

## Hepcidin and iron-loading anemias

**Elizabeta Nemeth, Tomas Ganz**

David Geffen School of Medicine, University of California, Los Angeles, USA.

E-mail: [enemeth@mednet.ucla.edu](mailto:enemeth@mednet.ucla.edu); [tganz@mednet.ucla.edu](mailto:tganz@mednet.ucla.edu)

Hepcidin is the key regulator of systemic iron homeostasis and a pathogenic factor in anemia of inflammation and hereditary hemochromatosis. Hepcidin inhibits iron influx into plasma from duodenal enterocytes that absorb dietary iron, from macrophages that recycle iron from senescent erythrocytes and from hepatocytes that store iron. Hepcidin acts by binding to the cellular iron exporter ferroportin and causing its internalization and degradation. Hepcidin production is increased by iron and inflammation and decreased by anemia and hypoxia, however, the molecular mechanisms of hepcidin regulation by iron, oxygen and anemia are still unclear. Iron-loading anemias are disorders in which hepcidin is regulated by opposing influences of ineffective erythropoiesis and concomitant iron overload. In this issue, Kattamis and colleagues report on hepcidin expression in patients with thalassemia major and provide further insight into the regulation of hepcidin by anemia and iron.

### Hepcidin peptide

Human hepcidin (Figure 1) was first identified in human blood ultrafiltrate<sup>1</sup> and in urine<sup>2</sup> as a result of a search for novel cysteine-rich, cationic antimicrobial peptides. Bioactive hepcidin indeed bears structural similarity to disulfide-rich antimicrobial peptides: out of 25 amino acids, ~30% are cysteines and these form four disulfide bonds. The hairpin-shaped peptide also shows an amphipathic distribution of residues, with hydrophobic residues on the convex side and positively charged ones on the concave side<sup>3</sup> (Figure 1). However, hepcidin only displayed modest antimicrobial properties *in vitro* apparent only at very high (10-30  $\mu$ M) concentrations,<sup>2</sup> and the relevance of hepcidin's direct antimicrobial activity *in vivo* remains to be determined. Hepcidin's evolutionary conservation, however, distinguishes it from typical antimicrobial peptides: closely related hepcidin sequences are found in vertebrates from fish to humans.

Hepcidin is synthesized in the liver as a propeptide and has a characteristic furin cleavage site immediately N-terminal to the 25-amino-acid peptide.<sup>2</sup> Little is known about the propeptide processing step or its importance in the post-translational regulation of hepcidin; however, the correlation between hepcidin mRNA and peptide levels<sup>4</sup> implies that prohepcidin cleavage is not a regulated rate-limiting process.

### Mechanism of hepcidin action

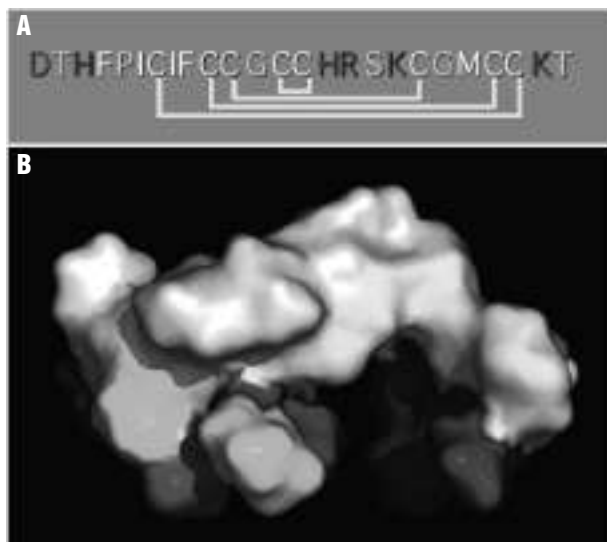
Hepcidin causes a decrease in serum iron. Injection of synthetic hepcidin into mice results in hypoferrremia already within 1 hour,<sup>5</sup> and a similar effect was seen

with acute induction of hepcidin expression in tetracycline-inducible transgenic mice.<sup>6</sup> The hypoferrremia develops because hepcidin blocks the supply of iron into plasma while the relatively small plasma iron pool is rapidly used up by erythrocyte precursors. Hepcidin blocks iron flows from macrophages recycling iron, from stores in the liver and from enterocytes absorbing dietary iron. The molecular mechanism centers on hepcidin's interaction with ferroportin.<sup>7</sup> Ferroportin is the only known cellular iron exporter in vertebrates, and is expressed in all the tissues handling major iron flows - reticulo-endothelial macrophages, hepatocytes and duodenal enterocytes.<sup>8</sup> Hepcidin binds to ferroportin and causes its internalization and degradation in lysosomes, thus effectively blocking the export of iron from the cells<sup>7</sup> (Figure 2). *In vitro*, the internalization of ferroportin occurs less than 1 hour after addition of hepcidin, consistent with the kinetics of hypoferrremia observed *in vivo*.<sup>5</sup> Likewise, injection of radiolabeled hepcidin in mice resulted in equally rapid accumulation of radioactive hepcidin in ferroportin-rich organs (spleen, duodenum and liver),<sup>5</sup> providing further support for the key role of hepcidin-ferroportin interactions in the regulation of iron transport.

### Hepcidin is the principal regulator of extracellular iron concentration

Hepcidin is increased by iron loading and this provides the homeostatic loop to maintain normal extracellular concentrations of iron. A rise in plasma iron (e.g. after a meal or an iron supplement) leads to increased hepcidin production. In turn, elevated hepcidin reduces the concentration of ferroportin molecules on the cell surface and inhibits the entry of iron into plasma, thus allowing the iron concentration to return to normal levels (Figure 5A). Conversely, in iron deficiency, hepcidin production decreases, allowing a greater export of iron through ferroportin into plasma; this results in an appropriate rise in circulating iron.

Chronic alterations of hepcidin expression result in systemic disorders of iron metabolism and maldistribution of iron in the body. Homozygous disruption of the hepcidin gene in humans or mice leads to severe iron overload.<sup>9,10</sup> Conversely, overexpression of hepcidin in transgenic mice resulted in severe microcytic, hypochromic anemia.<sup>11</sup> Mice with tumor xenografts engineered to overexpress hepcidin also developed hypoferrremia and anemia, with iron sequestration in the stores.<sup>12</sup> Similarly, overproduction of hepcidin by liver tumors in patients with type 1a glycogen storage disease caused iron-refractory anemia which resolved only after resection of the tumor, or after liver transplanta-



**Figure 1.** The sequence (A) and the nuclear magnetic resonance structure (B) of human hepcidin. Bioactive hepcidin is a 25-amino acid peptide, stabilized by four disulfide bonds. Positively-charged residues are shown in blue, negatively-charged residues in red, polar residues in green, hydrophobic cysteines in yellow, and other hydrophobic residues in white. Similar to amphipathic antimicrobial peptides, charged and polar side chains of hepcidin are spatially segregated from hydrophobic ones, suggesting that hepcidin may have evolved as an antimicrobial peptide that acquired a new function.

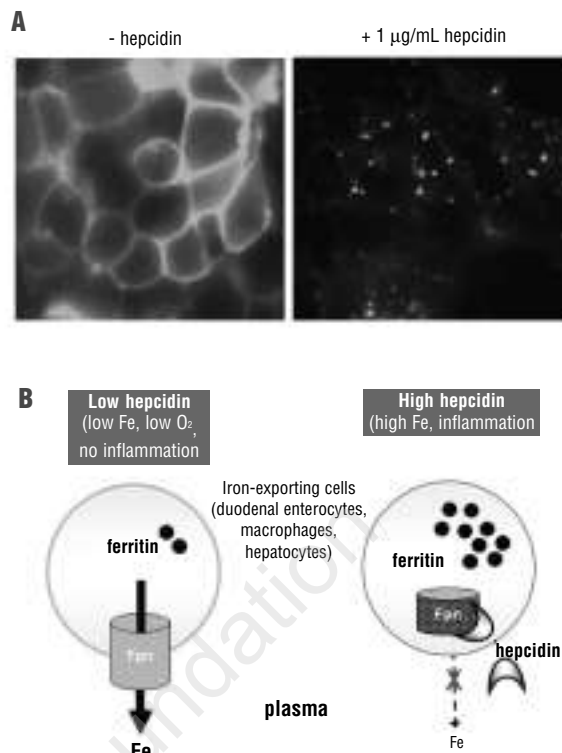
tion.<sup>13</sup> Altogether, these studies confirm the role of hepcidin as the negative regulator of iron absorption, recycling and release from stores.

**Regulation of hepcidin synthesis; implications for the disorders of iron metabolism**

As befits an iron-regulatory hormone, hepcidin synthesis is increased by iron loading and inflammation and is decreased by anemia and hypoxia. Except for inflammation, the molecular pathways underlying regulation of hepcidin are not well understood and are areas of intense research in the field. Dysregulation of hepcidin synthesis, however, appears to be the key factor in the pathogenesis of a spectrum of iron disorders, with hepcidin deficiency causing iron overload and elevated hepcidin mediating anemia of inflammation.

**Regulation by inflammation**

Hepcidin synthesis is markedly induced by infection and inflammation. In animal models, injection of turpentine, lipopolysaccharide, or Freund's adjuvant increased hepatic hepcidin mRNA expression,<sup>14-16</sup> and in humans, infusion of lipopolysaccharide resulted in a rapid increase in urinary hepcidin.<sup>17</sup> These effects are mediated by inflammatory cytokines including interleukin (IL)-6 and IL-1. IL-6 is sufficient for hepcidin induction since direct treatment of primary hepatocytes with IL-6 resulted in rapid upregulation of hepcidin mRNA and infusion of human volunteers with IL-6 resulted in increased urinary hepcidin excretion within just 2 hours after infusion.<sup>14,18</sup> IL-1 $\alpha$  and IL-1 $\beta$  are also able to induce hepcidin *in vitro*, however, in human primary hepatocytes, this effect was IL-6-dependent as the addition of anti-IL-6 antibody blocked the hepcidin



**Figure 2. Mechanism of hepcidin action.** A: Hepcidin causes internalization of green fluorescent protein (GFP)-tagged ferroportin in HEK293 cells stably expressing the ferroportin-GFP fusion protein. B: Hepcidin-induced ferroportin internalization and degradation results in decreased cellular iron efflux.

induction (*E. Nemeth and T. Ganz, unpublished*), whereas in mouse primary hepatocytes<sup>19</sup> and hepatoma cell lines HuH-7 and HepG2,<sup>20</sup> the effect of IL-1 was independent of IL-6. In an acute inflammatory model of turpentine-injected mice, IL-6 was required for hepcidin increase as hepcidin failed to be induced in IL-6 knockout mice (unlike control mice).<sup>14</sup> However, a mouse model of chronic inflammation indicated that an IL-6-independent pathway exists, because hepcidin was comparably increased in both IL-6 knockout and control mice after multiple turpentine injections or after chronic peritoneal *S. epidermidis* infection.<sup>21</sup>

**Hepcidin as a mediator of anemia of inflammation**

Regardless of the details of the cytokine pathways, hepcidin increase was associated with hypoferrremia in all the inflammatory models. Increased hepcidin appears to be the key factor in the development of anemia of inflammation. Hypoferrremia and anemia of inflammation have likely developed during evolution as a host defense strategy against infection, limiting the growth of invading microbes. However, the same strategy has become maladaptive with the increasing incidence of non-infectious diseases associated with excessive cytokine production, including rheumatologic diseases, inflammatory bowel disease, multiple myeloma and other malignancies. Anemia of inflammation is characterized by decreased serum iron and impaired

mobilization of iron from stores, evident from the presence of iron in bone-marrow macrophages and increased ferritin levels. These are the very features observed in mouse models with increased hepcidin. Intraperitoneal injection of synthetic hepcidin resulted in hypoferremia within 1 hour,<sup>5</sup> and chronic overexpression of hepcidin in tumors resulted in anemia and hypoferremia despite increased liver iron stores.<sup>12</sup> Furthermore, patients with infection or inflammatory disorders have elevated urinary excretion of hepcidin compared to healthy controls.<sup>18</sup>

Thus, the molecular pathway from inflammation to anemia centers on the elevated plasma hepcidin which causes the internalization and degradation of ferroportin in macrophages, hepatocytes and duodenal enterocytes, sequestering iron in these cells and blocking iron flows into plasma. As the bone marrow continues to utilize iron for hemoglobin synthesis, the small plasma iron compartment becomes rapidly depleted causing hypoferremia. Persistent hypoferremia, as in chronic inflammation, leads to iron-restricted erythropoiesis and anemia. However, it still remains to be established whether the increase in hepcidin is the essential factor in the development of this disorder, since inflammation may contribute to anemia by alternative hepcidin-independent mechanisms including decreased erythropoietin production, blunted response to erythropoietin and shortened erythrocyte lifespan.

#### **Regulation of hepcidin by iron and the lessons from hereditary hemochromatosis**

Even though hepcidin's response to iron was known since its discovery in 2000, when the increased hepcidin mRNA was described in livers of mice fed or injected with iron,<sup>22</sup> the molecular mechanisms underlying this response still remain obscure. The major obstacle to understanding these mechanisms is our inability to replicate the hepcidin response to iron *in vitro*. Treatment of primary mouse or human hepatocytes or hepatic cell lines with iron-transferrin or other forms of iron failed to increase hepcidin expression.<sup>18,22,23</sup> No response was seen in either co-cultures of primary hepatocytes and non-parenchymal cells, or mouse hepatic organoid cultures treated with iron (*unpublished data*). So far, the only clues about molecules involved in the pathway of hepcidin regulation by iron come from mutations causing hereditary hemochromatosis. In addition to juvenile hemochromatosis caused by inactivating mutations in the hepcidin gene itself, it appears that hepcidin deficiency is the unifying cause of most types of hereditary hemochromatosis. Measurements of urinary hepcidin excretion or hepatic mRNA expression showed that patients and animal models with homozygous disruption of HFE, transferrin receptor 2 (TfR2) and hemojuvelin (HJV) all had hepcidin levels inappropriately low for the systemic iron load.<sup>24-30</sup> While the precise function of the three molecules is not known, they likely participate in the sensing of iron or the consequent signal transduction that regulates hepcidin synthesis and release. Importantly, the degree of hepcidin deficiency appears to correlate with the severity of the disease. The most severe form, juvenile hemochromatosis, is

caused by mutations in either the hepcidin or HJV gene and these are phenotypically undistinguishable. Patients with HJV mutations have very low or undetectable urinary hepcidin<sup>28</sup> suggesting that HJV is the key regulator of hepcidin. HJV belongs to the family of repulsive guidance molecules (Rgm) which are involved in neuronal differentiation, migration, and apoptosis, but the expression pattern of HJV (skeletal muscle, the liver and the heart) differs from that of its neuronal Rgm relatives.<sup>28</sup> Neuronal Rgm are GPI-linked proteins that function as receptor ligands. The GPI-linked membrane protein hemojuvelin was also reported to interact with neogenin<sup>31</sup> and the bone morphogenetic protein signaling pathway,<sup>32</sup> but how this relates to hepcidin regulation by iron remains to be elucidated. Recent evidence indicates that HJV could be a part of the iron sensing complex since (i) the shedding of the GPI-linked form from the membrane is inhibited by iron, and (ii) membrane-bound and soluble HJV forms respectively increase and suppress hepcidin mRNA expression.<sup>33</sup>

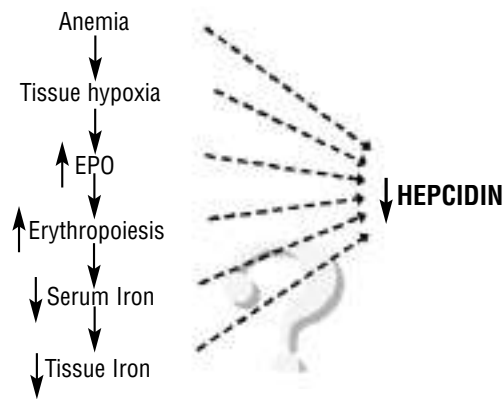
Humans and mice with homozygous HFE or TfR2 disruption have a milder form of hemochromatosis and their hepcidin levels are not as severely decreased as with HJV mutations.<sup>27,34</sup> The function of HFE, a homolog of MHC class I molecules, is still mysterious 10 years after its discovery, but the protein has been shown to interact with transferrin receptor 1 (TfR1) and to alter the cellular iron content.<sup>35</sup> TfR2, a homolog of the ubiquitously expressed TfR1, is predominantly expressed in the liver and its protein levels are regulated by transferrin saturation,<sup>36,37</sup> suggesting a possible role in iron sensing.

#### **Regulation of hepcidin by anemia and hypoxia**

Inadequate delivery of oxygen to tissues, which occurs in anemia or hypoxemia, would be expected to result in homeostatic decrease in hepcidin synthesis. The decrease in hepcidin levels would in turn allow increased iron mobilization from macrophages and hepatocytes, and increased iron absorption from the diet, making more iron available for erythrocyte production. Indeed, hepcidin was shown to be suppressed by anemia and hypoxia;<sup>16</sup> however, the molecular pathways that regulate this response are still unclear. Though anemia may act (at least in part) by causing liver hypoxia, it is also possible that the pathways of hepcidin regulation by oxygen and by anemia/erythropoiesis are independent.

Exposure to hypoxia decreased hepcidin mRNA expression not only in mice *in vivo*, but also in isolated hepatocytes *in vitro*.<sup>16</sup> In general, cellular oxygen sensing and the related transcriptional control are largely mediated by the hypoxia-inducible factor (HIF). However, unlike most of the target genes that are transcriptionally activated by HIF, hepcidin expression is negatively regulated by hypoxia. In addition, except for human hepcidin promoter, the promoters in other mammals do not contain the consensus binding sites for HIF, and direct involvement of HIF in transcriptional regulation of hepcidin remains to be explored.

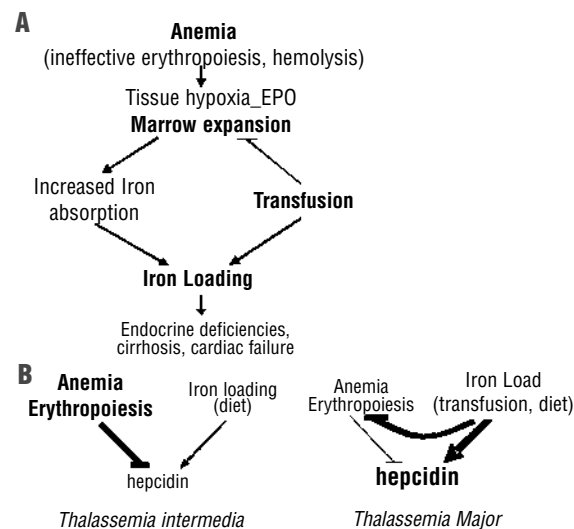
In mice, anemia caused by bleeding or phenylhydrazine-induced hemolysis suppressed hepcidin mRNA



**Figure 3. Possible pathways of hepcidin regulation by anemia.** Anemia reduces oxygen delivery to the tissues, which triggers the synthesis of erythropoietin (EPO) in the kidney. EPO stimulates the production of new erythrocytes which increases the demand for iron resulting in higher turnover of serum iron and depletion of iron stores. The decrease in hepcidin is critical for the mobilization of iron from the stores and increased iron absorption. While recent studies failed to detect a direct effect of EPO<sup>40</sup> on hepcidin production, other candidates for hepcidin-regulating signal include serum or tissue iron, hypoxia, or putative circulating factor(s) related to erythropoiesis or anemia.

levels.<sup>16,38,39</sup> Anemia initiates a cascade of adaptive responses aimed at boosting erythropoiesis and any of these could be involved in hepcidin regulation (Figure 3). In addition to tissue hypoxia, the candidate pathways include direct regulation of hepcidin by erythropoietin, changes in serum iron or tissue iron, or a circulating erythropoiesis-related factor. Vokurka *et al.*<sup>40</sup> attempted to shed light on the process by combining different stimulators and inhibitors of erythropoiesis. Stimulation of erythropoiesis with phenylhydrazine resulted in hepcidin suppression as expected, but the simultaneous inhibition of erythropoiesis by irradiation prevented hepcidin suppression despite severe anemia. In addition, irradiation prevented hepcidin suppression after erythropoietin administration, ruling out the direct effect of erythropoietin on hepcidin synthesis. Although the study evaluated only tissue iron but not the serum iron levels, it suggested that the changes in iron balance related to utilization of iron for erythropoiesis are the determining factor in the regulation of hepcidin in setting of increased erythropoiesis.

The new study by Kattamis *et al.* published in this issue does, however, challenge the iron-centered hypothesis. The authors evaluated hepcidin mRNA expression and urinary levels in 19 patients with  $\beta$ -thalassemia major. Hepcidin levels correlated with parameters of erythropoiesis and anemia but not with indices of iron load: a positive correlation was seen with hemoglobin and an inverse correlations with soluble transferrin receptor and erythropoietin, as expected, but no correlations was observed with liver iron concentrations, ferritin, serum iron or transferrin saturation. Non-transferrin-bound iron (NTBI) correlated negatively with hepcidin levels, suggesting that low hepcidin allows increased iron flows into plasma, leading to the saturation of transferrin and NTBI formation. The authors

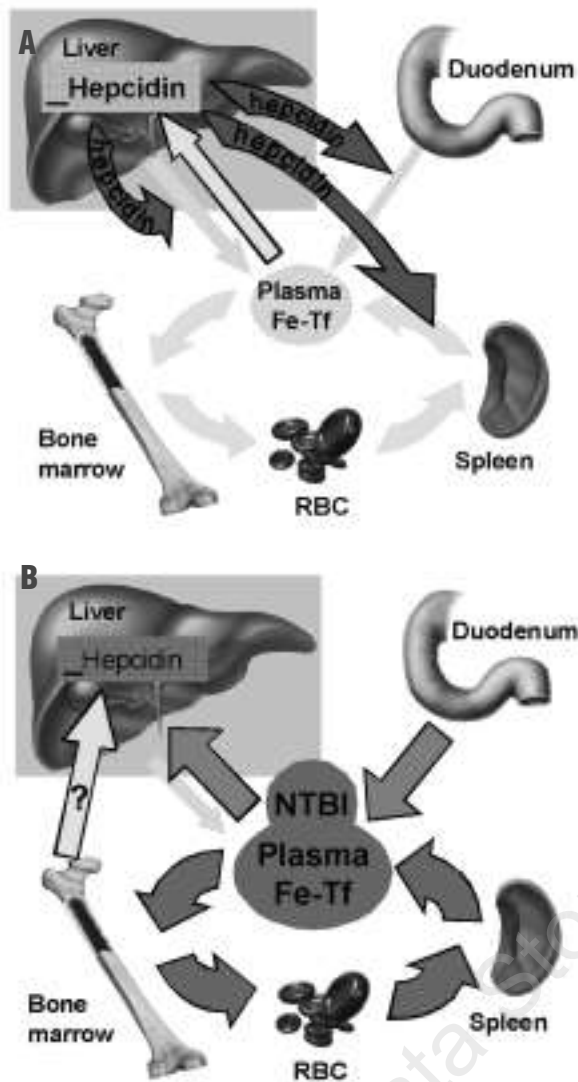


**Figure 4. A. Iron loading in thalassemia syndromes.** Thalassaemia can result in iron overload due to: a) increased dietary iron absorption driven by to the strong erythropoietic drive (primary iron overload), and b) blood transfusion (secondary iron overload). Transfusions, however, may indirectly reduce iron absorption by suppressing erythropoiesis. B. Regulation of hepcidin in thalassemia syndromes. In thalassemys, hepcidin is regulated by opposing influences of ineffective erythropoiesis, which suppresses hepcidin and iron load, which increases hepcidin. In thalassaemia intermedia (left panel), the strong erythropoietic drive is the dominant regulator of hepcidin; the resulting hepcidin deficiency may contribute to increased iron absorption in these patients. In thalassaemia major (right panel), hepcidin levels are higher than in thalassaemia intermedia, likely due to the transfusion-related relief of the erythropoietic drive and the increased body iron load.

hypothesize that “hypoxia and yet-undefined signals from the robust erythroid activity down-regulate hepcidin production” which then allows increased iron loading of patients with thalassemia.

Previous studies found a similar relationship between hepcidin, anemia and iron overload in hpx/hpx mice which have transferrin deficiency due to a point mutation in the transferrin gene.<sup>41</sup> The mice have a severe iron deficiency anemia, but also an increased pool of non-transferrin-bound iron and massive tissue iron overload in non-hematopoietic tissues (liver iron load 100-fold greater than in wild-type mice). Hepcidin mRNA expression in hpx mice was very low<sup>42</sup> supporting the notion of a dominant erythroid signal in hepcidin regulation as well as the importance of hepcidin deficiency in the development of iron overload. Measurements of urinary hepcidin in patients with thalassaemia intermedia<sup>43,44</sup> also corroborated the existence of a suppressive erythropoietic signal since hepcidin levels were very low despite high transferrin saturation and elevated serum ferritin.

One of the likely candidates for the circulating factor from the bone marrow was soluble TfR1 (sTfR1). sTfR1 is produced by proteolytic cleavage of TfR1, and circulates in blood as a monomer bound to transferrin. sTfR1 levels are high when iron availability is reduced and when erythropoiesis is stimulated.<sup>45</sup> In addition, urinary hepcidin levels in patients with thalassemia correlated



**Figure 5.** The role of hepcidin in normal iron homeostasis and iron-loading anemias. **A. Normal iron homeostasis:** an increase in plasma iron causes an increase in hepcidin production (yellow arrow). Elevated hepcidin inhibits iron flow into the plasma from macrophages, hepatocytes and the duodenum. As the plasma iron continues to be consumed for hemoglobin synthesis, the plasma iron levels decrease and hepcidin production abates, completing the homeostatic loop. **B. Iron-loading anemias:** greatly increased erythropoietic drive suppresses hepcidin production through as yet undefined pathway(s) (yellow arrow). Low hepcidin allows excessive iron absorption, leading to increased plasma iron levels, elevated transferrin saturation and accumulation of non-transferrin-bound iron (NTBI). Hepcidin regulation by iron remains blunted because of the dominating erythropoietic signal resulting in excessive iron loading, deposition of iron in vital organs and organ damage.

well with levels of sTfR1.<sup>44</sup> However, overexpression of sTfR1 in mice using a hydrodynamic gene transfer technique did not alter hepcidin expression,<sup>46</sup> and the putative erythropoietic regulator of hepcidin remains elusive.

Importantly, urinary hepcidin levels in the regularly-transfused thalassemia major patients, although very variable, were generally higher than hepcidin levels in untransfused thalassemia intermedia patients or in normal controls.<sup>44,47</sup> This was not surprising as thalassemia major and intermedia differ significantly in their degree

of anemia, erythropoietic drive and iron load due to transfusion therapy (Figure 4). Transfusions would be expected to affect hepcidin production by: a) relieving anemia and suppressing the erythropoietic drive which should lessen the suppression of hepcidin, and b) increasing body iron load which should upregulate hepcidin synthesis. Indeed, comparison of urinary hepcidin before and 3-4 days after transfusion showed that most patients responded by increasing hepcidin levels.<sup>47</sup> Nevertheless, when the ratio of urinary hepcidin to serum ferritin was analyzed as an index of appropriateness of hepcidin response to iron load, this ratio was still greatly decreased in thalassemia major patients when compared to normal subjects,<sup>44,47</sup> indicating the continued regulation of hepcidin by a suppressive factor. In sickle cell anemia, urinary hepcidin was low in pediatric patients<sup>47</sup> consistent with the suppression of hepcidin by increased erythropoietic drive but was elevated in adults (*E. Nemeth and T. Ganz, unpublished data*) perhaps due to the development of inflammatory complications and progressive transfusion induced iron overload that increased with age. It is possible that high hepcidin levels in some adults with sickle cell disease could protect them from the adverse consequences of iron overload by sequestering iron in macrophages where it is less toxic than in parenchymal cells.

The discovery of hepcidin has broadened our understanding of disturbances of iron homeostasis in iron-loading anemias. The findings suggest that anemia, especially when associated with increased and ineffective erythropoiesis, has a strong and dominant effect over iron on hepcidin production. The consequent low levels of hepcidin may be responsible for hyperabsorption and maldistribution of iron, thus contributing to systemic iron overload and associated organ damage (Figure 5).

Iron-loading anemias in humans and in animal models point out the importance of erythropoietic drive in hepcidin regulation and the need to understand its molecular basis. Moreover, the development of hepcidin-based diagnostics and therapies for iron-loading anemias may offer more effective approaches to prevent the toxicity associated with iron overload.

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