Evidence That Inhibition of Hemojuvelin Shedding in Response to Iron Is Mediated through Neogenin

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Hemojuvelin (HJV), encoded by the gene HFE2, is a critical upstream regulator of hepcidin expression. Hepcidin, the central iron regulatory hormone, is secreted from hepatocytes, whereas HFE2 is highly expressed in skeletal muscle and liver. Previous studies demonstrated that HJV is a GPI-anchored protein, bind the proteins neogenin and bone morphogenetic proteins (BMP2 and BMP4), and can be released from the cell membrane (shedding). In this study, we investigated the physiological significance and the underlying mechanism of HJV shedding. In acutely iron-deficient rats with disruptions of both HFE2 mRNA or protein levels in gastrocnemius muscle. Studies in both C2C12 (a mouse myoblast cell line) and HepG2 (a human hepatoma cell line) cells showed active HJV shedding, implying that both skeletal muscle and liver could be the source of serum HJV. In agreement with the observations in iron-deficient rats, HJV shedding in these cell lines was down-regulated by holo-transferrin in a concentration-dependent manner. Our present study showing that knockdown of endogenous neogenin, a HJV receptor, in C2C12 cells suppresses HJV shedding and that overexpression of neogenin in HEK293 cells markedly enhances this process, suggests that membrane HJV shedding is mediated by neogenin. The finding that neither BMP4 nor its antagonist, noggin, was able to alter HJV shedding support the lack of involvement of BMP signaling pathway in this process.

Iron is an essential nutrient required for a variety of biochemical processes such as respiration, metabolism, and DNA synthesis. Cells and organisms possess tightly regulated but poorly understood mechanisms for iron absorption and metabolism. Iron homeostasis in the body is regulated primarily at the level of iron uptake through the intestine. Mutations in the key iron homeostatic proteins result in hereditary hemochromatosis (HH). HH is a heterogeneous group of inherited iron overload disorders linked to mutations in several genes including HFE, HFE2, HAMP, and TFR2 (1).

Hemojuvelin (HJV) is the most recently discovered protein critical to iron homeostasis. HJV is encoded by the gene simultaneously cloned in humans as HFE2 and in mice as RGMc, the third member of the repulsive guidance molecule family (2–5). All three members of the RGM family are GPI-linked proteins and co-receptors for BMP2 and BMP4 (6–11). RGMa and RGMb are expressed primarily in the developing and adult central nervous system in distinct, mostly non-overlapping patterns (3–5). By contrast, HFE2 mRNA is found predominantly in skeletal muscle and to a lesser extent in the liver (2). RGMa is a neuronal guidance molecule critical for proper brain development and binds neogenin, a multifunctional transmembrane receptor. The interactions between RGMa and neogenin are involved in the regulation of neuronal survival (5, 6, 12–14). The underlying mechanisms are not known.

Homozygous or compound heterozygous mutations of HFE2 cause juvenile hemochromatosis (JH), a particularly severe form of HH (15, 16). The central role of HFE2 in body iron homeostasis is supported by the most recent findings in mice with disruptions of both HFE2 alleles (Hjv+/−), showing a marked increase of iron deposition in liver, pancreas, and heart (17, 18). The severe suppression of hepatic hepcidin expression observed in both HFE2 mutation-related JH patients and Hjv+/− mice implies that HJV is a key upstream regulator of hepcidin expression. Hepsin, a central iron-regulatory peptidase hormone, plays a pivotal role in maintaining body iron homeostasis through down-regulating the iron exporter ferroportin (Fpn) (19). Fpn is responsible for the uptake of iron into the body from the intestine (1). In this manner, increases in hepcidin levels result in decreased absorption of dietary iron. A
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recent study indicates that HJV induces hepatic hepcidin expression through BMP-mediated signaling pathway by being a co-receptor for BMP2 and BMP4 (10). BMPs are cytokines of the TGF-β superfamily, which exhibit multiple roles in a wide variety of processes through different signaling pathways (20, 21). The underlying mechanism by which BMP signaling regulates hepatic hepcidin expression in response to body iron status still remains unknown.

In addition to BMPs, our previous studies showed that HJV also interacts with neogenin (7). Neogenin is a transmembrane protein widely expressed in different tissues including skeletal muscle and liver (22–24). It is the classical receptor for netrins as well as RGMα (25, 26). In skeletal muscle cell lines, studies demonstrated the involvement of neogenin in myotube formation (27). The interaction of HIV with neogenin increases iron loading into HEK293 cells (7). The role of this interaction in the maintenance of body iron homeostasis still remains to be resolved.

HFE2 mRNA is expressed highly in skeletal muscle and at relatively lower levels in liver (2). Hepatocytes are the main source of both HIV and hepcidin in the liver (18, 28, 29). The finding that liver HFE2 mRNA levels do not respond to high body iron status in mice, imply that the induction of hepatic hepcidin does not occur through transcriptional control of HFE2 (30). Although recent studies show that endogenous HJV expression is induced during the differentiation of C2 cells, a mouse myoblast cell line (11, 31), the function and the regulation of HJV in skeletal muscle are not known. HIV does not appear to play a major role in muscle development because individuals with JH do not have any obvious muscle abnormalities (2). The extent to which dysregulation of muscle HFE2 affects systemic iron metabolism remains unexplored.

A recent study reported the presence of HIV in human serum, demonstrated an iron suppressed-membrane HIV release (commonly called shedding) in HFE2-transfected-cells, and found that the soluble form of HIV competitively inhibits the induction of hepcidin expression through the action of membrane bound form of HIV (32). However, the origin of serum HIV and its physiological significance are unclear. In this study we investigated the response of serum HIV as well as HFE2 expression in skeletal muscle to various extents of iron deficiency using rats as a model. The underlying mechanism of HIV shedding, in response to iron, was studied using a mouse myoblast cell line, C2C12 that can be induced to differentiate into myotubes, a human hepatoma cell line (HepG2), and HEK293 cells. Our results indicate that membrane HIV shedding is a transferrin-regulated and neogenin-mediated process.

EXPERIMENTAL PROCEDURES

Generation of Iron-deficient Rats—Rats were made iron-deficient as previously described (33). Briefly, weaning male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were randomly assigned to two different categories with free access to a control diet (50 mg iron/kg diet, group control) or were pair-fed an iron-deficient (ID) diet (less than 2 mg iron/kg diet, group ID). Pair-feeding involves providing the control group with the same amount of diet consumed by the animals fed the iron-deficient diet on the previous day with the two groups on a staggered schedule differing by 1 day. Pair-feeding is a necessary control because animals fed the iron-deficient diet exhibit reduced food intake. Pair-feeding ensures the two groups of animals have equivalent energy intake but different iron intake. All animals had free access to water. Animals fed the diets for 1, 2, 3, 7, 14, or 21 days were anesthetized with isoflurane, and blood was collected by heart puncture for serum preparation. The animals were euthanized while under anesthesia by incising the diaphragm. Skeletal muscles (gastrocnemius and soleus) and liver were rapidly removed and snap-frozen in liquid nitrogen and then stored at −80 °C for qRT-PCR and Western blot analysis. There are either 4 or 5 animals per each group as indicated in the text. All procedures for animal use met the requirements of the University of Wisconsin Research Animal Resource Center.

Serum Iron Analysis and Liver Non-heme Iron Assay—Serum iron, serum total iron binding capacity (TIBC), and transferrin saturation were analyzed by Cornell University Veterinary Diagnostic Service. Quantitative measurement of non-heme iron in liver tissues was performed as described previously (17). Results are expressed as microgram iron per gram wet tissue.

Quantitative Real-time RT-PCR (qRT-PCR)—Total RNA from rat skeletal muscle and liver was isolated using TRIzol reagents (Invitrogen, Carlsbad, CA). Contaminating genomic DNA was removed by RNase treatment, followed by another cycle of RNA purification using RNeasy kit (Qiagen). cDNA preparation and qRT-PCR analysis were conducted essentially the same as previously described (29). The primers for rat HFE2 are 5′-TTCCATCTTGCCTCTTTGAT-3′ (forward) and 5′-GGAAAAGTGCAGTTCTCACA-3′ (reverse). The primers for rat neogenin are 5′-GGCACAGCACTGGCTTC-3′ (forward) and 5′-TGCCCTTCTTCTGACACCAAAATCT-3′ (reverse). All other primers used are the same as previously described (29). The result for each gene of interest is expressed as the amount relative to that of GAPDH in each specific sample.

Membrane HJV Shedding Analysis in Transfected Cells—Mouse myoblast cell line, C2C12, was obtained from Dr. Matt Thayer, OHSU, Portland, OR. Human hepatoma cell line, HepG2, and human embryonic kidney cell line, HEK293, were purchased from ATCC. There was no detectable endogenous HFE2 mRNA or HIV protein in HepG2, HEK293 and un-induced C2C12 cells by qRT-PCR or Western blot analysis, respectively (data not shown). C2C12 cells were cultured in DMEM/15% FCS, and transiently transfected in 60-mm plates with either human HFE2 cDNA or the empty vector pcDNA3 using Lipofectamine 2000 reagent (Invitrogen). To avoid the possible differences in transfection efficiency of different plates, we pooled the transfected cells after about 24 h of transfection, followed by subculturing the cells into 12-well plates in 1 ml of DMEM/10% FCS. After another 24 h or 48 h of incubation in the presence of 30 μM transferrin (TF) with different ratios of holo- to apo-TF, ferric ammonium citrate (FAC), 50 ng/ml BMP4 (R&D system), or 1 μg/ml noggin (R&D system) as indicated in the text, the conditioned culture medium (CM) was collected and cell lysate prepared using NET-Triton buffer (150 mM NaCl, 5 mM EDTA, and 10 mM Tris (pH 7.4), 1% Triton X-100) supplemented with protease inhibitors (Protease Inhibi-
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RESULTS

Iron-deﬁcient Diet Results in a Rapid Depletion of Serum Iron—We wanted to determine if the iron status of animals resulted in altered levels of HJV in the blood and to test whether serum HJV had physiological signiﬁcance. Taking advantage of the high dietary iron requirement of weanling rats, we generated animals with various extents of iron deﬁciency by feeding an ID diet (containing less than 2 mg of the iron/kg diet), as previously described (33), for 1, 2, 3, 7, 14, and 21 days. The results from the analysis of serum iron, TIBC and serum Tf saturation are summarized in Table 1. In comparison to rats fed a regular iron diet (control), animals fed the ID diet exhibited a rapid decline of serum iron concentrations, showing a progressive and stable decrease in serum iron for up to 21 days. In agreement with the low iron status, TIBC in all ID groups were gradually increased. The levels of total liver non-heme iron, an
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### Table 1

| Days post-ID diet | Serum iron* | TIBC* | Tf iron saturation* |
|-------------------|-------------|-------|---------------------|
|                   | Control     | ID    | Control             | ID         |
| µg/dL              | µg/dL       | µg/dL | µg/dL               | %          |
| 1                  | 392 ± 136   | 141 ± 77 | 551 ± 35           | 71 ± 22   |
| 2                  | 302 ± 84    | 40 ± 11   | 518 ± 28           | 53 ± 14   |
| 3                  | 448 ± 7     | 29     | 529 ± 20           | 84 ± 2    |
| 7                  | 285 ± 47    | 23 ± 4.5 | 492 ± 40           | 56 ± 8.4  |
| 14                 | 367 ± 21    | 21 ± 1   | 476 ± 9            | 77 ± 5    |
| 21                 | 429 ± 33    | 21 ± 1.4 | 550 ± 10           | 78 ± 7    |

* Serum iron levels in all ID groups are statistically lower than those in the corresponding controls. p < 0.001.  
* TIBC levels in all ID groups are statistically higher than those in the corresponding controls. p < 0.001.  
* Tf iron saturation levels in all ID groups are statistically lower than those in the corresponding controls. p < 0.001.

indicator of liver iron storage, did not change as rapidly as serum iron levels. There was no statistical difference in the total liver non-heme iron level in the ID group compared with the control group at day 3 (ID: 62.3 ± 9.3 versus controls: 74.9 ± 9.8 µg iron/g tissue, p = 0.1117). However, a robust increase of TfR1 protein levels was observed in the liver tissues from the animals of ID group at this time point (supplemental Fig. S1), implying a decreased labile iron pool. By day 14, however, a dramatic difference of liver non-heme iron levels was detected (ID: 53.0 ± 7.8 versus controls: 100.8 ± 25.1 µg of the iron/g tissue, p = 0.0108). Because of the relatively low liver iron storage in these young rats, the difference in non-heme iron levels between days 3 and 14 is mainly derived from the lack of accumulation of storage iron in the ID group. The initial decrease in hepatic hepcidin mRNA levels correlated with the decrease in serum iron levels. Quantitative analysis of hepatic hepcidin mRNA by qRT-PCR showed ~60- and 270-fold decrease in ID days 3 and 14 groups, respectively, compared with their corresponding control groups (results not shown). These results indicate that the reduced serum iron and/or hepatic labile iron pool could markedly down-regulate hepatic hepcidin expression (ID day 3). Because HIV is thought to be an upstream regulator of hepcidin expression, we sought to determine the role that muscle HIV could play in this process.

Iron Deficiency Does Not Alter HFE2 Expression in Skeletal Muscle—Skeletal muscle, accounting for about 35–40% of body weight, is likely the major source of HIV found in blood since skeletal muscle has the highest expression of HFE2 mRNA (2). Skeletal muscle is also a significant iron consumer as much as 3–4% of body iron can be found in myoglobin (37). To elucidate the role of HIV in skeletal muscle in the regulation of body iron homeostasis, we first examined the response of HFE2 mRNA in gastrocnemius muscle to iron deficiency in ID day 3 and day 14 rats by qRT-PCR (Fig. 1, A and C). In agreement with serum iron deficiency (Table 1), ID day 3 and 14 animals showed a significant increase of TfR1 mRNA by ~2.5 and 3.1-fold, respectively, in comparison with their corresponding controls. In ID day 3 animals, we showed no statistical difference of HFE2 and neogenin mRNA levels compared with controls. Similar results were obtained in soleus muscle from these same animals (data not shown). In addition, mRNA levels of other genes implicated in iron homeostasis including TFR2, HFE, and hepcidin mRNA in these tissues were also analyzed. As expected, we failed to detect significant expression of these genes in both control and ID rats in muscle (data not shown). Therefore, HFE2 mRNA levels in skeletal muscle are independent of body iron status.

To further examine the regulation of HFE2, we analyzed the protein levels of HIV, neogenin, and TfR1 by Western blot analysis (Fig. 1, B and D). Our rabbit anti-human HIV antibody cross-reacts with human, rat, and mouse HIV (data not shown). In agreement with the low iron status, TfR1 levels were strongly increased in gastrocnemius muscle of all rats on ID day 3 and 14 in comparison with the corresponding controls. However, no significant change of HIV and neogenin protein levels in ID day 3 and 14 was observed. In addition, no detectable change of HIV and neogenin protein levels was seen in liver tissues from the same animals (data not shown). These results, therefore, suggest that the expression of both HIV and neogenin proteins is not influenced by body iron status. Analysis of HIV in both tissues showed a predominant band of full-length HIV migrating at about 50 kDa (Fig. 1, B and D). Results, therefore, indicate that low body iron does not significantly affect HIV protein level in skeletal muscle (Fig. 1, B and D).

Low Serum Iron Induces an Early Phase Increase of Serum HIV—HIV is a GPI-anchored protein and undergoes shedding in HFE2 cDNA-transfected cells (7, 32). To explore the physiological significance of this process, we measured the levels of HIV in rat sera as a function of iron status. As shown in Fig. 2A, Western blot analysis showed a single HIV band migrating at about 50 kDa under reducing conditions in sera from ID day 3 and 14 rats, similar to the molecular weight of the full-length HIV in the cell lysate from HEK293-HIV.neo cells. Intriguingly, as the serum iron concentration decreased, the serum HIV exhibited a gradual and steady increase in rats fed an ID diet at least for the first 3 days in comparison with the corresponding controls. Quantitative analysis of proteins on Western blots revealed an increase in HIV levels by ~13, 34, and 150% for ID day 1, 2, and 3, respectively (Fig. 2B). The increases observed for ID day 2 and 3 were statistically significant. During this period of time, the consequences of dietary iron deficiency were mainly detectable in serum (Table 1). However, as the extent of iron deficiency was intensified in rats on ID diet for 7 days or longer, when iron-deficient anemia appeared (33), serum HIV was found to return to the control levels. Our results, therefore,
suggest that serum HJV levels are negatively regulated by serum iron solely under the conditions of no anemia.

Analysis of HJV Shedding in Cell Lines Indicates That Serum HJV Could Arise from Both Skeletal Muscle and Hepatocyte—Skeletal muscle and hepatocytes are the major sites of HJV expression in the body (2, 18). To determine the possible tissue origin of serum HJV, we chose C2C12 cells, a mouse myoblast cell line, and HepG2, a human hepatoma cell line, as model systems for skeletal muscle and hepatocytes, respectively. C2C12 cells are a rapid growing cell line when maintained in DMEM medium supplemented with 15% FCS, and do not express detectable endogenous HJV by Western blot analysis (Fig. 3A). C2C12 cells can be induced to differentiate to form contractile myotubes and express characteristic muscle proteins (34). First, we examined the HJV shedding in C2C12...
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A. C2C12 cells

| neo | C HJV |
|-----|-------|
| Tfr1 | |
| HJV (L) | |
| actin | |
| HJV (CM) | |

B. Differentiated C2C12 cells

| ud | d C2C12 |
|-----|---------|
| TF(μM) | FAC(μg/ml) |
| 0 | 10 | 30 | 10 |
| neo | |
| Tfr1 | |
| HJV (L) | |
| actin | |
| HJV (CM) | |

C. HepG2

| C HepG2 |
|----------|
| holo-Tf(μM) | FAC(μg/ml) |
| 0 | 1 | 10 | 20 | 30 | 10 | 25 | 50 | 100 |
| neo | |
| HJV (L) | |
| actin | |
| ferritin | |
| HJV(CM) | |

D. HJV in muscle & liver of rats

| muscle | liver |
|--------|-------|
| HJV | |
| actin | |

FIGURE 3. HJV shedding from C2C12 and HepG2 cells. A, HJV shedding from transfected-C2C12 cells. C2C12 cells were transiently transfected with either HFE2 cDNA (HJV) or pcDNA3 empty vector (C). At about 48 h after transfection, cell lysate and a fraction of conditioned medium (CM) were collected and subjected to SDS-PAGE under reducing and denaturing conditions. The levels of HJV (HJV (L)), neogenin (neo), Tfr1 and actin in cell lysates as well as HJV in the CM (HJV (CM)) were immunodetected with the corresponding antibodies. HJV detected in CM represents the fraction shed from the cell membrane. B, endogenous HJV shedding from differentiated C2C12 cells. Differentiation induction of C2C12 cells was initiated by switching the culture medium to DMEM/2% horse serum. Holo-Tf (0, 10, and 30 μM holo-Tf plus apo-Tf) was added to make a constant 30 μM of final total Tf. FAC was added to make a constant 10 μg/ml of final total FAC. At 72 h after induction, the levels of HJV (HJV (L)), neo, Tfr1 and actin in cell lysates were determined by Western blot analysis. All the experiments were repeated for at least three times with consistent results. D, Western blot analysis of HJV and actin in gastrocnemius and liver tissues from six control rats, in which three animals were from day 3 control group and the other 3 from day 14 control group as described in the legend to Fig. 1B. Experiments were conducted as described in the legend to Fig. 18.
expressed HJV in the differentiated C2C12 cells to holo-Tf and ferric ammonium citrate (FAC). In the cells treated with holo-Tf (0, 10 and 30 μM plus apo-Tf to make a constant 30 μM of total Tf) for 24 or 48 h, a concentration-dependent decrease of HJV was detected in CM, whereas the HJV amounts in cell lysates remained relatively constant (Fig. 3B). Quantitative analysis of HJV in CM revealed ~2.4- and 4.2-fold inhibition by holo-Tf at 10 and 30 μM, respectively. When cells were treated with FAC (10 μg/ml), we detected an evident decrease of TR1 level, suggesting an increased cellular iron loading. FAC, a widely used non-Tf iron source, bypasses Tf-mediated iron uptake pathway and directly loads iron into cells through an undefined mechanism. However, we did not find significant change of HJV shedding in the presence of FAC (Fig. 3B). Morphology analysis and Western blot analysis of cellular HJV show that neither holo-Tf nor FAC treatment affects the differentiation of C2C12 cells and the cellular HJV levels (supplemental Figs. S2 and 3B). These findings imply that a small fraction of membrane-bound HJV is shed from the cells. These results, therefore, support the concept that holo-Tf, rather than the intracellular iron status, determines the amount of HJV shedding.

To seek insight into the regulation of HJV shedding in hepatocytes, we further analyzed the response of membrane HJV shedding to both Tf and non-Tf iron sources in HFE2-transfected-HepG2 cells (HJV-HepG2). Consistent with the results observed in the differentiated C2C12 cells (Fig. 3B), we only detected a concentration-dependent inhibition of HJV release into CM by holo-Tf, but not in response to FAC even when added a high concentration (100 μg/ml) (Fig. 3C). Quantitative analysis revealed ~2-, 4-, and 6-fold decrease in the presence of 10, 20, and 30 μM holo-Tf, respectively. The level of HJV in cell lysate was not affected by prior treatment of cells with either holo-Tf or FAC (Fig. 3C), implying that only a small fraction of the HJV is shed from the cells. The doublet bands in the CM might result from the heterogeneous glycosylation in this cell line. There exist three consensus sequences for N-glycosylation in the coding sequence of HFE2 cDNA (7). Because both holo-Tf and FAC treatment could increase the cellular ferritin levels in a similar profile at the examined concentrations (Fig. 3C), these results indicate that the levels of holo-Tf, rather than the intracellular iron level, play a determinant role in the regulation of HJV shedding in HepG2 cells. The similar pattern observed in both differentiated C2C12 and HepG2 cells implies that both skeletal muscle and hepatocytes might share a common machinery in the regulation of HJV shedding.

Because our previous study showed that HJV interacts with neogenin (7), we next determined whether C2C12 and HepG2 cells express endogenous neogenin. As shown in Fig. 3, A, B, and C (first lane from the left), neogenin was readily detected in cell lysates by Western blotting. Interestingly, expression of HJV down-regulates neogenin protein levels dramatically in HepG2 cells (Fig. 3C). These results along with the lack of change in neogenin mRNA levels (Fig. 1 and data not shown) imply that expression of HJV increases the rate of neogenin protein turnover in this cell line.

HJV Shedding Depends on Neogenin—To address whether neogenin is involved in the regulation of HJV shedding, we employed siRNA to knockdown the endogenous neogenin in C2C12 cells. Only the specific siRNA to neogenin (neo siRNA) and transfection of HFE2 into C2C12 cells were coordinately conducted as described under “Experimental Procedures.” The untransfected (C) and the control siRNA transfected C2C12 cells (C siRNA) were used as negative controls. The HJV (HJV (L)), neogenin (neo), and actin levels in cell lysate and the amount of HJV in conditioned medium (HJV (CM)) during the period from 24 to 48 h post-transfection of HJV was detected as described in the legend to Fig. 3A. B, HJV shedding in HEK293 cells. The following cells were generated for this study: HEK293 cells stably transfected with the empty vector pcDNA3 (C), HEK293 cells transiently transfected with HJV alone (HJV), HEK293 cells stably transfected with both HJV and neogenin (HJV + neo), HEK293 cells stably transfected with G320V HJV alone (HJV G320V), and HEK293 cells with a stable transfection of neogenin and a transient transfection of G320V HJV (HJV G320V + neo). Cells were incubated with fresh complete medium supplemented with either 30 μM holo-Tf or 50 μg/ml FAC for 24 h. The amount of HJV in both cell lysate (HJV (L)) and CM (HJV (CM)) was detected as described in the legend to Fig. 3A. Two exposures of the westerns from CM are illustrated to show the effects of iron treatment on hemojuvelin shedding. The levels of neogenin (neo), TR1, and β-actin in cell lysate were also detected by Western blot analysis as described under “Experimental Procedures.” All experiments were repeated at least three times with consistent results.

![Hemojuvelin Shedding and Neogenin](image-url)
Hemjuvelin Shedding and Neogenin

HJV traffics to the cell surface, it does not bind directly to neogenin as assessed by co-immunoprecipitation (7). First we confirmed the previous finding that HJV undergoes shedding in HEK293 cells with endogenous neogenin (lane 2, Fig. 4B) and that the shedding is inhibited by holo-Tf (lane 2 versus 3, Fig. 4B) (32). However, we failed to detect significant effect of FAC on shedding in this cell line (lane 2 versus 4, Fig. 4B), which is similar to HepG2 and differentiated C2C12 cells (Fig. 3, A and B). Complementarily to the knockdown study (Fig. 4A), our results showed a significant increase of HJV shedding when neogenin was co-expressed (lane 2 versus 5, Fig. 4B). However, in G320V HJV-expressing cells, a much lower amount of HJV was detectable in the conditioned medium than in the cells expressing wild-type HJV, and the shedding was not significantly enhanced when neogenin was co-expressed (lanes 8–13, Fig. 4B). Further studies in HEK293 cells with a stable expression of neogenin and a transient expression of either wild type or G320V HJV indicate that neogenin has no significant effect on cellular HJV levels (supplemental Fig. S3). Because G320V mutation disrupts the interaction of HJV with neogenin but does not affect its trafficking to cell plasma membrane (7), these results indirectly indicate the requirement of the HJV-neogenin interaction in the process of holo-Tf regulated-membrane HJV shedding.

HJV Shedding in HFE2-transfected Cells Is Not Mediated through the BMP Signaling Pathway—A recent study demonstrates that HFE2 is a co-receptor for BMP2 and BMP4 and that it regulates hepcidin expression through BMP signaling pathway in hepatocytes (10). BMP signaling is activated upon BMP binding to BMP receptor complexes on cell surface, which triggers the sequential phosphorylation of Smad1, Smad5, and Smad8 in the cytoplasm. The phosphorylated Smads form hexameric complexes with Smad4 and then translocate into the nucleus to modulate gene transcription (10). To elucidate whether this signaling pathway is involved in the iron-regulated HJV shedding in HJV-transfected C2C12 and HepG2 cells, we tested the effects of BMP4 and noggin, a specific and physiological BMP antagonist, on this iron-regulated process. Addition of BMP4 robustly enhanced the levels of phosphorylated Smad1, 5, and 8, thus indicating an intact BMP-responsive signaling machinery in both cell lines (supplemental Fig. S4, A and B). In agreement with the previous finding (10), we detected about 3-fold induction and a 7-fold inhibition of hepcidin mRNA when HJV-HepG2 cells were treated overnight with 50 nM BMP4 or with 1 μg/ml noggin, respectively (results not shown). However, we failed to detect any significant effect of either BMP4 or noggin on holo-Tf-mediated down-regulation of HJV shedding into medium after 24 or 48 h of incubation. Furthermore, addition of holo-Tf or FAC in the parallel controls did not show any evident activation of Smad phosphorylation (supplemental Fig. S4, A and B). Thus, these results suggest that the membrane HJV shedding is not mediated through BMP signaling pathway.

DISCUSSION

In this study we found that serum iron deficiency in rats results in an early phase increase of serum HJV, but has no significant effects on the expression of HFE2 in skeletal muscle despite a robust down-regulation of hepatic hepcidin mRNA. Studies in C2C12 and HepG2 cells imply that serum HJV could be derived from both skeletal muscle and hepatocytes. HJV shedding in both cells is negatively regulated by holo-Tf. This process does not seem to be regulated through BMP signaling pathway. Rather, our data support that neogenin plays an important role in shedding.

HJV is a GPI-anchored protein (7, 11). GPI-anchored proteins can be shed from the membrane by a membrane secretase-like proteolytic cleavage, phospholipase cleavage of the GPI anchor moiety, or by both, and released in a soluble form from the cell membrane (38–40). Previous studies have reported a detectable soluble HJV in human serum and found an inhibition of HJV shedding by both Tf and non-Tf-iron in HFE2-transfected Hep3B and HEK293 cells (32). To address the physiological significance of this process, we examined the serum HJV levels in rats with various extents of iron deficiency. Intriguingly, our results revealed a reverse correlation between serum HJV concentration and serum iron levels in animals fed an ID diet for the first 3 days (Fig. 2), when the iron deficiency is only detectable in serum (Table 1). Examination of HFE2 mRNA and HJV in gastrocneumius muscle and liver ruled out that it is derived from its up-regulation of its expression (Fig. 1). These results are consistent with the observation that high iron does not alter HFE2 mRNA in mouse liver (30). The failure to detect any significant change of HJV in both skeletal muscle and liver imply that only a small fraction of the HJV is shed into the circulation. This is in agreement with the observations in C2C12 and HepG2 cells (Fig. 3, B and C). When the extent of iron deficiency is intensified in rats fed an ID diet for 7 days or longer, under the conditions that iron deficient anemia appears (33), serum HJV was found to return to control level (Fig. 2). This might be either because of the tissue adaptation to iron deficiency or due to the involvement of other inhibitory factors induced by anemia or iron depletion in liver. Therefore, serum HJV levels appear to be negatively regulated by serum iron in response to acute iron deficiency.

HJV is highly expressed in skeletal muscle, which accounts for approximate one-third of body weight. Studies in C2C12 and HepG2 cells suggest that both skeletal muscle and liver could be contributors of serum HJV in vivo (Fig. 3). Our findings that there is much more HJV protein in gastrocneumius muscle than in liver are in agreement with the Northern blot analysis in a previous study showing a much higher level of HFE2 mRNA in the former than in the latter (2). Because of the much greater mass and a higher HJV message level in skeletal muscle than in liver (2), we speculate that serum HJV is mainly derived from the skeletal muscle. Consistent with the observation in ID rats, we detected a negative regulation of HJV shedding by holo-Tf in the examined cell types, including HepG2, HEK293, and the differentiated C2C12, cells (Figs. 3 and 4). The failure of non-Tf iron to inhibit the membrane HJV shedding in HepG2, HEK293, and differentiated C2C12 cells imply that the HJV shedding is not regulated by the intracellular iron status, rather, it is a Tf-mediated process. Our results are consistent

A.-S. Zhang, unpublished data.
with the observations in a previous study that holo-Tf inhibits HJV shedding, but not in agreement with the findings that FAC, a non-Tf iron source, has a similar inhibitory effect (32). These discrepancies might be partially due to the different conditions used in the studies. Our findings support that the levels of holo-Tf determine the amount of HJV shedding. We propose that the HJV shedding from skeletal muscle and hepatocytes are negatively regulated by the holo-Tf levels in the circulation. HJV released from skeletal muscle could indirectly modulate hepatic hepcidin expression and the consequent body iron homeostasis, whereas the modulation of membrane HJV on hepatocytes membrane is assumed to directly modulate hepcidin expression.

Although HJV has been demonstrated as a critical upstream regulator of hepatic hepcidin expression (2, 17, 18), how it regulates hepcidin expression in response to body iron status remains unknown. In the liver, HIV is mainly expressed in hepatocytes (18). Most recent studies demonstrated that TGF-β/SMAD4 is essential in regulating hepcidin expression and that HIV is involved in this process by being a co-receptor for both BMP2 and BMP4 to positively regulate the BMP signaling pathway (10, 41). In addition, another study demonstrated that the predominantly liver-specific BMP9 is also able to robustly induce hepatic hepcidin expression through this pathway although it has not been defined whether or not HIV is also a co-receptor for BMP9 (42).

The activity of BMP signaling could be regulated at multiple stages (20, 21). Apparently the bioavailability of BMPs would be a critical limiting factor to the function of HIV on hepatocyte membrane. BMPs are cytokines synthesized in many tissues (20, 21). As a result, we propose that serum HIV functions to compete with membrane HIV on hepatocytes for the limited BMP2, BMP4, and BMP9 in serum, and thereby to negatively regulate BMP signaling in hepatocytes (Fig. 5). In this model, skeletal muscle functions as a sensor for serum iron status, and serum iron supply negatively regulates HIV shedding in this tissue. Low serum iron enhances HIV shedding and consequently elevates its level in serum. Serum HIV would compete with the HIV anchored on hepatocyte membrane for the limited BMPs in serum. The resulting consequence would be a decreased BMP signaling that in turn inhibits hepcidin expression. Decreased hepcidin levels would result in an increased iron uptake from duodenum to increase dietary iron absorption, and an increased mobilization of iron from hepatocytes and Kupffer cells into circulation to meet the body iron requirement. In contrast, high transferrin saturation would lead to lower serum HIV levels and result in up-regulation of hepcidin expression. In the case of hepatocytes, however, the negative regulation of HIV shedding by holo-Tf would directly control the amount of membrane HIV and the consequent BMP signaling for the regulation of hepcidin expression. The combined regulation of serum HIV concentration and hepatocyte membrane HIV by serum iron would result in an adequate level of hepatic hepcidin expression. On the basis of the findings in this study, this model does not exclude the involvement of other possible regulatory machineries from other tissues, such as liver, in regulating hepatic hepcidin expression at the same time.

Further studies of the underlying mechanism for membrane HIV shedding revealed that neogenin, rather BMP signaling, is involved in this process. Neogenin is a membrane protein widely expressed in different tissues including skeletal muscle and liver (22–24). It is the classical receptor for netrins as well as repulsive guidance molecule a (RGMa), a close family member of HIV (3–5). Studies in mice showed that RGMa is mainly expressed in central nervous systems and possesses a distinct pattern of tissue expression from RGMc, the ortholog of human HIV (3–5). More recent studies have demonstrated that the interaction of RGMa with neogenin is critical in the regulation of neuronal survival as well as neural development (14, 26). Our finding that neogenin is required for HIV shedding provides an important clue to further elucidate the regulation of RGMa through neogenin. However, the underlying mechanism by which neogenin regulates HIV shedding in response to holo-Tf is the subject of future studies.

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**FIGURE 5. A model for the regulation of acute membrane HJV shedding through neogenin.**
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