
DOI: 10.1371/journal.pone.0014553

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http://hdl.handle.net/10536/DRO/DU:30062867
BMP Signaling Modulates Hepcidin Expression in Zebrafish Embryos Independent of Hemojuvelin

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Abstract

Hemojuvelin (Hjv), a member of the repulsive-guidance molecule (RGM) family, upregulates transcription of the iron regulatory hormone hepcidin by activating the bone morphogenetic protein (BMP) signaling pathway in mammalian cells. Mammalian models have identified furin, neogenin, and matriptase-2 as modifiers of Hjv’s function. Using the zebrafish model, we evaluated the effects of hjv and its interacting proteins on hepcidin expression during embryonic development. We found that hjv is strongly expressed in the notochord and somites of the zebrafish embryo and that morpholino knockdown of hjv impaired the development of these structures. Knockdown of hjv or other hjv-related genes, including zebrafish orthologs of furin or neogenin, however, failed to decrease hepcidin expression relative to liver size. In contrast, overexpression of bmp2b or knockdown of matriptase-2 enhanced the intensity and extent of hepcidin expression in zebrafish embryos, but this occurred in an hjv-independent manner. Furthermore, we demonstrated that zebrafish hjv can activate the human hepcidin promoter and enhance BMP responsive gene expression in vitro, but is expressed at low levels in the zebrafish embryonic liver. Taken together, these data support an alternative mechanism for hepcidin regulation during zebrafish embryonic development, which is independent of hjv.

Introduction

Bone morphogenetic proteins (BMPs), originally identified for their ability to induce bone differentiation, are members of the TGF-β superfamily. Binding of a BMP molecule to a BMP receptor complex results in phosphorylation of Smad1, 5, and 8. These proteins then activate the human hepcidin promoter and enhance BMP responsive gene expression in vitro, but is expressed at low levels in the zebrafish embryonic liver. Taken together, these data support an alternative mechanism for hepcidin regulation during zebrafish embryonic development, which is independent of hjv.

Although hjv expression is not iron responsive,[8,11], iron deficiency induces production of soluble hjv,[8], while iron loading inhibits release of soluble hemojuvelin.[12,13] It has been proposed that soluble Hjv, produced via a Furin-mediated proteolysis of membrane-bound Hjv[13,14], antagonizes the function of membrane-bound Hjv[12,15] resulting in low levels of hepcidin expression. Recently another membrane-bound cell surface serine protease, Matriptase-2 (Mtp2, also known as TMPRSS-6) has been shown to decrease hepcidin transcription[16] and to bind and cleave Hjv[17] in vitro.

We have been developing the zebrafish embryo (Danio rerio) as a model to study the developmental regulation of hepcidin. We have demonstrated that hepcidin expression begins at 36 hpf in the zebrafish embryo and that the zebrafish ortholog of Transferrin, transferrin-a, is required for hepcidin expression during embryonic development.[18] While the BMP pathway has been studied for its effect on embryonic symmetry and patterning,[19] its effect on hepcidin regulation during embryonic development has not been characterized previously. Furthermore, the effects of hjv and related genes on hepcidin expression have not been evaluated previously during embryonic development.

In this report, we demonstrate that activation of the BMP pathway increased the intensity and extent of hepatic hepcidin expression during embryonic development, and suppression of
BMP signaling by the chemical inhibitor dorsomorphin eliminated hepcidin expression. In contrast, knockdown of hjv reduced the size of the liver, but failed to eliminate hepcidin expression. While knockdown of mmp2 increased hepcidin expression, relative to liver size, this effect was independent of hjv. As experimental overexpression of hjv in zebrafish embryos failed to increase hepcidin expression, we propose that the regulation of hepcidin expression in zebrafish embryos is hjv-independent.

### Results

**Induction of bmp2b at 48 hpf stimulates hepcidin expression in zebrafish embryos**

BMP signaling has been shown to modulate hepcidin expression in adult mammals[9,20] and in adult zebrafish.[21] As BMP2 has been demonstrated to stimulate hepcidin transcription in mammalian cell culture[20], we exploited the tg(hsp70:bmp2b) line of zebrafish[19] to assess whether BMP signaling regulates hepcidin expression in the zebrafish embryo. Tg(hsp70:bmp2b) transgenic zebrafish carry the bmp2b gene, one of two zebrafish orthologs of BMP2, under the control of the hsp70 promoter. Transgenic animals were incrossed to generate embryos, which were subjected to heat shock, or no heat shock, at 48 hours post-fertilization. Pools of embryos were harvested at 2, 6, and 24 hours post-treatment and assayed for bmp2b expression (Figure 1A) in comparison to nontransgenic embryos at 48 hpf, which were not subjected to heat shock. Quantitative real-time RT-PCR revealed a 2000-fold increase in bmp2b expression in the transgenic embryos two hours after heat shock compared to nontransgenic embryos subjected to heat shock (4866.2 ± 3556 vs 1.12 ± 0.184, p < 0.001) or 100-fold increase compared to transgenic embryos not subjected to heat shock (4866.2 ± 3556 vs 44.85 ± 14.85, p < 0.01). In the transgenic embryos, bmp2b expression remained significantly elevated 6 hours after heat shock, but 24 hours after heat shock bmp2b expression declined to non heat-shock levels of expression (58.8 ± 12.2).

Induction of hepcidin expression corresponded with induction of bmp2b expression in the tg(hsp70:bmp2b) embryos. Two hours after the start of the heat shock, hepcidin expression levels increased ten-fold (Figure 1B), compared to untreated WT embryos (10.1 ± 3.86 vs 1.00 ± 0.03) or compared to untreated transgenic embryos (10.1 ± 3.86 vs 1.15 ± 0.398). As elevations in hepcidin expression persisted in the transgenic embryos 6 hours after the start of heat shock (54 hpf) and were not associated with an increase in hepcidin expression in WT embryos subjected to heat shock, this time point was selected for subsequent experiments. At 24 hours post heat shock, or 72 hpf, hepcidin transcript levels increased in both the heat shock and non heat shock treated WT and non heat shock treated transgenic embryos. This is consistent with a developmental increase in hepcidin expression from 54 to 72 hpf, which we have observed previously[18]. To confirm that heat shock activated the BMP signaling pathway in tg(hsp70:bmp2b) embryos, we performed whole mount immunohistochemistry (Figure 1C–E) for phosphorylated Smad1, 5, and 8 proteins, which revealed increased staining for these phosphoproteins in the liver, somites, and head 6 hours after heat shock (55 hpf).

**Inhibition of BMP type I receptors decreases hepcidin expression in zebrafish embryos**

To evaluate further the role of BMP signaling in hepcidin regulation during embryogenesis we selectively inhibited BMP type I receptors using the recently identified BMP signaling inhibitor, dorsomorphin.[21] Dorsomorphin was previously shown to doralize embryos, expanding structures derived from the dorsal pole, when added before 12 hpf.[21] By delaying the addition of dorsomorphin until 28 hpf, and then maintaining them in the chemical until 55 hpf, we found that the embryos exhibited normal embryonic patterning, but exhibited a dose-dependent decrease in hepcidin expression by quantitative realtime RT-PCR from 1 to 40 µM (data not shown). We chose to use 40 µM dorsomorphin, which produced near complete inhibition of hepcidin expression. We then incubated pools of tg(hsp70:bmp2b) embryos in 40 µM dorsomorphin/0.3% DMSO or in 0.3% DMSO alone from 28-55 hpf. Half the pools were subjected to heat shock at 48 hpf to induce bmp2b expression. The embryos were fixed at 55 hpf for quantitative real-time RT-PCR. In the absence of dorsomorphin (Figure 1F), he shock significantly increased hepcidin expression (3.17 ± 1.01 vs 0.702 ± 0.154, p < 0.01). In the absence of heat shock, dorsomorphin exposure reduced hepcidin expression 20-fold (0.032 ± 0.012 vs 0.702 ± 0.154, p < 0.001). In the presence of heat shock, dorsomorphin diminished the effect of bmp2b induction on hepcidin expression six-fold, but failed to abrogate it. Immunohistochemical staining demonstrated that dorsomorphin decreased, but did not eliminate phospho-smad1,5,8 staining, in transgenic embryos treated with heat shock (Figure S1). Thus it appears that the 2000-fold increase observed in BMP2b expression following heat shock of transgenic embryos partially overcomes the inhibitory effects of dorsomorphin on BMP signaling.

We found further support that BMP signaling regulates hepcidin expression in zebrafish embryos, by using transgenic zebrafish that express the BMP signaling antagonist noggin3 under the control of the hsp70 promoter [19]. We crossed these tg(hsp70:noggin3) zebrafish to WT fish producing progeny in which 50% of the embryos carried the transgene. We then heat shocked these progeny at 48 hpf and fixed at 55 hpf for quantitative real-time RT-PCR. Heat shocked embryos exhibited a significant reduction (Figure 1G) in the hepcidin transcript levels (1.78 ± 0.39 vs. 0.467 ± 0.126, p = 0.027), consistent with inhibition of hepcidin expression by noggin3.

**Knockdown of hjv causes notochord and somite defects**

To assess whether hjv is required to induce hepcidin expression during zebrafish embryogenesis, we injected antisense morpholinos (MOs) at the one-cell stage to knock down the hjv gene. Hjv MO1 targets the 5′-UTR of hjv and is designed to impair translation of hjv, while hjv MO2 is a non-overlapping morpholino targeting the second exon donor site of the coding sequence. Injection of either morpholino at 0.5 mM was associated with severe growth retardation, which impaired the ability of the embryo to develop past 18 hpf (data not shown) to the expected time of onset of hepcidin expression[18] (36 hpf). At a lower injection concentration, 0.2 mM, injected embryos were able to develop past somitogenesis. Compared to uninjected embryos (Figure 2A,F), embryos injected with either hjv MO1 (Figure 2B,G) or hjv MO2, (Figure 2C) exhibited undulating notochord and body axis at 15–18 hpf, visible on light microscopy or by whole mount in situ hybridization for the notochord specific marker, no tail (Figure 2F,G). Co-injecting hjv MO1 and hjv MO2 exacerbated notochord distortion (Figure 2D), however injection of a mismatch control morpholino (hjv MM02) did not distort the notochord (Figure 2E). While zebrafish embryonic somites exhibited a well-delineated V-shape at 24 hpf in uninjected or control morpholino injected embryos (Figure 2H), the somites were decreased in the anterior-posterior dimension and U-shaped in hjv morphants (Figure 2J).

**Induction of bmp2b increases the intensity and extent of hepcidin expression without affecting liver size**

As Hjv has been shown to function as a BMP co-receptor in mammalian models, we assessed the effect of BMP signaling and hjv on hepcidin expression. In comparison to uninjected WT embryos at 55 hpf (Figure 3A), induction of bmp2b by heat shock at 48 hpf in tg(hsp70:bmp2b) resulted in increased intensity and extent...
Figure 1. The BMP pathway regulates hepcidin expression in zebrafish embryos. A–B. Time course of bmp2b and hepcidin expression following induction of BMP2b expression. Tg(hsp70: bmp2b) is a transgenic line of zebrafish, which carries the BMP2b gene under the control of the hsp70 promoter. At 48 hours post-fertilization (hpf), WT or tg(hsp70: bmp2b) groups of embryos (n = 20 embryos per group) were subjected either to heat shock (+HS) at 37°C for 40 min or maintained at the usual temperature (28°C) (-HS). Pools of embryos were obtained for RNA extraction at 2, 6, and 24 hours after the start of heat shock, corresponding to 50, 54, and 72 hpf. Quantitative real-time RT-PCR was performed to measure transcript levels of bmp2b (A) or hepcidin (B), normalized to β-actin transcript levels and measured as fold increase over control, WT, -HS at 2 hours post-treatment. Data shown are means ± SE. * indicates p<0.05, compared to control. N = 2 pools per group. WT, -HS (pink circles), WT, +HS (orange squares), transgenic, -HS (light green triangles), transgenic, +HS (dark green triangles). C–E. Immunohistochemistry for P-Smad1/5/8. Compared to zebrafish embryos without BMP2b induction (C), P-Smad1/5/8 staining is increased in the liver (arrow) in tg(hsp70: BMP2b) embryos following heat shock (D). Omitting the primary antibody (anti-P-smad1/5/8), but including the biotinylated anti-Rabbit IgG/streptavidin horseradish peroxidase resulted in very low levels of background staining (E). N = 20 embryos per group. F,G. Inhibition of hepcidin expression by dorsomorphin (F) or noggin3 (G). F. From 28–55 hpf, pools of tg(hsp70: BMP2b) embryos were treated with the BMP inhibitor, 40 μM dorsomorphin (+Dorso), or treated with an equivalent amount of DMSO vehicle alone (+DMSO). Half the pools of embryos were subjected to heat shock at 48 hpf to induce bmp2b expression, followed by fixation at 55 hpf for quantitative real-time RT-PCR. G. Pools of embryos carrying tg(hsp70:noggin3) were subjected to heat shock or no heat shock at 48 hpf. The embryos were fixed at 55 hpf for quantitative real-time RT-PCR. Data shown are means ± SE. N = 4–5 pools per group. * indicates p<0.05, compared to no heat shock and no dorsomorphin treatment. # indicates p<0.05 compared with previous column. doi:10.1371/journal.pone.0014553.g001
of hepcidin expression in the liver and foregut (Figure 3B) by whole mount in situ hybridization, while treatment with dorsomorphin from 28–55 hpf (Figure 3C) abrogated hepcidin expression. Transgenic induction of the BMP antagonist noggin3 at 48 hpf produced an equivalent effect (data not shown). While early BMP signaling is important for embryonic liver development,[22], induction of bmp2b at 48 hpf, which is after specification of the liver, did not increase liver size (Figure 3D,E), as assessed by whole mount in situ hybridization for foxa3 [forkhead box a3], a gene expressed in zebrafish embryonic liver tissue.[23]. Treatment with the BMP signaling inhibitor dorsomorphin from 28–55 hpf, also failed to decrease liver size (Figure 3F).

Knockdown of hjv fails to impair hepcidin expression at 55 hpf

To evaluate whether hjv is required for hepcidin expression, we injected hjv MO1 or hjv MO2, and assessed hepcidin expression by in situ hybridization. Compared to uninjected controls (Figure 3A), neither hjv MO1 nor hjv MO2 (Figure 3G,H) exhibited decreased hepcidin expression, although the liver was slightly reduced in size (Figure 3J,K). As knockdown of hjv produced developmental defects, we evaluated the effects of hjv deficiency on hemoglobin production and found that hjv knockdown did not produce anemia (Figure S2). To test whether hjv is required for the stimulatory effect of bmp2b on hepcidin expression, we injected hjv MO2 in tg(hsp70b:bmp2b) embryos at the one cell stage, followed by heat shock at 48 hpf and fixation at 55 hpf. Induction of bmp2b still enhanced hepcidin expression, despite knockdown of hjv (Figure 3I). Quantitative realtime RT-PCR (Figure 3M) revealed an 8-fold increase in hepcidin transcript levels (8.41±2.54.99 vs 10.14±4.93, p = 0.029) in hjv morphants following heat shock compared to morphants without heat shock. To confirm that the hjv gene was effectively knocked down in the hjv zebrafish morphant embryos, we extracted RNA from hjv-MO2 injected embryos and amplified the predicted splice site by RT-PCR. We found that the amplified region in the hjv morphants was shorter than in uninjected controls (Figure 3N). We cloned and sequenced the amplified product from the morphants and uninjected controls and confirmed that the morphant transcript bypasses the exon donor targeted by hjv-MO2 in favor of an aberrant splice from nucleotide 25 to 104 of the coding sequence. The predicted translation of this aberrant spliceform lacks amino acids 9 through 35, which is the majority of the signal peptide, as predicted by the algorithm PrediSi[24].

Neoerin and furin have been shown to interact with hjv to regulate hepcidin transcription in mammalian models.[8,14] In zebrafish embryos, neoerin has previously been shown to be required for normal somite development,[25], while the two zebrafish furins, furina and furinb participate in pharyngeal cartilage development.[26] We generated knockdowns of neoerin or of both furina and furinb, which did not exhibit impaired hepcidin expression or abnormal liver size at 55 hpf (Figure S3), although these knock downs reproduced the published developmental phenotypes (Figures S4 and S5).

Hjv is weakly expressed in the zebrafish embryonic liver

To determine why knockdown of hjv- or related genes did not impair hepcidin expression, we evaluated a time course of hjv expression in zebrafish embryos by whole mount in situ hybridization. As previously reported,[27] we found that hjv is strongly expressed in the notochord at 11 hpf (Figure 4A) and in the developing somites at 18 hpf (Figure 4B), prior to the onset of liver development. We discovered that hjv was not detectable by in situ hybridization at 50 hpf, 72 hpf, or 7 days post-fertilization (dpf) (Figure 4C-E), in contrast to hepcidin (Figure 3) or transferrin-a ([18] and Figure 4F), which are evident in the liver.

By bioinformatic analysis, we identified three other members of the repulsive guidance molecule family in the zebrafish, RGMα, RGMβ, and RGMδ. We found that none of these paralogs of hjv were expressed in the zebrafish embryonic liver (Figure S6). Knockdown of each of them failed to impair hepcidin expression at 55 hpf (Figure S7). At 72 hpf (Figure S8), hepcidin transcript levels were normal in the RGM morphants, although liver development was impaired in RGMβ and RGMδ morphants.

To verify whether there was weak hjv expression in the liver during embryogenesis, which was undetected by in situ hybridization, we used a fluorescence activated cell sorter to sort hepatocytes from transgenic embryos, which expressed GFP under the control of the liver specific liver fatty acid binding protein (L-FABP) promoter. RNA was obtained from sorted (GFP+ and GFP−) and from unsorted cells for RT-PCR. GFP+ cells strongly expressed L-FABP, relative to β-actin (Figure S9A). In the sorted cells, hjv expression was below the detection level in a quantitative real-time PCR assay. We performed semi-quantitative RT-PCR, which

Figure 2. Morpholino knockdown of hjv results in notochord and somite abnormalities. A–E. Light microscopy of zebrafish embryos at 15 hpf in dorsal view. Compared to uninjected embryos (A) or embryos injected with a mismatch control morpholino (E), embryos injected with either single hjv morpholinos (B, C) or a combination of hjv MO1 and hjv MO2 (D) exhibited a distorted notochord (arrows). N = 30 per group. F, G. Whole mount in situ hybridization at 18 hpf with no tail, which stains the notochord, illustrates the bent shape in the hjv MO1 injected morphants. N = 15 per group. H–J. Light microscopy of the tail at 24 hpf lateral view (top) with additional 3.5x enlargement of area labeled in red (below). Somites (arrows) in uninjected (H) and control morpholino-injected embryos (I) appeared V-shaped, while somites appeared U-shaped with decreased anterior-posterior dimension (distance between each pair of arrows) in hjv morphants (J). N = 20 per group.

doi:10.1371/journal.pone.0014553.g002
revealed weak expression of hjv in both GFP+ and GFP− cells (Figure 4G). Comparing GFP+ to unsorted cells, the hjv expression was diminished in a similar proportion to that for the hepcidin transcript, and is thus consistent with hepatic expression, although at low levels. In contrast, hepcidin expression was evident only in the GFP+ cells and transferrin was detectable in both populations. In adult zebrafish, hjv transcripts were detected by RT-PCR in both skeletal muscle and liver (Figure 4G), similar to the adult human hjv expression pattern.[3] We also found that neogenin and the zebrafish paralogs of hjv were expressed in the adult zebrafish liver (Figure S9B). These data indicate that, hemojuvelin is a developmentally regulated gene, which exhibits low levels of expression in zebrafish embryonic hepatocytes, consistent with the hjv-independent regulation of hepcidin that we observed in the zebrafish embryo (Figure 3).

Overexpression of zebrafish hjv fails to increase hepcidin expression in zebrafish embryos

To test the hypothesis that zebrafish hjv fails to regulate hepcidin expression during embryonic development because it is only weakly expressed in the embryonic liver, we injected zebrafish hjv cRNA at the one-cell stage and assessed hepcidin and foxa3 expression at 55 hpf. Compared to uninjected embryos, hjv overexpression failed to increase hepcidin expression (Figure 5A–D). Quantitative real-time RT-PCR for hepcidin expression at 72 hpf normalized to β-actin (Figure 5E) or to LFABP (Figure 5F) failed to show an increase in hepcidin expression in embryos injected with hjv cRNA. To overcome concerns about potential degradation of the cRNA during development, we also injected at the one-cell stage a DNA construct (pHjv-CS2) containing the zebrafish hjv gene in the pCS2 vector under the control of a ubiquitous promoter. Similar to the results in Figure 5E, we found no significant increase in hepcidin expression in the transgenic embryos compared to embryos injected with the pCS2 vector alone (Figure S10).

Zebrafish hjv induces hepcidin expression in human hepatocytes

As overexpression of zebrafish hjv failed to increase hepcidin expression in the zebrafish embryos, we questioned whether zebrafish hjv functions as a BMP co-receptor. To evaluate this, we
Knockdown of *matriptase-2* increases *hepcidin* expression in a BMP dependent manner

As zebrafish *hvj* functioned as a BMP co-receptor in vitro and the message appeared to be present at a low level in embryonic hepatocytes, we hypothesized that *matriptase-2* (*mtp2*) may be inhibiting the effect of *hvj*. Morpholino knockdown of *mtp2* has previously been shown to induce anemia in zebrafish embryos,[17] although *mtp2*‘s effect on *hepcidin* expression and the genetic interaction between *mtp2* and *hvj* in zebrafish embryos have not been evaluated previously. Compared to un.injected embryos (Figure 6A), we found that *mtp2* morphants exhibited decreased hemoglobin staining (Figure 6B) at 72 hpf. We also observed a delay in development in the *mtp2* morphants, characterized by a large yolk, decreased embryo size, and decreased melanocyte pigmentation (Figure 6B).

To evaluate the potential interaction of *mtp2* with the BMP pathway and *hvj*, embryos were injected at the one cell stage with *mtp2* MO, *hvj* MO2, or co-injected with *hvj* MO2 and *mtp2* MO, and fixed at 55 hpf for whole mount *in situ* hybridization with probes for *hepcidin* or *foxa3*. Compared to uninjected embryos (Figure 6C) or *hvj* morphants (Figure 6D), *mtp2* morphants exhibited increased staining intensity for *hepcidin* in the foregut, but a smaller area of staining in the liver (Figure 6E). Co-injection of *hvj* MO1 and *mtp2* MO exacerbated the growth retardation, but the embryos exhibited similar *hepcidin* expression in the liver and foregut (Figure 6F) to *mtp2* MO alone. Dorso morphin treatment from 28–55 hpf abrogated *hepcidin* expression in both uninjected embryos (Figure 6G) and in *mtp2* morphants (Figure 6H) indicating that *mtp2* knockdown stimulates *hepcidin* expression in a BMP-dependent manner. Staining with *foxa3* revealed decreased liver size in all the embryos injected with *mtp2* MO (Figure 6I, J, and L), compared to dorso morphin treatment alone (Figure 6K) or no treatment (Figure 5C).

Knockdown of *mtp2* increases *hepcidin* expression relative to liver size

As knockdown of *hvj* and *mtp2* altered embryonic development, we evaluated the effects at 72 hpf to verify if they were similar to those observed at 55 hpf. In comparison to uninjected embryos (Figure 7A), *hvj* morphants (Figure 7B) and *mtp2* morphants (Figure 7C) exhibited smaller areas of *hepcidin* staining at 72 hpf, which correlated with decreased liver size in the morphants, particularly of *mtp2* (Figure 7D–F). The decrease in liver size was supported by quantitative real-time RT-PCR for the liver specific marker, *LFABP* (Figure 7G), which revealed that *LFABP* levels were <10% of normal in *mtp2* morphants (0.08±0.036 vs 1.02±0.069, p = 0.025). In contrast, knockdown of *furina* and *furinb* failed to reduce *LFABP* expression, while knockdown of *hvj* or *neogenin* produced approximately 50% reduction. Quantitative real-time RT-PCR at 72 hpf to assess *hepcidin* transcript levels relative to β-actin revealed a decrease in *hepcidin* expression in the *mtp2* morphants (Figure 7H), consistent with the small size of the liver. Normalizing to the liver specific gene, *LFABP*, however, revealed that *mtp2* morphants exhibited a significant increase in *hepcidin* transcript levels compared to uninjected (Figure 7I) (5.31±2.3 vs 0.96±0.04, p<0.05), consistent with increased transcript levels of *hepcidin* in a smaller number of hepatocytes. In contrast, transcript levels of *hepcidin*, normalized to *LFABP*, for morphants of *hvj*, *neogenin*, and *furina*/*furinb* were not significantly different from uninjectected controls. Co-injection of morpholinos for *hvj* and *mtp2* failed to reduce *hepcidin* transcript levels relative to *LFABP* (Figure 7I), indicating that *mtp2*‘s effect on *hepcidin* expression does not require *hvj*.

cotransfected human hepatocytes (Hep3B cells) with increasing doses of zebrafish *hvj* cRNA and a reporter construct containing the human *hepcidin* promoter upstream of Firefly luciferase. Increasing doses of zebrafish *hvj* were associated with stronger induction of the human *hepcidin* promoter, which was potentiated by the addition of BMP6 (Figure 5G). Similarly, cotransfection of zebrafish *hvj* cRNA with a reporter construct containing a BMP response element upstream of luciferase, revealed a dose dependent increase in promoter activity, which was enhanced by the addition of BMP6 (Figure 5H).
Hjv knockdown fails to increase embryonic nonheme iron stores

Ferroportin is localized to the yolk syncytial layer in the zebrafish embryos, where it facilitates the transfer of iron from the yolk into the embryo.[28] We expected that if hjv has a significant effect on zebrafish embryonic iron homeostasis, hjv morphants would exhibit decreased hepcidin protein levels, which would result in increased ferroportin activity at the yolk syncytial layer and increased embryonic iron stores. Conversely, we expected hjv overexpressing embryos or mtp2 morphants to exhibit decreased embryonic iron stores, secondary to elevated hepcidin levels. We performed staining for nonheme iron at 55 hpf to evaluate these hypotheses (Figure 7J–Q) and found that knock down of hjv resulted in a normal level of iron staining (Figure 7J,L), while overexpressing hjv increased iron staining in the terminal gut (proctodeum) and somites (Figure 7M), rather than decreasing iron staining. Interestingly, we observed increased iron staining in the
somites, proctodeum, brain, and dorsal spinal cord of the mtp2 morphants (Figure 7J,N) in a pattern of iron accumulation resembling that seen in the erythroid transferrin receptor mutant chianti (Figure 7J,O), which has a defect in erythroid iron assimilation. The iron accumulation in the mtp2 morphants differed from the decreased embryonic iron staining observed in transferrin-a deficient gavi (Figure 7J,P) and ferroportin deficient weissherbst (Figure 7J,Q) mutants. Furthermore, treatment with dorsomorphin from 28–55 hpf abrogated hepcidin expression in both uninjected embryos (G) and mtp2 morphants (H). I–L. Whole mount in situ hybridization for foxa3 demonstrated smaller liver size (arrowhead) in embryos injected with mtp2 MO (I,J,L), compared to dorsomorphin alone (K) or untreated embryos (compare with Figure 3D). N = 20–30 embryos per group.

doi:10.1371/journal.pone.0014553.g006

Discussion

We have performed the first detailed analysis of embryonic regulation of hepcidin and the role of hjv during embryonic development. Previously we demonstrated that hepcidin transcript levels in zebrafish embryos increase in response to iron loading[6] and that onset of hepcidin expression requires the function of transferrin-a and transferrin receptor 2[18]. In this study, we found that, as in mammalian models[9,15,20], hepcidin regulation was responsive to BMP signaling, however, hjv (a BMP co-receptor), and the putative hjv interacting genes, furin and neogenin, were not required for hepcidin expression in zebrafish embryos. We discovered that knockdown of matriptase-2 (mtp2), a protease which cleaves membrane-bound hjv[17], produced anemia, accumulation of intraembryonic iron, and increased hepcidin expression in zebrafish embryos, however, surprisingly, mtp2’s effect on hepcidin expression was independent of hjv. Thus the zebrafish embryonic model of hepcidin regulation (Figure S12) differs from the mammalian model, which was derived from in vitro studies, human patients, and post-natal animal models. Further studies will be needed to determine if hepcidin regulation in mammalian embryos resembles that observed in zebrafish embryos.

BMP signaling is required for hepcidin expression in zebrafish embryos

Using a heat shock inducible transgenic zebrafish, we found that induction of bmp2b increased hepcidin expression and phosphory-
but increased iron staining (black arrows) in the somites and proctodeum (terminal gut) of weissherbst (hjv) or to increase intraembryonic iron stores. Further supporting an hybridization at 72 hpf for decreased intraembryonic iron staining was observed in the regulation of bmp2b functions as a BMP co-receptor, can activate the human hepcidin promoter in vitro, and is expressed, together with hjv in the zebrafish adult liver. We do not have a model for hjv deficiency in adult zebrafish to test this hypothesis. The effect of a morpholino injection dissipates after 4 days of development.

Mtp2 knockdown increases hepcidin expression independent of hjv

We found that the zebrafish mtp2 morphant embryo exhibits increased hepcidin transcript levels relative to the size of its liver and that this effect on hepcidin expression is not impaired by knockdown of hjv. This contrasts with mouse models in which crossing mice deficient in matriptase-2 with mice deficient in hjv suppresses elevated hepcidin (HAMP) transcript levels and the microcytic anemia associated with matriptase-2 deficiency in mice 9–15 weeks of age.[42]

Anemia in mtp2 morphant zebrafish embryos has been attributed to the effect of excessive hepcidin production[17], however we found that abrogation of hepcidin expression by treatment with dorsomorphin failed to reverse anemia in mtp2 morphants (Figure S11A–H). Furthermore, mtp2 morphants exhibited decreased gata1 staining, consistent with a decrease in the number of erythroid progenitor cells (Figure S11I,J). The mtp2 morphants also displayed increased intraembryonic iron staining, particularly in the somites, brain, and spinal cord, consistent with the erythroid transferrin receptor deficient phenotype (Figure 7O), which is characterized by normal iron transport from the yolk to the embryo, but ineffective transport to the erythrocyte.[43] Thus it seems likely that mtp2 knockdown produces anemia in zebrafish embryos by decreasing erythroid progenitor development. This, in turn, impairs erythroid iron assimilation, which results in intraembryonic iron loading and an increase in hepcidin transcript levels.

The regulation of hepcidin has clinical importance for patients with hemochromatosis and thalassemia, who exhibit inappropriately low levels of hepcidin despite the presence of iron overload[44–46]. Improving our understanding of hepcidin regulation holds promise for better therapies for these patients. The zebrafish embryo has proved a useful tool for identifying and characterizing the function of genes involved in iron metabolism[28,47–49] and elucidating the role of transferrin and transferrin
As hjv does not appear to play a role in hepcidin regulation in zebrafish embryos, the system will be most useful in identifying hjv-independent regulators of hepcidin transcription. Future studies will be needed to determine if hjv regulates hepcidin expression during mammalian development.

Materials and Methods

Ethics statement

Ethical approval was obtained from the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center (Animal Welfare Assurance #A3153-01) in accordance with national and international guidelines. Beth Israel Deaconess Medical Center maintains full accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care. Zebrafish strains, maintenance and determination of genotype. Zebrafish were maintained as described,[50] Tg(hsp70:bmp2b) and tg(hsp70:noggin3) zebrafish are described elsewhere[19]. Heterozygote carriers of tg(hsp70:bmp2b) or tg(hsp70:noggin3) were identified by crossing with WT zebrafish, subjecting the progeny embryos at the shield-stage to heat shock at 37 °C for 40 min, and assessing the percentage of ventralized or dorsIALIZED embryos produced.[19] Hypochromic anemia mutants used included chianti (zia1[Ta26]), gati (gat[Tt025]), and weissherbst (zeh[Ta35c]) [6,18,28,43].

Bioinformatics

Alignments were generated using ClustalW and Muscle[51,52], followed by manual refinement using SeaView[53] to remove redundant and improperly annotated sequences. For additional details, please see Figure S6.

Morpholino Injection, cRNA injection, and Heat Shock

Antisense morpholino oligonucleotides[54], obtained from Gene Tools, Inc. (Philomath, OR), were designed either to interfere with translation or to impair appropriate splicing of transcripts. Morpholinos for hjv, RGMa, RGMb, RGMd, noggin[25], faru[h26], farub[26], and matriptase-2[17] (Table S1) were injected at the one-cell stage with 3 nl in 1x Danieau medium, supplemented with phenol red. The aberrant splice produced by injection of hjv MO2 was cloned by PCR amplification with the primers (5'-TCAGTTGGCTCGAGCTT-CAG-3' and 5'-CCAAACCTGGCGACTATTAT-3'), cloned into the plasmid pCR2-TOPO (Invitrogen, Carlsbad, CA), digested with NotI and sense-antisense riboprobes were generated for use in the in situ hybridizations: hemojuvelin, hepcidin[18], transferrin-α[19], foxa3[19], RGMa[27], RGMb[27], RGMd, no tail (gift of G. Begemann), myoD (gift of V. Laudet) and gata1[29]. Representative embryos were photographed at 100x magnification with a BX51 compound microscope (Olympus) and a Q-capture 5 digital camera (QImaging, Surrey, BC, Canada). Images were processed using Adobe Photoshop software. Scale bars represent 100 microns, unless otherwise indicated.

Whole mount embryo staining for cartilage, hemoglobin, and iron

Staining for cartilage was performed with Alcian blue at 5 days post-fertilization following fixation in 4% paraformaldehyde-PBS, as described.[57] Live anesthetized embryos were stained for hemoglobin with o-dianisidine, as described.[30] Diaminobenzidine (DAB) enhanced-staining for ferric iron was performed as described[59] following fixation in 4% paraformaldehyde-PBS. Photomicrographs of representative embryos were obtained using an SZX51 zoom stereomicroscope (Olympus) at 40x magnification with a DP-71 camera (Olympus).

Quantitative analysis of gene expression

At specified time points, embryos were pooled in groups of 20, anesthetized with tricaine, and placed in RNAlater (Ambion). RNA extraction, generation of cDNA, and quantitative real-time RT-PCR assay were performed as previously described.[6,60] Detection and analysis were performed on an ABI 7000 and an ABI 7700 (Applied Biosystems, Inc.). Data presented are the means and standard errors. N=2–8 pools per time point or condition. For additional details, please see supplemental Methods S1.

Flow cytometry

Transgenic embryos expressing green fluorescent protein (GFP) under the control of the zebrafish liver fatty acid binding protein (L-EBP) promoter (tg(LEBP::GFP)) were a gift from W. Goessling. The embryos were manually dissociated in 0.9% PBS and sorted for fluorescence using a 488 nm laser with a FACSAria II (BD Biosciences, San Jose, CA). N=80–100 embryos for each sorting.

Biostatistical Analysis

Heterogeneity among cohorts was analyzed by ANOVA using Prism 5 (GraphPad Software, Inc., San Diego, CA). Tests for
heterogeneity used the natural log for assessment of transcript levels. All estimates and standard errors presented have been converted back to the original units. When the global P-value obtained from the ANOVA analysis was statistically significant, pairwise comparisons between the cohorts were performed using two-tailed Student’s t-tests with a Bonferroni correction for multiple comparisons. P values less than 0.05 were deemed statistically significant and are indicated by an asterisk.

Luciferase Assays

Human hepatoma (Hep3B) cells were cultured in Dulbecco’s Modification of Eagle’s Medium (Cellgro, Mediatech Inc., Virginia) supplemented with 10% Fetal Bovine Serum at 37°C in 5% CO2. All transfections were performed with Lipofectamine-2000 (Invitrogen Life Technologies, Carlsbad, CA). Hep3B cells were transiently transfected with zebrafish hjv cRNA (0–5000 ng) and pGL2-2.7 Hepc, a 2.7 kb fragment of the human hepcidin promoter upstream of the Firefly luciferase reporter gene, or a plasmid containing the BMP response element (BRE) upstream of a Firefly luciferase reporter gene.[9] A control pRL-TK Renilla luciferase reporter (Promega, Madison, NY) was also transiently transfected simultaneously, to control for transfection efficiency. The cells were incubated in the presence or absence of BMP6 (5 ng/ml) (R&D Systems, Minneapolis, MN) for sixteen hours and then lysed. The luciferase activity was determined with the Dual Reporter Assay (Promega, Madison, NY).

Supporting Information

Table S1

Found at: doi:10.1371/journal.pone.0014553.s001 (0.07 MB PDF)

Methods S1

Found at: doi:10.1371/journal.pone.0014553.s002 (0.20 MB PDF)

Figure S1

Treatment with dorsomorphin decreases BMP2b-induced phospho-smad1,5,8 staining in zebrafish embryos. Tg(hsp70:bmp2b) embryos were fixed at 55 hpf for immunohistochemical staining for phospho-smad1,5,8 following (A) no heat shock, (B) no chemical treatment (−HS, −dorso), (C) heat shock, but treatment with dorsomorphin (−HS, +dorso), (D) heat shock and no chemical treatment (+HS, −dorso), (E) heat shock and treatment with dorsomorphin (+HS, +dorso), representative embryos lateral view. Heat shock was performed at 48 hpf. Dor somorphin treatment was performed from 28–55 hpf at a concentration of 40 μM. For enhanced sensitivity, a fluorescently-labeled secondary antibody was used (Alexa Fluor® 488 goat anti-rabbit IgG, Invitrogen, #A-11008). Embryos were illuminated with an X-cite Series 120 PC microscope lamp (Exfo Life Sciences and Industrial Division, Quebec, Canada) and emitted light was filtered with a green fluorescent protein (GFP) filter set. N = 15–22 embryos per group.

Found at: doi:10.1371/journal.pone.0014553.s003 (2.47 MB TIF)

Figure S2

Knock down of hjv fails to produce anemia in zebrafish embryos. O-dianisidine staining for hemoglobin in uninjected (A) and hjv morphants (B,D). Whole mount in situ hybridization for hjv at 50 hpf (B) and 72 hpf (C, black arrows). At 50 hpf (D) and 72 hpf (E), hjv is faintly expressed in the mid and hindbrain (black arrows). At 50 and 72 hpf, hemojuvelin is no longer detected in the developing liver. Strong staining was detected in the mid and hindbrain for RGMa at 50 hpf (B) and 72 hpf (C, black arrows). At 50 hpf (D) and 72 hpf (E), RGMb is faintly expressed in the mid and hindbrain (black arrows). At 50 and 72 hpf, hemojuvelin is no longer detected in the developing embryo by in situ hybridization (F,G). At 50 hpf, RGMd transcripts were detected in the pharyngeal arches (H, black arrow). RGMd expression was no longer detected at 72 hpf (I). N = 20 embryos per group. [J] Phylogenetic tree of the RGM gene family constructed with all available vertebrate sequences. Note that hjv is expressed in a wide range of mammals, fish, and in Xenopus. We have identified hjv in the genome of a bird, the zebra finch (arrow), for the first time. RGMd has only been identified in fish. To generate the tree shown, we downloaded the protein sequences of the RGM gene families defined in the Ensembl database version 52 (as of December 2008) (<http://www.ensembl.org/>) which includes the hjv sequences. In addition to the Ensembl data, which also includes the Uniprot

Figure S3

Knock down of hjv interacting proteins, neogenin or furin, fails to decrease hepcidin expression. Whole mount in situ hybridization for hepcidin (A,D, blue arrow) and foxa3 (D-F, black arrowhead) in uninjected embryos (A,D), compared to embryos injected with neogenin MO (B,E) or morpholinos directed against both zebrafish furins (furina and furinb) (C,F), dorsolateral view. N = 20 embryos per group.

Found at: doi:10.1371/journal.pone.0014553.s005 (0.98 MB TIF)

Figure S4

Neogenin knockdown reproduced the reported defect in somitogenesis associated with neogenin deficiency. A,B. Whole mount in situ hybridization for myod1 to stain the somites in uninjected (A) and neogenin morphants (B) at the 20 somites’ stage of development (dorsal view) confirmed that injection of the neogenin morpholino at 0.15 mM produced elongation of the somites, manifest by increased distance between the two arrowheads. This is characteristic of the neogenin deficient phenotype, as described by [4]. Scale bar represents 100 microns. C,D. Whole mount in situ hybridization for hepcidin at 72 hpf in uninjected control embryos (C) and neogenin morphants (D) (lateral view) revealed a shortened body axis with a curved tail and flattened somites (arrowhead) in the neogenin morphants. Hepcidin expression is present in the liver (arrow) of the neogenin morphant, although the expression domain of hepcidin is smaller than in the uninjected control. Scale bar represents 200 microns. N = 20 embryos per group. Embryos were photographed at 100x magnification with an axio imag 1 compound microscope (Carl Zeiss Micr Imageing, Inc., Thornwood, NY) and an AxioCam ICc1 digital camera (Carl Zeiss MicroImaging, Inc.)(A,B) or a BXS1 compound microscope (Olympus, Center Valley, PA) and a Q-capture 5 digital camera (QImaging, Surrey, BC, Canada) (C,D).

Found at: doi:10.1371/journal.pone.0014553.s006 (3.73 MB TIF)

Figure S5

Whole mount Alcian blue staining for cartilage in zebrafish embryos at 5 days post-fertilization confirms a branchial arch phenotype in furin morphants. Doros lateral view of the head of an uninjected control embryo (A) and an embryo injected with morpholinos to knock down furina and furinb (B) reveals an open mouth phenotype (arrow in B) in the furina/furinb morphant. Lateral view of an uninjected control (C) and a furina/furinb morphant showing the fused cartilage elements (arrowhead in D) characteristic of furin morphants. N = 20 embryos per group.

Found at: doi:10.1371/journal.pone.0014553.s007 (3.46 MB TIF)
group. The liver (arrowhead) revealed a slight reduction of liver size in the
(A), knockdown of RGMa (B), RGMb (C), or RGMd (D) failed to
inhibit hepcidin expression (arrow). E–H. Expression of foxa3 in the
liver (arrowhead) revealed a slight reduction of liver size in the
morphants (F–H) compared to control (E). N = 20 embryos per
group. 

Figure S7 Effect of morpholino knockdown of RGM genes at
55 hpf. Whole mount in situ hybridization for hepcidin (A–D) or
fox3 (E–H), dorsolateral views. Compared to uninjected controls
(A), knockdown of RGMa (B), RGMb (C), or RGMd (D) failed to
inhibit hepcidin expression (arrow). E–H. Expression of foxa3 in the
liver (arrowhead) revealed a slight reduction of liver size in the
morphants (F–H) compared to control (E). N = 20 embryos per
group. 

Figure S8 Effect of knockdown of RGM genes at 72 hpf. Whole
mount in situ hybridization for hepcidin (A–D) or fox3 (E–H),
dorsolateral views. Compared to uninjected controls (A), knock-
down of RGMa (B), RGMb (C), or RGMd (D) failed to
inhibit hepcidin expression. E–H. Expression of foxa3 in the liver
revealed a significant reduction of liver size in the RGMb and
RGMd morphants (G, H). N = 20 embryos per group. I. Quantitative real-time RT-PCR revealed no significant decrease
in hepcidin transcript levels relative to liver fatty acid binding
protein (LFABP). N = 3 pools of embryos per group. Data shown
are means ± SE. 

Figure S9 Additional expression data for zebrafish embryonic
hepatocytes and zebrafish adult tissues. A. Quantitative real-time
RT-PCR to assess transcript levels of LFABP (liver fatty acid
binding protein) relative to β-actin in hepatocytes sorted from
pools of 80–100 transgenic zebrafish embryos at 72 hpf. N = 2
pools per group. Data shown are means ± SE. * indicates
p<0.05 compared to unsorted. B. Semiquantitative RT-PCR for
hepcidin, RGMa, RGMb, hjv, RGMd, and neogenin performed with
RNA from adult zebrafish liver and skeletal muscle. Hepcidin
expression was detected in the adult liver, but not in adult skeletal
muscle. All RGM genes and neogenin were detected in the adult
liver and skeletal muscle. 

Figure S10 Effect of injecting zebrafish hjv cDNA in zebrafish
embryos, pHJv-CS2 or pCS2 vector only (50 ng/microliter) were
each injected into zebrafish embryos at the one cell stage.
Quantitative real-time RT-PCR for hepcidin transcript levels
normalized to β-actin expression revealed no significant increase
in hepcidin expression at 55 hpf in embryos injected with pHJv-
CS2 cDNA compared to pCS2 vector alone. N = 5–6 pools per
group. Data shown are means +/- SE. 

Figure S11 Effect of dorsomorphin on anemia and iron loading
in mtp2 deficient embryos. Embryos were injected with mtp2
morpholino at the one cell stage, followed by treatment with
dorsomorphin from 28 hpf until fixation for either o-dianisidine
staining at 50 hpf (A–D) or whole mount nonheme iron staining at
55 hpf (E–H), lateral views. Uninjected controls (A) and embryos
treated with dorsomorphin (B) exhibited normal hemoglobin
staining, while mtp2 morphants (C) manifest decreased hemoglo-
bin staining, which failed to improve when mtp2 morphants were
treated with dorsomorphin (D). N = 54–99 embryos per group.
Compared to uninjected controls (E), embryos treated with
dorsomorphin (F), mtp2 morphants (G), or mtp2 morphants
-treated with dorsomorphin (H) exhibited increased iron staining in
the somites, brain, and dorsal spinal cord. N = 32–45 embryos per
group. (I,J) Whole mount in situ hybridization for gata1 (lateral
views) when embryos have developed 24 somites, about 22 hpf,
demonstrated decreased numbers of gata1-staining erythroid
precursors in mtp2 morphants compared to uninjected embryos.
N = 21–36 embryos per group. 

Figure S12 Comparison of the role of hemojuvelin in the
mammalian model of hepcidin regulation with the zebrafish
embryonic model. A. In the mammalian model of hepcidin
regulation, which is based on in vitro studies, human patients, and
post-natal animal studies[10–25], hjv acts as a BMP co-receptor to
promote BMP signaling, which results in increased hepcidin
transcription. Cleavage of membrane-bound hjv by matriptase-2
or furin results in the release of soluble hjv, which acts as a
competitive inhibitor for BMP signaling. B. In the zebrafish
embryonic model, which we have developed, BMP signaling
promotes hepcidin transcription independent of hjv. Matriptase-2
exhibits a BMP-dependent, but hjv-independent effect on hepcidin
expression. Stimulatory effects are shown by arrows. Repressive
effect is shown by |-.

Acknowledgments
We acknowledge the assistance of Jason Holzheimer, Sarah Burnett, Diana
Miao, Dr. Teresa Bowman, John Tigges and Vasilia Tocaxidhi of the Beth
Israel Deaconess Medical Center/Harvard Stem Cell Institute Research
Flow Cytometry Core, and Dr. Victoria Petkova of the Beth Israel
Deaconess Medical Center Real-time PCR Core. 

Author Contributions
Conceived and designed the experiments: YG PF. Performed the
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PF. Contributed reagents/materials/analysis tools: JB HL MH PF. Wrote
the paper: PF. 

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