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Regulation of systemic iron homeostasis: how the body responds to changes in iron demand

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Abstract The iron that is required to meet the metabolic needs of cells and tissues is derived from the plasma. Plasma iron in turn reflects the release of iron from various body cells, principally the macrophages of the reticuloendothelial system, and the absorption of dietary iron by the proximal small intestine. This iron donation is highly regulated and the liver-derived peptide hepcidin has emerged as the key modulator of cellular iron export. Following its synthesis and secretion from the liver, circulating hepcidin reduces iron export into the plasma by binding to the iron efflux protein ferroportin1 on the surface of enterocytes, macrophages and other cell types and causing its internalization. The level of hepatic hepcidin expression is influenced by HFE, transferrin receptor 2 and hemojuvelin, and the signal transduction pathway(s) linking these proteins to hepcidin are only beginning to be revealed. Hemojuvelin has recently been shown to signal through the bone morphogenetic protein pathway, ultimately activating receptor SMAD/SMAD4 complexes to alter hepcidin transcription. Circulating differic transferrin has emerged as a possible upstream regulator of the liver-based hepcidin regulatory pathway. In addition to being regulated by body iron requirements, hepcidin expression can be modulated by pro-inflammatory cytokines such as interleukin-6. The continuing analysis of inherited disorders of iron metabolism combined with biochemical analysis of signal transduction pathways is essential to fully define this important regulatory system.

Keywords Hemojuvelin · HFE · TfR2 · Hepcidin · Ferroportin1

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Iron, by virtue of its ability to accept or donate electrons, is essential for many of the biological reactions carried out by living systems. This same characteristic, however, allows free iron in solution to form highly reactive free radicals that can lead to cell damage. The appropriate regulation of systemic iron homeostasis, therefore, is crucial for the survival and wellbeing of all complex organisms, including humans. While the precise mechanism by which the body achieves this is the subject of intense research, recent discoveries have greatly increased our understanding of this vital process.

The compartmentalization of iron

In order to understand how systemic iron homeostasis is maintained, it is prudent to examine the

movement of iron between the various functional compartments of the body (Fig. 1). The average male human contains approximately 4 grams of iron (Brittenham 1994). Under normal conditions approximately 1–2 mg of iron per day enters the body via the enterocytes of the proximal small intestine. This newly absorbed dietary iron is released into the circulation and binds to the serum protein transferrin, each molecule of which can bind two atoms of iron. Approximately 3 mg of iron circulates bound to transferrin and, although this represents only a small proportion of total body iron, evidence is accumulating that this iron compartment is vital for the maintenance of body iron homeostasis, as will be discussed in detail below. Transferrin bound iron is taken up by cells by transferrin receptor 1 (TfR1)-mediated endocytosis (Huebers and Finch 1987). Most of the transferrin bound iron in the circulation is destined for the developing erythrocytes of the bone marrow, where it is taken up at a rate of approximately 22 mg of iron per day (Brittenham 1994), and used in the production of haemoglobin. About 65–70% of body iron exists in this form in circulating red blood cells. Old or damaged red blood cells are removed from the circulation by the macrophages of the reticuloendothelial (RE) system, where iron is released

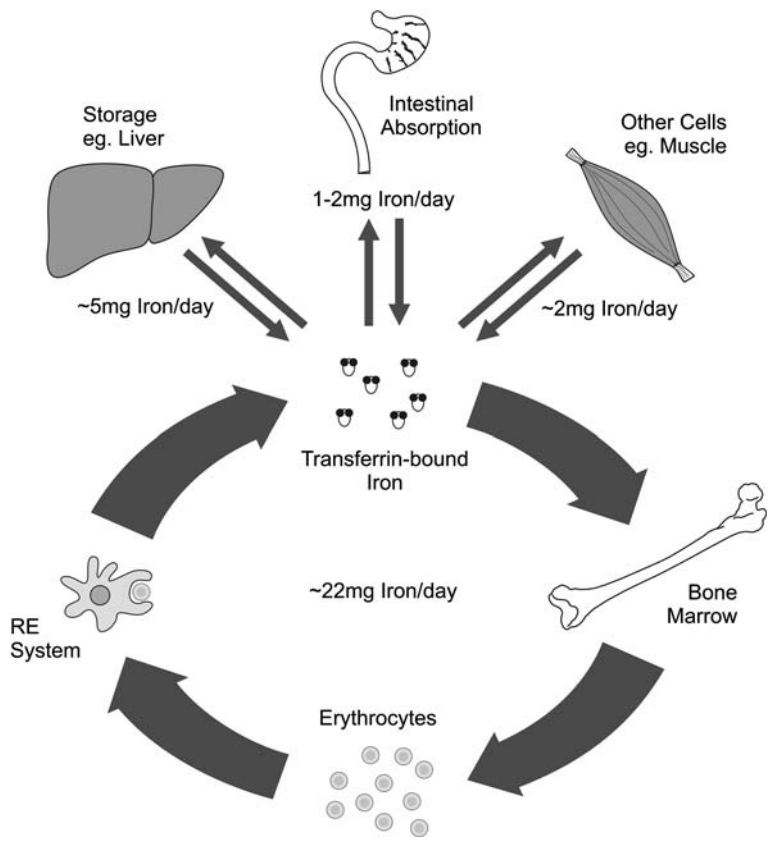

Fig. 1 The movement of iron through body compartments. Iron is transported around the body bound to transferrin in the circulation. Most of this iron is taken up by the developing erythrocytes in the bone marrow and incorporated into haemoglobin. Old or damaged erythrocytes are removed from the bloodstream by the macrophages of the RE system and the iron is recycled back to plasma transferrin. Smaller quantities of iron are exchanged with the body's storage tissues, predominantly the hepatocytes of the liver. Other tissues also take up iron for their metabolic needs, for example, the synthesis of myoglobin in muscle. Dietary iron enters the body via the proximal small intestine in amounts equalling the iron lost from the body by the shedding of epithelial cells, particularly from the gastrointestinal tract, thus maintaining the body's iron homeostasis

from haemoglobin and either stored in the intracellular iron storage protein ferritin, or released back into the circulation as transferrin-bound iron. The cells of the RE system release about 22 mg of iron per day, thus replacing the amount taken up by the bone marrow. Other body cells also take up iron from the circulation via TfR1-mediated endocytosis although in far lower amounts. This iron can be used to synthesize a wide range of iron containing intracellular proteins, such as the haem containing cytochromes, and proteins containing iron-sulfur clusters. Approximately 10–15% of body iron is present in such proteins, with up to 80% of this found in muscle cell myoglobin. The remaining 20% of body iron is present as storage iron, predominantly located in the macrophages of the RE system and the hepatocytes of the liver.

The movement of iron between these compartments is a tightly regulated process that can be modulated according to the body's iron requirements. Individual cells maintain appropriate intracellular iron levels by altering the expression of TfR1 on the cell surface (Huebers and Finch 1987). The more iron each cell requires, the higher the expression of TfR1. While the uptake of iron by cells is predominantly controlled locally by intracellular iron levels, iron release, particularly from the cells of the RE system, liver and proximal small intestine, appears to be regulated by systemic signals. Evidence now suggests that the control of cellular iron efflux is the major regulatory point for the maintenance of systemic iron homoestasis (Frazer and Anderson 2003). For example, the stimulation of erythropoiesis following blood loss increases the iron requirements of the bone marrow. To cope with this demand, the body increases cellular iron release, making available iron stored in the macrophages of the RE system and hepatocytes of the liver. At the same time, the increase in iron release by intestinal enterocytes allows iron taken up from the diet to enter the circulation and replenish the body's iron stores. Once erythropoiesis and the demand for iron have reduced to normal levels, cellular iron release also decreases to maintain iron homeostasis.

The importance of tightly regulated cellular iron release is evident from a number of pathological conditions in humans. Patients with the genetic disorder HFE-associated haemochromatosis may develop severe iron loading that can often result in tissue damage and possibly organ failure (Fleming et al. 2005). This appears to be caused by inappropriately high iron release by enterocytes and macrophages. As a result, iron normally stored in the RE system or lost from the alimentary canal is released into the circulation, overwhelming the iron binding capacity of transferrin, and allowing the formation non-transferrin bound iron (Batey et al. 1980). This form of iron is very rapidly taken up by cells, particularly hepatocytes, leading to iron overload (Brissot et al. 1985). Another pathological condition involving altered iron release from cells is the anaemia of inflammation. During an inflammatory response, iron release from cells decreases, lowering the level of transferrin-bound iron in the circulation (Weiss and Goodnough 2005). This response probably evolved to assist the immune system to overcome infection by withholding iron (a well recognised virulence factor for microorganisms) from invading pathogens. However, in chronic inflammatory conditions, such as rheumatoid arthritis, the transferrin saturation is reduced for long enough to compromise iron delivery to the developing erythrocytes in the bone marrow, resulting in anaemia (Weiss and Goodnough 2005). In both of the above pathological states, cellular iron release does not reflect the body's iron requirements. Due to its physiological importance, the mechanism by which the body regulates iron release is the focus of intense scrutiny.

Molecular basis of iron homeostasis

A major advance in our understanding of cellular iron release came with the discovery of the iron export molecule, ferroportin1 (Abboud and Haile 2000; Donovan et al. 2000; McKie et al. 2000). This multi-spanning membrane protein is expressed in most tissues, with the highest levels found in the villus enterocytes of the proximal small intestine, the macrophages of the RE system and the hepatocytes of the liver, the very tissues known to play a major role in regulating

the amount of iron that enters the circulation. Indeed, ferroportin1 knockout mice accumulate iron in these tissues, demonstrating the essential role played by this molecule in iron release (Donovan et al. 2005). However, key insights into the way the body regulates iron release from cells came with the discovery of the iron regulatory peptide hepcidin (Nicolas et al. 2001). This small antimicrobial peptide is synthesized by hepatocytes and secreted into the circulation where it inhibits the efflux of iron from macrophages, intestinal enterocytes and other cell types. Hepcidin production is decreased in response to stimuli known to increase cellular iron release (e.g. iron deficiency, increased erythropoiesis) and increased under conditions where iron release is inhibited (e.g. iron loading, inflammation) (Pigeon et al. 2001, Nicolas et al. 2002). Further studies have shown that hepcidin interacts directly with ferroportin1 at the cell surface of HEK293 cells in culture, causing the internalization and subsequent degradation of the ferroportin1 protein (Nemeth et al. 2004b). If this process also occurs in vivo, it provides a mechanism for the hepcidin-mediated regulation of cellular iron release. Support for this model is provided by a study showing that radiolabelled hepcidin injected into mice accumulates in ferroportin1-rich tissues (Rivera et al. 2005). It is also possible that there are other receptors for hepcidin, although the ferroportin1/hepcidin model can adequately explain the observed regulation of systemic iron homeostasis.

Since the discovery of hepcidin, it has become apparent that this molecule is the major, if not the only, regulator of iron delivery to the plasma, and so the precise regulation of hepcidin synthesis is of vital importance to survival. Despite this, the mechanism by which hepcidin production is regulated by body iron requirements remains incompletely understood. Many of the advances in this area have come from the examination of inherited iron loading disorders in humans and mice.

The first molecule to be associated with the regulation of systemic iron homeostasis was HFE, which is mutated in the most common form of hereditary haemochromatosis (Feder et al. 1996). As noted above, affected patients develop iron loading in various tissues due to increased iron absorption and iron release from reticuloendothelial macrophages, despite adequate or high body iron stores. The HFE protein is a non-classical MHC class I molecule that is expressed in most tissues but at particularly high levels in the liver (Frazer et al. 2001). HFE has also been shown to interact with TfR1, leading to suggestions that it modifies the amount of iron taken up by tissues (Parkkila et al. 1997). Both the phenotype of affected patients and the high hepatic expression of HFE in the liver suggested a link between the liver and the regulation of iron absorption. Indeed, in 2003 we provided a mechanism for this by reporting that mutations in HFE were associated with inappropriately low hepcidin mRNA levels in the liver of both humans and mice (Bridle et al. 2003). Further studies by Nicolas et al. showed that iron loading in HFE null mice could be corrected by constitutively expressing hepcidin, confirming that HFE acts upstream of hepcidin in the iron regulatory pathway (Nicolas et al. 2003). HFE, like hepcidin, is most strongly expressed in hepatocytes (Zhang et al. 2004) suggesting that it is in these cells that HFE is acting to regulate hepcidin expression. It has been suggested that Kupffer cells, the resident macrophages of the liver, may be the site of HFE activity (Bastin et al. 1998; Makui et al. 2005). However, several studies have shown that ablation of macrophages does not interfere with the regulation of hepcidin expression (Lou et al. 2005; Montosi et al. 2005).

The second protein to be associated with hepcidin expression was transferrin receptor 2 (TfR2). Also highly expressed in hepatocytes, this protein, like its homologue TfR1, can take up transferrin-bound iron from the circulation via receptor-mediated endocytosis (Kawabata et al. 2000). However, mutations in the *TfR2* gene lead to hepatocyte iron loading making it unlikely that cellular iron uptake is its primary role (Camaschella et al. 2000). Indeed, TfR2 mutations, although much less common than those of HFE, result in an iron loading disease with symptoms very similar to those of HFE-associated hemochromatosis. Furthermore, subsequent studies have shown that TfR2 mutations lead to the same inappropriately low hepcidin levels seen when

HFE is disrupted (Nemeth et al. 2005), suggesting that TfR2 and HFE are part of the same regulatory pathway.

The most recently discovered member of this regulatory network is hemojuvelin. This molecule is expressed at high levels in muscle and heart and to a lesser extent in liver (Papanikolaou et al. 2004). The mutations in the *HFE2* gene, which encodes the hemojuvelin protein, lead to an early onset, severe from of iron loading known as juvenile hemochromatosis (Papanikolaou et al. 2004). Juvenile hemochromatosis patients do not produce detectable levels of hepcidin despite their high iron load. The lack of hepcidin expression is also seen in hemojuvelin knockout mice (Huang et al. 2005; Niederkofler et al. 2005) and suggests that hemojuvelin is essential for the production of hepcidin and, along with HFE and TfR2, lies upstream of hepcidin in the pathway regulating iron homeostasis.

Plasma signals and cellular responses

The precise role played by HFE, TfR2 and hemojuvelin in the regulation of hepcidin expression in response to changes in body iron demands is not yet known. We have previously hypothesised that HFE, by virtue of its ability to bind to TfR1, and TfR2 are involved in detecting the levels of the diferric form of transferrin in the circulation (Frazer and Anderson 2003). Diferric transferrin is an ideal indicator of body iron demand as it is preferentially taken up by cells requiring iron (Huebers and Finch 1987). This is because TfR1 has a higher affinity for diferric transferrin than monoferric or apo-transferrin. When cellular iron demand is high, circulating diferric transferrin levels will decrease, while the opposite occurs when iron demand is low. Our studies have demonstrated a close correlation between diferric transferrin levels and hepatic hepcidin mRNA expression in rats following hemolysis (Frazer et al. 2004a) or the switch from a control to an iron deficient diet (Frazer et al. 2002). Recent evidence from the haemoglobin deficit (*hbd*) mouse also supports a role for diferric transferrin in hepcidin regulation. The gene affected in these mice is *Sec15l1* (Lim et al. 2005) which, when disrupted, alters the recycling of TfR1 containing endosomes (Zhang et al. 2006). This leads to a decrease in TfR1-mediated iron uptake, limiting the iron supply to the bone marrow and resulting in anaemia. The reduction in iron uptake leads to an increase in the level of diferric transferrin in the circulation of *hbd* mice (Wilkins et al. 2006). Further investigation revealed that these animals have higher hepcidin expression than control mice. We have suggested that the higher level of diferric transferrin signalled the hepcidin regulatory machinery to increase hepcidin levels despite the presence of anaemia, which would be expected to decrease hepcidin production (Wilkins et al. 2006).

Our model involves a detection mechanism for diferric transferrin that involves both HFE and TfR2 on the hepatocyte plasma membrane (Frazer and Anderson 2003). HFE and transferrin bind to overlapping sites on TfR1 (West et al. 2001). We have suggested that diferric transferrin and HFE compete for binding to TfR1 and that the amount of HFE bound depends on the level of diferric transferrin in the circulation, an indicator of body iron demand, and the amount of TfR1 on the cell surface, an indicator of hepatocyte intracellular iron levels. Evidence in favour of such a competition between HFE and diferric transferrin has been reported using tagged HFE constructs transfected into cell lines (Giannetti and Björkman 2004). The addition of diferric transferrin to the cell culture medium resulted in the movement of HFE from the TfR1-containing endosomes to the plasma membrane, indicating that diferric transferrin had outcompeted HFE for TfR1 binding. In our model, changes in body iron requirements would alter the composition of the HFE/TfR1/diferric transferrin complex which, in turn, would modify the signal transduction pathway controlling hepcidin expression.

TfR2 also seems to be able to respond to circulating diferric transferrin levels as the TfR2 protein is stabilised by the binding of diferric transferrin (Jonhson and Enns 2004; Robb and Wessling-Resnick 2004). If a signal to increase hepcidin expression is produced by TfR2, the stabilisation of this molecule by diferric transferrin would maintain this signal. When diferric

transferrin levels drop, TfR2 protein levels would decrease, reducing the signal to the nucleus and, subsequently, hepcidin expression. Whether TfR2 and HFE feed into the same signal transduction pathway or produce separate signals is not known.

The role played by hemojuvelin in the regulation of hepcidin is proving particularly intriguing. It is a member of the repulsive guidance molecule (RGM) family of proteins (Papanikolaou et al. 2004). In cell culture models, hemojuvelin is a GPI-linked membrane protein located on the extracellular surface of the plasma membrane (Niederkofler et al. 2004), however, no localisation studies have been carried out in vivo. Surprisingly, hemojuvelin message is most highly expressed in skeletal muscle and heart (Papanikolaou et al. 2004), suggesting that these tissues may play a role in the regulation of body iron homeostasis. Lower levels have also been detected in the liver. As there are no reports of hemojuvelin protein expression levels in vivo, it is unclear whether the mRNA expression pattern reflects the level of protein in each of these tissues. However, most research groups have focussed on hemojuvelin expression in hepatocytes as being most relevant to the regulation of hepcidin production.

Insight into a possible link between hemojuvelin and hepcidin expression has come from a recent study by Babitt et al., which revealed that hemojuvelin can act as a bone morphogenetic protein (BMP) co-receptor (Babitt et al. 2006). Using cells in culture, they showed that hemojuvelin can bind to the type I BMP receptor and enhance the signal produced by the binding of BMP2 and BMP4. The BMPs are members of the TGFβ superfamily of cytokines and they play a role in a number of important biological processes, such as cell proliferation, differentiation and apoptosis, particularly during development (Nohe et al. 2004). The binding of BMPs to their receptors results in the phosphorylation of a subset of receptor SMADs (SMADs 1, 5 and 8), which then bind to SMAD 4. This complex migrates to the nucleus where it binds to specific DNA motifs and regulates gene transcription. Babitt et al. showed that hemojuvelin was able to enhance the phosphorylation of SMAD 1/5/8 in response to BMP stimulation and that this leads to an increase in hepcidin expression in cultured cells (Babitt et al. 2006). Furthermore, mutations in hemojuvelin corresponding to known pathological mutations in human juvenile haemochromatosis patients abrogated this effect. This group also showed a decrease in the phosphorylation of SMAD 1/5/8 in hemojuvelin knockout mice, demonstrating that the cell culture studies were likely to reflect the in vivo function of hemojuvelin. As further support for a role of the BMP pathway in the regulation of hepcidin expression, mice with a liver specific knockout of the *SMAD4* gene develop iron overload and express little if any hepcidin (Wang et al. 2005). In addition, hepcidin expression in these mice cannot be stimulated by iron loading or inflammation, as it is in wild-type mice, suggesting that SMAD4, and possibly the entire BMP pathway, is essential for hepcidin production.

The role of the BMP/SMAD pathway in regulating the expression of hepcidin is still incompletely understood. It is not yet known whether the SMAD-mediated signal from hemojuvelin simply maintains a basal level of hepcidin expression which is then modulated by signals coming from HFE or TfR2, or whether HFE and TfR2 exert their effects on hemojuvelin to manipulate SMAD phosphorylation and subsequently hepcidin expression. How the BMPs themselves relate to iron homeostasis is also unknown. A recent study has shown that BMP-2, 4 and 9 stimulates hepcidin mRNA expression in isolated hepatocytes taken from wild-type, *HFE* knockout, *TfR2* knockout and *interleukin-6 (IL-6)* knockout mice (Truksa et al. 2006). The strongest stimulation was seen with BMP-9, which is predominantly expressed in the liver, and this suggests a possible autocrine/paracrine role for this BMP in hepcidin regulation. To add to the complexity of this regulatory pathway, studies by Lin et al have indicated that soluble hemojuvelin can modulate hepcidin expression in cultured cells (Lin et al. 2005). They suggest that the regulation of hepcidin is a function of the competition between membrane bound hemojuvelin on hepatocytes and soluble hemojuvelin, possibly originating from skeletal muscle and heart, released in response to some unknown signal. Hemojuvelin has also been shown to interact with

neogenin, a netrin receptor involved in neuronal development, and this interaction leads to iron accumulation in cultured cells (Zhang et al. 2005). Clearly there is much more to learn about this complex pathway (Fig. 2).

Unlike the normal physiological regulation of hepcidin expression by iron demand, the increased hepcidin production during inflammation highlights how this peptide can be utilized to control iron levels in a pathological state. Inflammatory stimuli trigger an increase in hepcidin expression, decreasing iron release and subsequently diferric transferrin levels (Roy and Andrews 2005). In this case, however, low diferric transferrin levels do not lead to lower hepcidin levels, but rather are caused by high hepcidin production. This hypoferraemia is thought to assist the immune system by withholding iron from invading pathogens. To achieve this, it would appear that inflammatory stimuli override the normal regulatory pathway involving HFE, TfR2 and hemojuvelin. Indeed, recent studies have shown that the increase in hepcidin expression during inflammation is equally strong in wild-type, HFE knockout, TfR2 knockout and hemojuvelin knockout mice (Lee et al. 2004; Frazer et al. 2004b; Niederkofler et al. 2005). This implies the existence of a separate hepcidin

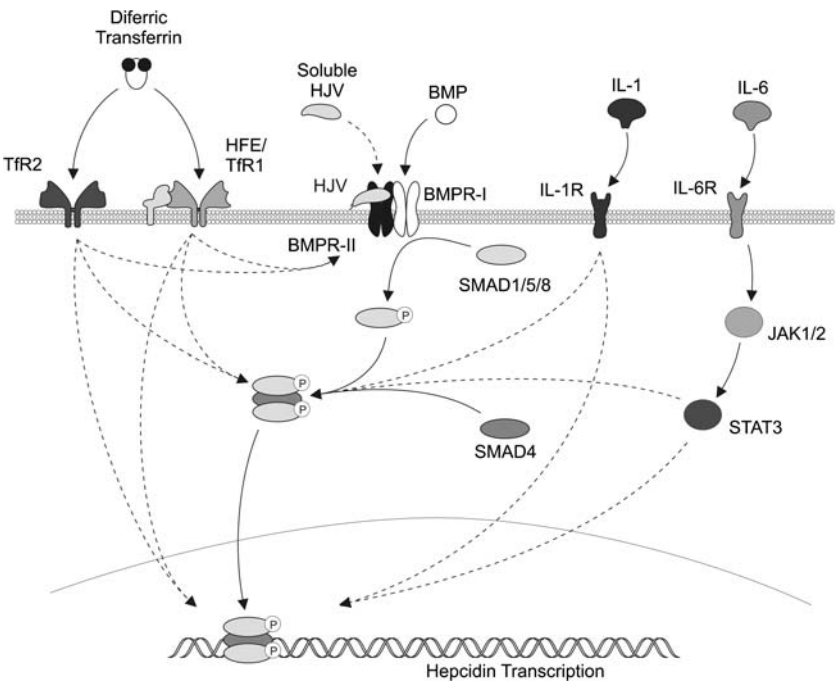

Fig. 2 Pathways of hepcidin regulation. The regulation of hepcidin expression is potentially very complex and is currently not well understood. The BMP pathway has emerged as an important component of this regulatory network. BMPs bind to the BMP receptors, BMPR-I and BMPR-II, resulting in the phosphorylation of SMADs 1, 5 and 8. The phosphorylated forms of these SMADs bind to SMAD4 in the cytoplasm, resulting in the complex being translocated to the nucleus where it can induce hepcidin transcription. GPI-linked hemojuvelin (HJV) enhances the signal produced by BMP binding. Soluble HJV may also be able to modify this signal. TfR2 and the HFE/TfR1 complex can both bind and potentially detect diferric transferrin levels in the circulation. Whether these complexes modify hepcidin expression by signalling through HJV, by interacting with a part of the SMAD pathway, or by directly affecting the hepcidin promoter is not yet known. IL-1 and IL-6 can also affect hepcidin expression following binding to their cell surface receptors. They do not require HJV, but may increase hepcidin expression by interacting with the SMAD pathway or by independently affecting the hepcidin promoter. The solid lines indicate known pathways, whereas broken lines indicate pathways where the links have yet to be proven or where information is incomplete

regulatory pathway. However, the inactivation of SMAD4 in the liver prevents the upregulation of hepcidin by inflammation (Wang et al. 2005), suggesting that the two pathways must converge at some point at or before the involvement of SMADs. Evidence from cell culture models, and from studies in mice and humans, suggests that IL6 is at least one inflammatory cytokine affecting hepcidin expression during inflammation (Nemeth et al. 2003; Nemeth et al. 2004a). IL6 signals via the JAK/STAT signalling pathway (Heinrich et al. 2003). There is a published report of cross talk between this pathway and the SMAD pathway (Nakashima et al. 1999), and, although this was observed in fetal brain tissue, it is possible that similar cross talk also occurs in the liver to regulate hepcidin expression. Further studies have also implicated interleukin 1 (Lee et al. 2005) in the inflammatory regulation of hepcidin. To complicate the matter further, several studies have suggested that extra-hepatic hepcidin expression by macrophages and neutrophils may also be involved in the regulation of iron homeostasis during inflammation (Peyssonnaux et al. 2006).

In recent years there have been enormous advances in our understanding of how iron traffic into and around the body is controlled. The emergence of hepcidin as a central player in these processes has provided the focus for a large number of ongoing studies, however, much remains to be learned. For example, we still do not know how body requirements are relayed to the liver. Diferric transferrin has emerged as a potential signalling molecule, but how it provides its signal is unclear. Similarly we know almost nothing about the signal transduction pathways linking HFE and TfR2 to hepcidin. Some information is available on how hepcidin exerts its effects on target cells, but many details of its interaction with ferroportin1, and particularly how the process operates in vivo, have yet to be revealed. Another conundrum is why hemojuvelin is expressed so strongly in skeletal and cardiac muscle. Recent work has revealed many unexpected and intriguing facets of the regulation of body iron homeostasis and the future promises to equally, if not more, exciting.

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