1 ng/mL of mRGMa.Fc and mRGMb.Fc proteins plotted with measured OD 450 nm readings. (B) Six-week old female *Hjv*$^{-/-}$, *Hjv*$^{+/-}$, and *Hjv*$^{+/+}$ mice were analyzed for liver iron content (LIC). Error bars represent standard deviations. (C) Serum sHJV concentrations from *Hj*$^{-/-}$, *Hjv*$^{+/-}$, and *Hjv*$^{+/+}$ mice are shown in a box plot. The data were generated from five to seven animals per genotype. *P* values between the groups were calculated using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test.

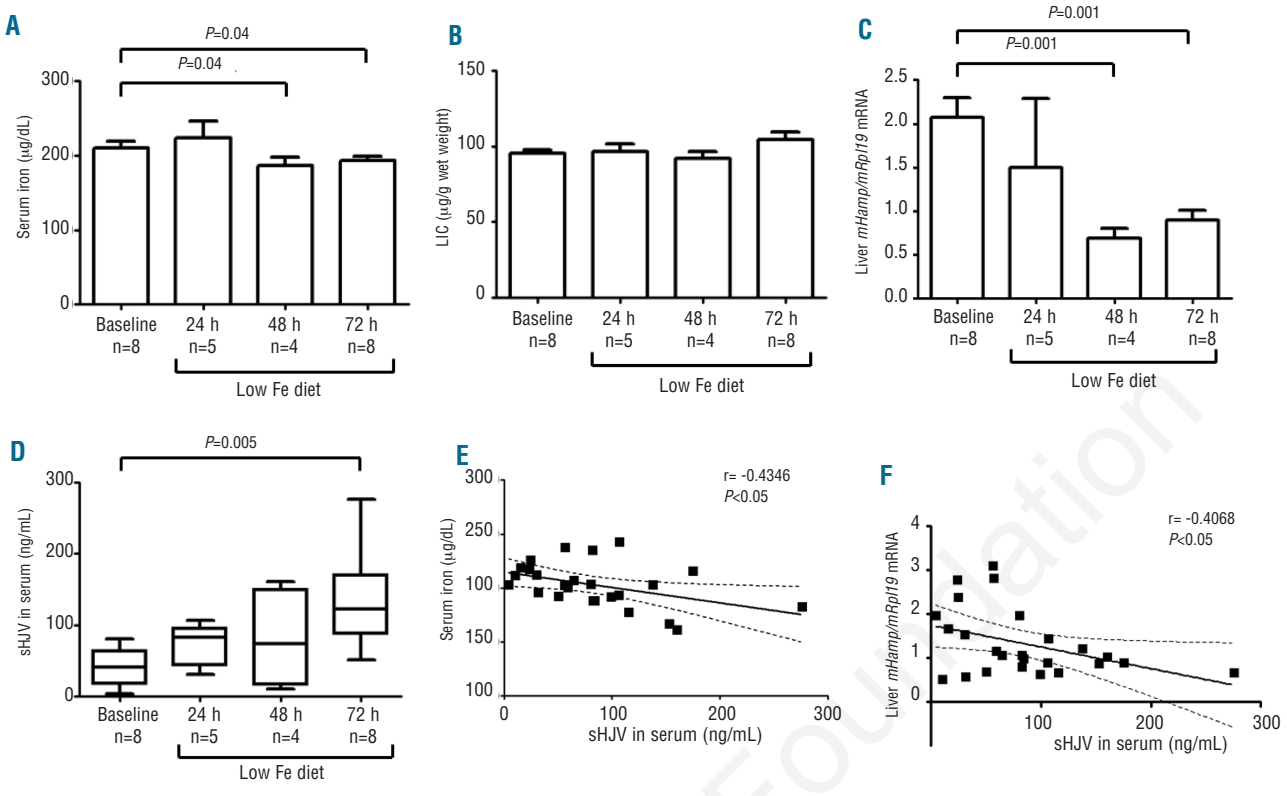


Figure 3. Quantitation of serum sHJV in mice after acute low iron diet treatment. (A-C) Nine-week old C57BL/6J female mice receiving a low iron diet for 0, 24, 48, and 72 h (N=4-8 per group) were analyzed for serum iron concentration (A), liver iron content (LIC) (B), and liver *Hamp1* (hepcidin gene) normalized to *Rpl19* mRNA expression (C). Error bars represent standard deviations. (D) Serum sHJV concentrations from each group are shown in a box plot. One-way ANOVA with Dunnett's post-hoc test was used to calculate the *P*-values between multiple groups. (E-F) Scatter plots show the correlation between serum sHJV concentrations and serum iron levels (E) and the correlation between serum sHJV levels and liver *Hamp1* mRNA expression (F). r is Pearson's correlation coefficient. The correlation line (middle black line) is given with the 95% confidence interval (upper and lower dashed lines).

serum sHJV levels, which may not correlate directly with protein activity levels. A second possibility could be that serum sHJV increases with age, and the mice we tested were relatively young compared to the healthy human volunteers in the published studies. A third possibility could be related to inherent differences between the assays. For example, the competitive one-site ELISA measured 300 ng/mL of serum sHJV in a *HJV*-null patient (healthy volunteer range 780-1140 ng/mL), indicating that this assay has a high background.[16,28,29] The commercially available two-site ELISA was not validated for specificity with serum from an *HJV*-null patient.[30,31] Since both the capture and detection antibodies used in our mouse sHJV ELISA can efficiently detect human HJV (*unpublished data*), our two-site assay may be suitable for quantitation of serum sHJV in humans in future studies.

Based on *in vitro* studies, it is thought that TMPRSS6 plays a role in cleaving HJV; if this is so, lack of TMPRSS6 should result in a decrease in sHJV levels compared to levels in wild-type controls. We did not detect significant differences in serum sHJV levels between *Tmprss6*$^{-/-}$, *Tmprss6*$^{+/-}$ and *Tmprss6*$^{+/+}$ mice. It is possible that TMPRSS6-cleaved sHJV fragments are degraded immediately, suggesting furin or other proteases may have a more prominent role in generating circulatory serum sHJV. It is also possible that the iron deficiency and anemic condition of *Tmprss6*$^{-/-}$ mice increased furin-mediated cleavage of serum sHJV and concealed the decrease of serum sHJV in *Tmprss6*$^{-/-}$ mice, thereby leading to no change in total amount of serum sHJV compared to the amount in wild-type mice. To test this hypothesis, serum sHJV levels in *Tmprss6*$^{-/-}$ mice should be compared to those in mice with similar levels of iron deficiency and anemia, which is the subject of future studies.

We observed an inverse correlation between increased serum sHJV concentrations and lowered serum iron levels in mice challenged with a low iron diet (Figure 3E). This inverse correlation between serum sHJV and serum iron was also identified in healthy individuals and in patients with anemia of chronic disease.[16] In hemodialysis patients, an inverse correlation between serum sHJV and total iron-binding capacity was also identified.[31] These findings are consistent with the negative regulation of sHJV release by either iron-saturated transferrin or non-transferrin bound iron in cell cultures and rats.[18,19,22,23] In addition, Silvestri *et al.* reported an increase in furin expression during iron deficiency conditions *in vitro*.[22] Moreover, hepatic TMPRSS6 expression was increased in rats receiving an iron-deficient diet.[40] The increased expression of furin and TMPRSS6 under these iron deficiency conditions could lead to increased sHJV release into the serum.

We also demonstrated an inverse correlation between elevated serum sHJV concentrations and lowered hepatic hepcidin mRNA expression levels in mice receiving a low

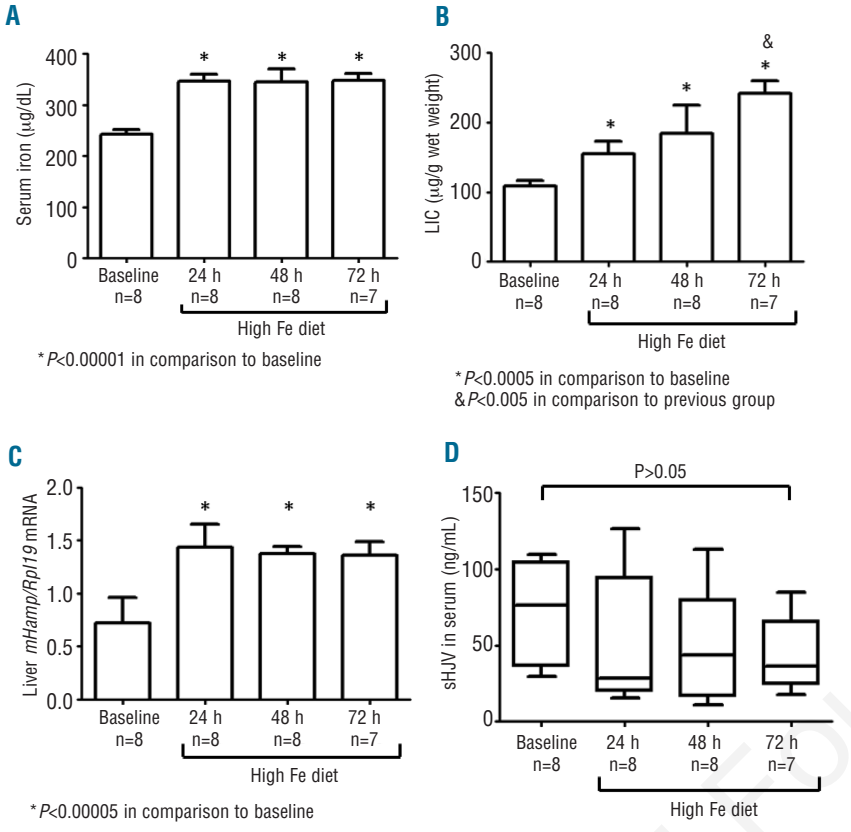


Figure 4. Quantitation of serum sHJV in mice after acute high iron diet treatment. (A-C) Nine-week old C57BL/6J female mice receiving a high iron diet at 0, 24, 48, and 72 h (N=7-8 per group) were analyzed for serum iron concentration (A), liver iron content (LIC) (B) and liver *Hamp1* normalized to *Rpl19* mRNA expression (C). Error bars represent standard deviations. (D) Serum sHJV concentrations from each group are shown in a box plot. One-way analysis of variance (ANOVA) with the Dunnett's post-hoc test was used to calculate *P* values between multiple groups.

iron diet (Figure 3F). A similar inverse correlation between serum sHJV and hepcidin concentrations was also identified in pregnant women.[28] These findings would appear to support the hypothesis that elevated sHJV could play a role in mediating lowered hepcidin levels. Further investigation is needed to confirm whether there is a suppressive role of endogenous serum sHJV in the regulation of hepcidin.

Patients with anemia of chronic disease and patients on hemodialysis have decreased serum iron levels and increased serum sHJV levels. The mice given a low iron diet in this study also had low serum iron levels and elevated serum sHJV levels. However, while there was an inverse correlation between elevated serum sHJV and lowered hepcidin expression in these mice, the inverse correlation was not observed in patients with anemia of chronic disease and in those undergoing hemodialysis. One possibility is that in patients with anemia of chronic disease and hemodialysis patients, hepcidin is stimulated by interleukin-6 or other inflammatory factors, which may mask the inhibitory effects of serum sHJV on hepcidin through the BMP/SMAD signaling pathway.

HJV is predominantly expressed in the liver and cardiac and skeletal muscle.[10] Although cardiac and skeletal muscle have been previously hypothesized to serve as a source of serum sHJV to inhibit hepcidin expression in response to iron deficiency and hypoxia, mice with specific knockout of cardiac and skeletal muscle *Hjv* do not have abnormal iron metabolism.[41,42] Liver-specific *Hjv* knockout mice did recapitulate the iron overload phenotype found in *Hjv*-null mice. To investigate whether the increase in serum sHJV seen in the mice under low iron diet conditions is due to increases in mRNA expression or cleavage of membrane HJV in HJV-expressing tissues, we measured *Hjv* mRNA and membrane HJV protein expression in the liver, and cardiac and skeletal muscle. No quantifiable changes in *Hjv* mRNA or membrane HJV in the liver were found between mice given low iron and normal iron diets. There was also no difference in *Hjv* mRNA expression in cardiac or skeletal muscle under the same conditions. We were unable to detect membrane HJV in cardiac and skeletal muscle by western blot analysis (*data not shown*). These findings suggest that increased serum sHJV after low iron diet treatment is not associated with a decrease of liver membrane HJV or an increase of *Hjv* mRNA expression. Further investigation is required to determine the tissue source and mechanism underlying sHJV release in response to low iron diet treatment.

In summary, we have developed and validated a novel two-site ELISA to quantify serum sHJV in mice. We showed that serum sHJV concentrations in mice are much lower than those reported for humans. After acute treatment with a low iron diet, the serum sHJV concentration in mice was increased and negatively correlated with serum iron levels and liver hepcidin mRNA expression. There was no significant decrease in serum sHJV in mice after acute high iron diet treatment. This assay will be a useful tool to further elucidate the source and physiological role of endogenous serum sHJV in the regulation of hepcidin and iron metabolism using well-established mouse models of iron-related diseases.

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Authorship and Disclosures

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