Review Article

The regulation of iron transport

David M. Frazer*
Gregory J. Anderson

Iron Metabolism Laboratory, QIMR Berghofer Medical Research Institute, Brisbane, Qld, Australia

Abstract

Iron is an essential nutrient, but its concentration and distribution in the body must be tightly controlled due to its inherent toxicity and insolubility in aqueous solution. Living systems have successfully overcome these potential limitations by evolving a range of iron binding proteins and transport systems that effectively maintain iron in a nontoxic and soluble form for much, if not all, of its time within the body. In the circulation, iron is transported to target organs bound to the serum iron binding protein transferrin. Individual cells modulate their uptake of transferrin-bound iron depending on their iron requirements, using both transferrin receptor 1-dependent and independent pathways. Once inside the cell, iron can be chaperoned to sites of need or, if in excess, stored within ferritin. Iron is released from cells by the iron export protein ferroportin1, which requires the ferroxidase activity of ceruloplasmin or hephestin to load iron safely onto transferrin. The regulation of iron export is controlled predominantly at the systemic level by the master regulator of iron homeostasis hepcidin. Hepcidin, in turn, responds to changes in body iron demand, making use of a range of regulatory mechanisms that center on the bone morphogenetic protein signaling pathway. This review provides an overview of recent advances in the field of iron metabolism and outlines the key components of the iron transport and regulation systems. © 2013 BioFactors, 40(2):206–214, 2014

Keywords: *transferrin; transferrin receptor; ferroportin 1; hepcidin; BMP6*

1. Introduction

The importance of iron in biological systems stems from its ability to cycle between two relatively stable oxidation states (Fe^{2+} or ferrous iron and Fe^{3+} or ferric iron) allowing it to readily act as a co-factor in one electron transfer reactions [1]. This ability of iron, along with its coordination chemistry, has been utilized by evolution in a wide variety of ways, from the electron transport chains that are central to energy production, to the transfer and storage of oxygen by hemoglobin and myoglobin, and the diverse enzymatic reactions requiring iron, including those involved in DNA synthesis, lipid metabolism, and free radical scavenging. It is not surprising, therefore, that iron is an obligate requirement for almost all organisms.

Volume 40, Number 2, March/April 2014, Pages 206–214
**Address for correspondence: David M. Frazer, Ph.D., Iron Metabolism Laboratory, QIMR Berghofer Medical Research Institute, 300 Herston Road, Herston, Brisbane 4006, Australia. Tel.: +61-7-3845-3063, Fax: +61-7-3362-0191; E-mail: david.frazer@qimrberghofer.edu.au*
Received 16 July 2013; accepted 6 September 2013
DOI 10.1002/biof.1148
Published online 17 October 2013 in Wiley Online Library (wileyonlinelibrary.com)

Despite its obvious importance, living systems have had to overcome several difficulties associated with the use of iron. Ferrous iron in solution has the ability to catalyze the formation of toxic oxygen radicals via Fenton chemistry [1]. These highly reactive molecules can readily damage DNA, proteins and lipid membranes. For this reason, all organisms must very tightly regulate their internal iron levels to ensure that they have enough for their biological requirements without leading to iron-induced oxidative stress. In addition, aqueous iron in the presence of oxygen forms insoluble ferric oxide/oxyhydroxide precipitates, rendering it biologically inaccessible. Therefore, if iron is to be utilized by living systems, it must be maintained in a form that is not only nontoxic, but is also bioavailable. Complex organisms face the additional challenge of maintaining iron in this form while transporting it to various sites within a multicellular body. This review will focus on how mammals have overcome these challenges to effect the safe transportation of iron and how this process is regulated.

2. Body Iron Homeostasis

The adult human male contains about 3–5 g of iron [2]. Of this, ~65–70% is present as hemoglobin iron in circulating erythrocytes. A further 20% is intracellular storage iron, predominantly in macrophages and hepatocytes, and the

remaining 10–15% acts as a cofactor for various proteins, including many enzymes, in all cells. These pools of iron are not static, however, and there is a constant flux of iron between them. For example, newly absorbed dietary iron from the small intestine may first be deposited in hepatocytes, and then subsequently travel to the bone marrow to be incorporated into hemoglobin during red blood cell synthesis.

The transport of iron between the various compartments of the body is a highly regulated process. Under normal conditions, 1–2 mg of iron a day is absorbed from the diet, enough to replace obligatory losses from the skin and gastrointestinal tract and to keep body stores in balance [3]. Approximately 3 mg of iron, or less than 0.1% of total body stores, circulates in the plasma [4]. However, this iron is rapidly turned over with ~10 times this about, or 30 mg, being removed from the bloodstream each day [4], predominantly to supply the iron necessary for hemoglobin synthesis in developing erythrocytes. To maintain serum iron levels, a similar amount must be released back into the bloodstream, the bulk of which comes from macrophages as they recycle the iron from old or damaged red blood cells [5]. The dynamic nature of the serum iron pool necessitates a transportation system that maintains iron in a redox inactive and soluble form, but one that is easily accessible to cells requiring iron. Transferrin provides such a system.

2.1. Transferrin

Transferrin is the major extracellular iron binding protein. It is a single chain bilobal protein with a molecular weight of ~80,000 [6]. The lobes appear to have arisen from a gene duplication event and each lobe is separated by a short loop region. Each lobe also has a single iron-binding site with an extremely high affinity for ferric iron (K_d 10^{-23}/M) [7]. Despite this, the binding is reversible, a fact that the body makes use of in the effective and controlled delivery of iron to cells. The binding of ferric iron to transferrin requires carbonate as a synergistic anion [8] and causes the protein to undergo a conformation change from the "open" apo form to the "closed" holo form in which the ferric ions are buried deep within the each lobe [4,6]. This acts to keep the iron in a soluble but redox inactive state so that it can be safely transported around the body.

Transferrin is produced predominantly by the liver and is one of the most abundant proteins in the plasma, being present at 2–4 mg/mL in humans [9]. Under normal conditions, essentially all of the iron found in the circulation is bound to this protein, although it is typically only 30% saturated with iron [4]. As such, four species of transferrin are normally present: diferric transferrin; two monoferric transferrins; and apotransferrin [10]. The additional iron binding capacity is thought to provide a buffer in the event of a sudden influx of iron into the circulation. Despite this, the amount of iron in the circulation is maintained at a relatively constant level. There is good reason for this. If serum iron levels become too low, the iron supply to the erythroid marrow can be compromised resulting in anemia. Likewise, if circulating iron levels exceed the binding capacity of transferrin, highly toxic nontransferrin bound iron can form. Two highly regulated processes control the amount of iron fluxing through the serum iron pool—the rate of iron uptake by cells and the rate of cellular iron release.

3. Iron Transport and Its Regulation

3.1. Cellular Uptake of Transferrin-Bound Iron

3.1.1. Transferrin receptor-mediated uptake

Although there are several pathways for the delivery of transferrin-bound iron to cells, the best characterized involves uptake by transferrin receptor 1 (TFR1). TFR1 is expressed by most body cells, although levels are particularly high in those with elevated iron requirements, such as rapidly dividing cells and developing erythrocytes [11]. TFR1 is a type II transmembrane glycoprotein consisting of two identical subunits linked by disulfide bonds [4,10]. Each subunit consists of a large extracellular region that can bind one molecule of transferrin, a single transmembrane domain, and a cytoplasmic domain containing a consensus internalization motif [10]. The extracellular domains of TFR1 preferentially bind diferric transferrin, although they will also bind monoferric transferrin with a 10-fold lower affinity, and apo-transferrin with a 2000-fold lower affinity [12]. This preferential uptake of diferric transferrin ensures that iron is delivered as efficiently as possible to cells in need.

The binding of transferrin to TFR1 causes the protein complex to be taken into the cell by receptor-mediated endocytosis (Fig. 1). The resulting endosome is acidified to a pH of ~5.5 by a proton-pumping ATPase [10,13]. Acidification, along with the conformational change that occurs upon transferrin binding to TFR1, opens up the transferrin structure allowing the iron to be released [4,6]. The iron is then transported across the endosomal membrane and into the cell by divalent metal–ion transporter 1 (DMT1, also known as solute carrier family 11 (proton-coupled divalent metal-ion transporters), member 2, (SLC11A2)), a member of the natural resistance-associated macrophage protein (NRAMP) family of metal transporters, in a proton-coupled process [14]. However, DMT1 is a ferrous iron transporter, so the ferric iron bound to transferrin must be reduced before export from the endosome. Whether this reduction step occurs before or after the release of iron from transferrin is not known. In immature erythroid cells, the reduction of endosomal ferric iron is carried out by six-transmembrane epithelial antigen of the prostate 3 (STEAP3), a multispanning membrane protein with NADPH and flavin-dependent ferric reductase activity [15]. As STEAP3 is expressed in a range of different tissues, it may mediate the reduction in endosomal iron elsewhere in the body, although there are a number of STEAP family members, which may also play a role in transferrin-bound iron delivery. A recent study has suggested that iron can also be transported out of the endosome by ZRT/IRT-like protein 14 (ZIP14), a member

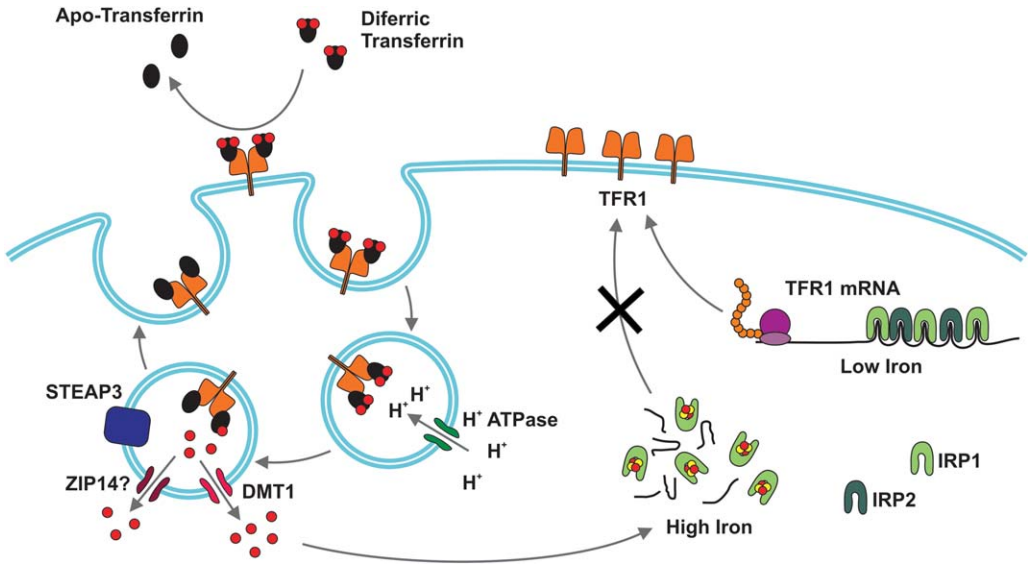

Fig 1

TFR1-mediated iron uptake and its regulation. The TFR1 pathway is the best characterized cellular iron uptake system. Transferrin binds to TFR1 on the cell surface, with diferric transferrin having the highest affinity. The ligand/receptor complex is taken into the cell by receptor-mediated endocytosis and the endosome acidified by proton pumping ATPases. The iron is then released from transferrin, reduced by STEAP3 or other reductases, and transported across the endosomal membrane and into the cell by DMT1 (and possibly ZIP14). Apo-transferrin remains bound to TFR1 in the endosome and is recycled back to the cell surface where it is released and the cycle begins again. The rate of iron uptake by TFR1 is regulated by IRPs, which bind to IRE sequences in the 3′ UTR of the TFR1 mRNA when iron levels are low. This stabilizes the message allowing TFR1 protein to be synthesized, increasing iron uptake. When iron levels increase, IRP2 is degraded and IRP1 gains an iron-sulfur cluster and can no longer bind IRE motifs. Under these conditions, the TFR1 message is degraded, reducing TFR1 protein synthesis and, as a consequence, iron intake.

of the ZIP family of metal iron transporters [16]. ZIP14 was shown to increase the uptake of both transferrin-bound iron and nontransferrin-bound iron in cultured cells although the importance of this protein in the delivery of iron to cells *in vivo* is yet to be determined.

Once the iron has been released from transferrin and imported into the cytoplasm, the apo-transferrin remains bound to TFR1 and the transferrin/receptor complex is recycled back to the plasma membrane [9]. Transferrin is then released into the circulation, leaving TFR1 free to repeat the iron uptake cycle.

3.1.2. The regulation of TFR1-mediated iron uptake

Individual cells are able to very tightly regulate the amount of transferrin-bound iron they take up by varying the expression of TFR1. This regulation is mediated by the binding of iron regulatory proteins (IRPs) to specific RNA stem-loop structures called iron-responsive elements (IREs) [17]. The RNA binding activity of IRPs is strongly induced by cellular iron deprivation; however, the consequence of this binding depends on the location of the IRE. The classic example of this is the opposing regulation of TFR1 and the iron storage protein ferritin by intracellular iron [17]. Five IRE motifs are present in the 3′ UTR of the TFR1 mRNA. The binding of IRPs when intracellular iron levels are low stabilizes the transcript and prevents it from being degraded by endonucleases, resulting in an increase in TFR1 protein expression (Fig. 1). In contrast, IRP binding to the single IRE situated in the 5′ UTR of ferritin mRNAs prevents ribosome attachment and the subsequent translation of the ferritin protein. The opposite occurs when the cell is iron replete, with decreased IRP binding destabilizing the TFR1 message, which reduces TFR1 protein levels and, consequently, iron uptake, while simultaneously allowing the translation of ferritin to safely store excess iron. In this way, the cell can very finely regulate intracellular iron levels to suit its requirements.

Two IRPs have been identified—IRP1 and IRP2. Both proteins bind IRE sequences with similar affinity; however, the proteins respond to cellular iron in different ways. IRP1 contains an iron–sulfur cluster under iron replete conditions, and in this form is unable to bind IRE motifs [17]. When iron is limiting, the iron–sulfur cluster breaks down and IRP1 converts to its RNA binding form. In contrast, IRP2 does not contain an iron–sulfur cluster and is constitutively able to bind IREs [17]. Instead, the SCF (SKP1-CUL1-F-box) E3 ubiquitin ligase complex is recruited in iron replete conditions to promote IRP2 ubiquitination and proteasomal degradation [18].

3.1.3. TFR1-independent pathways for transferrin-bound iron uptake

Transferrin bound iron is taken up exclusively by the TFR1-dependent pathway in some cells, such as immature erythrocytes. However, other cells, such as intestinal enterocytes and hepatocytes, are able to utilize both TFR1-dependent and

TFR1-independent uptake processes [9]. It has even been suggested that, at the normal levels of diferric transferrin found in the circulation, the TFR1-independent pathway should predominate in some cells [9]. While the mechanism by which transferrin bound iron is delivered to cells independently of TFR1 is poorly understood, several candidate transferrin binding proteins have been identified. TFR2 is a homolog of TFR1 and is expressed highly in hepatocytes [19,20]. It can bind transferrin and can facilitate the delivery of iron to cells, although its affinity for diferric transferrin is 25-fold lower than that of TFR1 [21]. However, the restricted, predominantly hepatocyte, expression pattern of TFR2 makes it unlikely to contribute to TFR1-independent iron uptake in other cell types such as enterocytes. Indeed, studies using knockout mice suggest that the major function of TFR2 is in the regulation of iron homeostasis [22] rather than cellular iron delivery (see Section 3.5.3.). Other candidate transferrin binding proteins include cubulin, cell surface glyceraldehyde-3-phosphate dehydrogenase, and proteoglycans [2], although whether these molecules make significant contributions to cellular iron uptake *in vivo* has not been determined.

Iron can also be taken up by cells as low molecular weight nontransferrin-bound iron in certain pathological conditions, and in other forms such as ferritin, heme bound to hemopexin, and hemoglobin bound to haptoglobin [1,9]. While these forms of iron are important for the maintenance of body iron homeostasis, transferrin-bound iron is quantitatively the most significant iron source for cells. Due to space limitations, the transport of nontransferrin bound iron species will not be discussed further in this review.

3.2. Iron Uptake by Specialized Cells

Although the mechanisms of iron uptake described above are common to most body cells, intestinal enterocytes, and erythrophagocytosing macrophages must utilize iron from very specific sources. As such, they exhibit unique pathways for iron uptake and metabolism as described below.

3.2.1. Intestinal enterocytes

The important task of absorbing iron from the diet is performed by the mature villus enterocytes of the duodenum and the upper jejunum. Dietary iron is predominantly in the form of ferric iron and must be reduced to the ferrous form before it can be absorbed. DCYTB (encoded by the *cytochrome b reductase 1* (*CYBRD1*) gene) [21] is a ferric reductase on the brush border membrane, but the evidence that it plays a significant role in intestinal iron absorption is limited [23]. The STEAP family member STEAP2 has also been suggested to be a duodenal ferric reductase [24], but again data on the involvement of this protein remain very limited.

Once reduced, ferrous iron is transported across the brush border membrane and into the enterocyte by DMT1 [21]. Both DMT1 and DCYTB are highly regulated in intestinal tissue, with the expression of both proteins increasing when body iron requirements are high. The DMT1 gene encodes several alternatively spliced transcripts, with the species predominating in enterocytes containing an IRE motif in the 3′ UTR [25], facilitating the iron-dependent expression of the DMT1 protein by IRPs. No IRE has been found in the DCYTB sequence, however, so similar control by IRPs is unlikely and its regulation remains poorly understood. Recent studies have suggested a role for hypoxia inducible factor 2α in the regulation of both DCYTB and DMT1 in intestinal cells [25,26].

Enterocytes also take up dietary iron in the form of heme, but their mechanism of heme uptake is not well understood. The only candidate heme transporter is heme carrier protein-1, although the major role of this protein is in the absorption of dietary folate [1]. Once inside the cell, iron is released from heme by heme oxygenases and thereafter follows the same pathway as recently absorbed non-heme iron.

Dietary iron taken up by enterocytes either can be stored within ferritin if the body is iron replete, or it can be exported across the basolateral membrane where it enters the circulation. Any iron stored as ferritin is lost within a few days, as the enterocyte migrates up the villus, and is sloughed into the intestinal lumen at the end of its life.

3.2.2. Macrophages

In humans, $\sim 2 \times 10^{11}$ old or damaged erythrocytes are removed from the circulation each day, predominantly by the macrophages in the spleen, and the iron is recycled and returned to the plasma [27]. Following erythrophagocytosis, the engulfed erythrocyte is exposed to hydrolytic enzymes and reactive oxygen species, which release heme from its protein components. Recent evidence suggests that heme is exported intact from the phagosome by the heme transporter heme-responsive gene 1 [28]. The iron is then removed from the heme moiety by heme oxygenase 1, although precisely where this occurs in the cell is unclear. Similar to enterocytes, the iron released from hemoglobin breakdown can either be stored within ferritin or released into the circulation depending on the body's iron requirements.

3.3. Cytoplasmic Iron Trafficking

Once iron has been delivered to the cytoplasm, it must be incorporated into the various iron containing proteins or, if it is not immediately required, stored within ferritin. How iron is transported from its entry point to its ultimate destination within the cell, while being maintained in a redox inactive and soluble form, is poorly understood. However, several studies have identified the poly (rC) binding proteins (PCBPs) as iron chaperones which can facilitate the incorporation of iron into several metalloproteins [29,30]. There are four members of this family and recent data indicate that they can all function as iron chaperones, with PCBP-1 and -2 having the ability to incorporate iron into ferritin, prolyl hydroxylases and asparagyl hydroxylases, although the precise mechanism by which the iron is delivered is poorly understood [30]. It is possible that other iron chaperones exist, each with the ability to provide iron to specific proteins, enabling the precise intracellular delivery of iron, while maintaining it in a redox inactive and bioavailable form.

3.4. Cellular Iron Release

The controlled release of iron from cells is crucial for the regulation of body iron homeostasis. This is particularly important for the enterocytes of the duodenum as the export of newly absorbed iron from these cells is normally the rate-limiting step in iron absorption [21]. It is the release of iron from enterocytes, therefore, that ultimately dictates the amount of iron in the body. Of equal importance is the controlled release of iron from hemoglobin breakdown by reticuloendothelial macrophages, as this accounts for around 70–80% of iron entering the plasma [5,11]. If the release of iron from these cells is too low, the body risks becoming iron deficient, with plasma iron levels decreasing to the point where red blood cell production is compromised. If iron release is too high, the excess iron saturates transferrin in the circulation and leads to tissue iron loading and eventual organ damage.

3.4.1. Ferroportin 1

The only known plasma membrane iron export protein in mammals is ferroportin 1 [3]. Ferroportin 1 is an integral membrane protein with 12 predicted membrane-spanning regions [31]. It is expressed in most cell types, but is most highly expressed in those with a major function in iron release such as enterocytes and macrophages [3]. It is also highly expressed in placental tissue where it probably plays a role in iron transfer to the fetus [9].

Ferroportin 1 likely exports iron in the ferrous state [32]. Therefore, an oxidation step is required before the exported iron can be loaded onto circulating transferrin. In most tissues, this is achieved by the copper-containing ferroxidase ceruloplasmin [3]. This protein is found both in a soluble form in the circulation and as a GPI-linked protein on the plasma membrane of some cells, particularly those of the central nervous system [33]. Regardless of the form, a direct interaction between ceruloplasmin and ferroportin 1 at the plasma membrane is required to facilitate iron release [34]. In the absence of ceruloplasmin, the iron remains bound to ferroportin 1 and the export protein is targeted for degradation by ubiquitination. There is also evidence for a transient interaction between ceruloplasmin and transferrin in the circulation [35], suggesting that ceruloplasmin acts not only as a ferroxidase to enable the release of iron from ferroportin 1, but also acts to shuttle iron directly to transferrin. While complexes between ceruloplasmin and transferrin have not been directly detected in the plasma, it makes biological sense for the body to keep iron continuously bound to proteins during cellular export, and indeed at all other times, to reduce the potential for problems associated with the inherent redox activity and insolubility of iron.

The amount of ceruloplasmin in the serum is well in excess of that needed to facilitate the release of iron under normal conditions. In fact, the ferroxidase activity of ceruloplasmin must drop below 5% of normal levels before iron export is affected [9]. The reason for the extra ferroxidase capacity is not known, although it may allow the body to rapidly mobilize stored iron in response to increases in iron demand, for example, following blood loss.

While circulating or GPI-linked ceruloplasmin is sufficient to facilitate iron release from most tissues, some cells require a specialized ferroxidase for this purpose. In intestinal enterocytes, this role is fulfilled by the membrane-bound ceruloplasmin homolog hephestin. Hephestin consists of an extracellular domain, which shares 50% identity with ceruloplasmin, attached to a single transmembrane domain and a short cytoplasmic tail [9]. The disruption of this protein in mice leads to the accumulation of iron within enterocytes and a mild anemia due to reduced iron absorption [36], indicating that circulating ceruloplasmin cannot fully compensate for the lack of hephestin. Although the primary role of hephestin appears to be the facilitation of dietary iron absorption, there is some overlap of function between it and ceruloplasmin, with the disruption of both proteins resulting in more severe iron loading in the central nervous system than does the deletion ceruloplasmin alone [37]. Ceruloplasmin has also been shown to play a role in intestinal iron absorption during times of high iron demand [38]. Why a specific intestinal oxidase is necessary for iron absorption is unclear, but it may be related to the unique role of enterocytes in regulating total body iron intake. Another ceruloplasmin homolog, zyklopen, is expressed in the placenta, but little is known of its biology [32].

3.5. Hepcidin and the Regulation of Iron Export

Unlike cellular iron uptake, which is regulated by the local iron demands of the cell, the regulation of iron release occurs predominantly at the systemic level and is mediated by the peptide hormone hepcidin (encoded by the *HAMP* gene). This 25 amino acid peptide is produced predominantly by the hepatocytes of the liver and secreted into the circulation [3]. It binds to ferroportin 1 on the surface of cells, causing the iron exporter to be internalized and degraded [39]. Thus, hepcidin has an inhibitory effect on iron release. The consequence of an increase in hepcidin production is a decrease in iron export from all body cells, particularly those expressing high levels of ferroportin 1, such as enterocytes and macrophages. This leads to a reduction in dietary iron absorption and causes recycled iron in macrophages to be stored within ferritin. The opposite occurs when hepcidin levels decrease, with iron absorption increasing and stored iron in macrophages being released into the circulation. For this reason, hepcidin is often regarded as the central regulator of iron homeostasis.

Although the precise mechanism of hepcidin-induced ferroportin 1 internalization has not been fully characterized, there have been several recent studies examining this process. It is now known that the binding of hepcidin to ferroportin 1 results in the ubiquitination of critical lysine residues in the third intracellular loop of the iron export protein, causing it to be directed to the proteasome for degradation [40]. Hepcidin remains bound and is degraded along with ferroportin 1, and it has been suggested that a disulfide bond exchange occurs between one of the four disulfide bridges in hepcidin and a

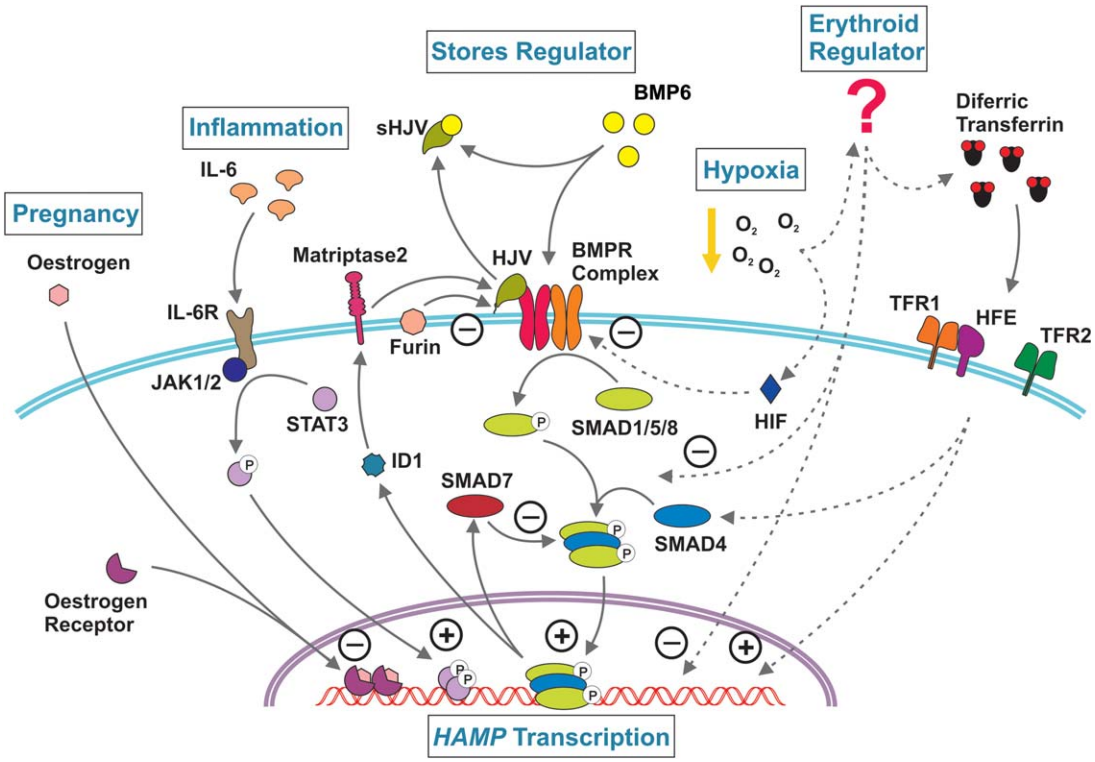

Fig 2

The regulation of hepcidin production. The expression of hepcidin by hepatocytes is regulated by a complex network of signaling pathways. The best characterized is the BMP6/SMAD pathway. BMP6 production is induced by increasing iron stores. BMP6 binds to its receptor complex (BMPR) on the surface and induces the phosphorylation of SMADs 1, 5, and 8. These bind to SMAD4 and translocate to the nucleus where they induce hepcidin transcription. HJV acts as a co-receptor and forms part of the BMPR complex. The SMAD pathway also induces the expression of SMAD7, which interferes with SMAD signaling, and ID1, which induces the expression of matriptase 2, a protease that degrades HJV. Both of these systems act as negative feedback loops to reduce hepcidin production. HJV is also cleaved by furin to produce a soluble form that competes with membrane HJV for BMP6 binding. Inflammation induces hepcidin expression predominantly via IL-6 binding to its receptor (IL-6R) and stimulating the JAK/STAT pathway. Hepcidin is decreased during pregnancy, at least in part, by the binding of the estrogen/estrogen receptor complex to an estrogen responsive element in the hepcidin promoter. The decrease in hepcidin expression by the erythroid regulator is not well characterized, but it interferes with the BMP6/SMAD pathway, possibly via the detection of diferric transferrin levels by TFR1/HFE and TFR2. While hypoxia has been proposed to decrease hepcidin expression by affecting BMP6 signaling, it now seems likely that its effects are mediated by the erythroid regulator and are secondary to the effect of hypoxia on erythropoiesis.

crucial cysteine residue located in the fourth extracellular loop of ferroportin 1, covalently linking the two molecules together [41].

3.5.1. The regulation of hepcidin production

Given the importance of hepcidin in the maintenance of iron homeostasis, it is not surprising that the regulation of hepcidin production has been the focus of extensive research since its discovery in 2000 [42], although many aspects remain poorly understood (Fig. 2). Hepcidin expression is decreased with iron deficiency, in response to stimulated erythropoiesis when iron supply to the marrow is limiting, hypoxia and during pregnancy, resulting in an increase in both dietary iron absorption and the release of storage iron [9]. In contrast, hepcidin production is increased when body iron stores rise and during inflammation, so that iron absorption is limited and body iron is directed toward storage in ferritin.

3.5.2. Body Iron Status and Hepcidin Regulation

The best understood and most extensively researched mechanism for hepcidin regulation is the bone morphogenetic protein (BMP)/SMAD pathway. Studies have shown that, at least in rodent models, increasing body iron stimulates the production of BMP6, which binds to a complex of type I and II BMP receptors on the plasma membrane of hepatocytes [43,44]. This leads to the phosphorylation of SMADs 1, 5, and 8 in the cytoplasm, which allows the binding of SMAD4. The entire complex is then translocated into the nucleus where it binds to BMP responsive elements in the hepcidin promoter, stimulating transcription. The GPI-linked membrane protein hemojuvelin binds BMP6 and acts as a co-receptor for the BMP receptor complex [11]. Hemojuvelin is essential for BMP6 signaling as the disruption of the protein, as occurs in the juvenile form of the iron loading disorder hemochromatosis, leads to the complete loss of hepcidin production [45]. Hemojuvelin can

also be cleaved from the plasma membrane by furin to produce a soluble form that retains the ability to bind BMP6 [46]. This sequesters the cytokine away from the cell surface receptor, inhibiting BMP6 signaling and, as a consequence, hepcidin expression. The proteolytic activity of furin is increased in iron deficiency and hypoxia [46], both of which are associated with reduced hepcidin levels.

Activation of the SMAD pathway also induces the expression of inhibitor of DNA binding 1 (ID1), which in turn induces the expression of matriptase 2 (also known as TMPRSS6) [47]. This plasma membrane protease cleaves cell surface hemojuvelin [48]. In this case, however, the soluble product does not effectively compete for BMP6 binding. Instead, cleavage of hemojuvelin by matriptase 2 prevents hemojuvelin from acting as a co-receptor for BMP6 binding, decreasing SMAD signaling and hepcidin production. Thus, the induction of ID1 and matriptase 2 by SMAD signaling acts as a negative feedback loop to modulate hepcidin production. A similar negative feedback loop involves SMAD7, which, like ID1, is induced by the BMP6/SMAD pathway [49]. This inhibitory SMAD interferes with SMAD signaling, thereby reducing hepcidin expression. Both the SMAD7 and ID1 negative feedback loops are thought to prevent the overexpression of hepcidin following stimulation, although their roles *in vivo* need further clarification.

3.5.3. Stimulated erythropoiesis suppresses hepcidin

BMP6 signaling appears to regulate hepcidin expression in response to body iron stores (the stores regulator) as BMP6 production closely follows hepatic iron levels. The other major regulator of hepcidin expression is the adequacy of iron supply to developing erythrocytes (the erythroid regulator) [50]. During times of stimulated erythropoiesis or, more specifically, when the iron demands of developing erythroid precursors exceed the supply of transferrin bound iron, the erythroid regulator signals a decrease in hepcidin production in the liver, allowing more iron to be released into the circulation. Unfortunately, while extensive research has revealed much about the BMP6/SMAD pathway, significantly less is known about the molecular basis of the erythroid regulator. In a mouse model of β-thalassemia, the signal to decrease hepcidin expression reduces the ability of BMP6 to activate SMAD signaling [51]. However, this does not explain the nature of the signal relaying erythroid iron usage to the liver. Two proteins, growth differentiation factor 15 (GDF15) [52] and twisted gastrulation homolog 1 (TWGS1) [53] have been proposed to perform this task. Both are members of the transforming growth factor-β family and, as such, have the potential to modulate BMP6 signaling, and both can decrease hepcidin expression in cultured cells [52,53]. However, neither protein has been shown to affect hepcidin expression *in vivo*. Indeed, the GDF-15 knockout mouse has no obvious iron phenotype and responds to stimulated erythropoiesis by down regulating hepcidin expression as normal [54].

Another potential mediator of the erythroid regulator is the level of diferric transferrin in the circulation. Diferric transferrin is taken up preferentially by TFR1 [12], making it an ideal indicator of body iron usage. And, as most circulating iron is taken up by developing erythroid cells, diferric transferrin levels would accurately reflect changes in erythroid iron demand. A potential detection mechanism for diferric transferrin has been recognized, and it involves the proteins HFE and TFR2 located on the plasma membrane of hepatocytes [21]. These proteins are known regulators of hepcidin expression, and the disruption of either one leads to insufficient hepcidin production and the iron overload condition hemochromatosis. How these proteins regulate hepcidin expression is unclear, however, recent studies have suggested that signaling both by HFE and TFR2 and by diferric transferrin is mediated by the SMAD pathway [55,56].

Both HFE and TFR2 have the potential to detect diferric transferrin levels. TFR2 binds diferric transferrin and this binding stabilizes the receptor [57,58], possibly allowing it to signal changes in hepcidin expression. Although HFE does not interact directly with diferric transferrin, it binds with high affinity to TFR1 at a site that overlaps with the transferrin binding site [21], suggesting a possible competition between the two proteins for TFR1. Indeed, studies using cultured cells have provided direct evidence for this [59]. However, even if the detection of diferric transferrin by HFE and TFR2 does represent the erythroid regulator, it cannot be the sole mediator of this signal. This is because serum iron levels are often elevated in conditions where the erythroid regulator is strongly inhibiting hepcidin expression, such as in β-thalassemia [60].

3.5.4. Inflammation, pregnancy, and hypoxia

Inflammation is a strong inducer of hepcidin expression [11] and the ensuing hypoferremia caused by reduced cellular iron release is thought to limit the availability of iron to invading pathogens. During prolonged inflammation, however, the iron supply to the marrow can become limiting, resulting in the anemia of inflammation [27]. The increase in hepcidin during inflammation is primarily due to the stimulation of the janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) pathway by cytokines such as interleukin 6 [61]. Although distinct STAT3 binding sites have been found in the *HAMP* promoter, a functional SMAD signaling pathway is also required for the induction of hepcidin expression by inflammation as demonstrated by the lack of hepcidin response to interleukin 6 in SMAD4 knockout mice [62]. This is most likely due to the requirement of the BMP/SMAD signaling pathway for basal hepcidin production. However, a recent study has shown that activin B, a member of the TGF-β family induced by inflammation, can up-regulate hepcidin expression by activating the SMAD pathway [63]. This occurs independently of JAK/STAT3 signaling.

Hepcidin expression is suppressed during pregnancy [64,65], increasing dietary iron absorption to provide the extra iron necessary for the developing fetus. Both hepatic iron stores and transferrin saturation decrease progressively throughout pregnancy suggesting that the decrease in hepcidin production is due to the increase in body iron needs during

this time. However, in both rodents and human, hepcidin returns to normal levels almost immediately following birth and before any significant recovery in iron status can occur [64,65]. This implies that the increased iron utilization by the fetus cannot be the sole regulator of hepcidin during pregnancy. A recent study has shown that estrogen has an inhibitory effect on hepcidin via an estrogen responsive element half site in the *HAMP* promoter [66]. It is, therefore, likely that the down-regulation of hepcidin during pregnancy is due to the combined effects of the iron demands of the growing fetus and the inhibitory effect of rising estrogen levels.

Hypoxia is associated with a decrease in hepcidin expression. Several studies have indicated that hypoxia may have a direct role on the suppression of hepcidin via the hypoxia-inducible factors (HIFs) [25,67]. However, more recent data suggest that the HIFs have an indirect effect on hepcidin synthesis by regulating erythropoietin expression [68,69]. During hypoxia, the HIFs upregulate erythropoietin expression, stimulating erythropoiesis, which feeds back via the erythroid regulator to decrease hepcidin expression. The increase in erythropoiesis was shown to be essential for hepcidin down-regulation in hypoxia, indicating that the HIFs did not directly regulate hepcidin.

4. Conclusions

The inherent redox activity and insolubility of iron has necessitated the evolution of highly specific binding proteins and complex regulatory networks to handle it in biological systems. The proteins and pathways described in this review share the common characteristic of keeping iron bound to protein at almost all times. It is highly likely that other iron binding proteins will be discovered, realizing a scenario in which iron is shuttled from one protein to another on its journey around the body, never being present in a low molecular weight form. This would make evolutionary sense for such an essential but potentially toxic element.

Acknowledgements

This work was supported by a Project Grant from the National Health and Medical Research Council of Australia (1051764) (GJA and DMF). GJA is supported by a Senior Research Fellowship from the National Health and Medical Research Council of Australia and DMF by an Australian Liver Foundation-Hospitality Industry Career Development Research Fellowship.

References

[1] Pantopoulos, K., Porwal, S. K., Tartakoff, A., and Devireddy, L. (2012) Mechanisms of mammalian iron homeostasis. Biochemistry 51, 5705–5724.

[2] Gkouvatsos, K., Papanikolaou, G., and Pantopoulos, K. (2012) Regulation of iron transport and the role of transferrin. Biochim. Biophys. Acta 1820, 188–202.

[3] Anderson, G. J. and Wang, F. (2012) Essential but toxic: controlling the flux of iron in the body. Clin. Exp. Pharmacol. Physiol. 39, 719–724.

[4] El Hage Chahine, J. M., Hemadi, M., and Ha-Duong, N. T. (2012) Uptake and release of metal ions by transferrin and interaction with receptor 1. Biochim. Biophys. Acta 1820, 334–347.

[5] Chen, J. and Enns, C. A. (2012) Hereditary hemochromatosis and transferrin receptor 2. Biochim. Biophys. Acta 1820, 256–263.

[6] Mizutani, K., Toyoda, M., and Mikami, B. (2012) X-ray structures of transferrins and related proteins. Biochim. Biophys. Acta 1820, 203–211.

[7] Aisen, P., Leibman, A., and Zweier, J. (1978) Stoichiometric and site characteristics of the binding of iron to human transferrin. J. Biol. Chem. 253, 1930–1937.

[8] Harris, W. R. (2012) Anion binding properties of the transferrins. Implications for function. Biochim. Biophys. Acta 1820, 348–361.

[9] Anderson, G. J. and Vulpe, C. D. (2009) Mammalian iron transport. Cell. Mol. Life. Sci. 66, 3241–3261.

[10] Steere, A. N., Byrne, S. L., Chasteen, N. D., and Mason, A. B. (2012) Kinetics of iron release from transferrin bound to the transferrin receptor at endosomal pH. Biochim. Biophys. Acta 1820, 326–333.

[11] Darshan, D., Frazer, D. M., and Anderson, G. J. (2010) Molecular basis of iron-loading disorders. Expert. Rev. Mol. Med. 12, e36.

[12] Tsunoo, H. and Sussman, H. H. (1983) Characterization of transferrin binding and specificity of the placental transferrin receptor. Arch. Biochem. Biophys. 225, 42–54.

[13] van Renswoude, J., Bridges, K. R., Harford, J. B., and Klausner, R. D. (1982) Receptor-mediated endocytosis of transferrin and the uptake of fe in K562 cells: identification of a nonlysosomal acidic compartment. Proc Natl Acad Sci U S A 79, 6186–6190.

[14] Fleming, M. D., Romano, M. A., Su, M. A., Garrick, L. M., Garrick, M. D., et al. (1998) Nramp2 is mutated in the anemic Belgrade (b) rat: evidence of a role for Nramp2 in endosomal iron transport. Proc. Natl. Acad. Sci. USA. 95, 1148–1153.

[15] Ohgami, R. S., Campagna, D. R., Greer, E. L., Antiochos, B., McDonald, A., et al. (2005) Identification of a ferrireductase required for efficient transferrin-dependent iron uptake in erythroid cells. Nat. Genet. 37, 1264–1269.

[16] Zhao, N., Gao, J., Enns, C. A., and Knutson, M. D. (2010) ZRT/IRT-like protein 14 (ZIP14) promotes the cellular assimilation of iron from transferrin. J. Biol. Chem. 285, 32141–32150.

[17] Anderson, C. P., Shen, M., Eisenstein, R. S., and Leibold, E. A. (2012) Mammalian iron metabolism and its control by iron regulatory proteins. Biochim. Biophys. Acta 1823, 1468–1483.

[18] Vashisht, A. A., Zumbrennen, K. B., Huang, X., Powers, D. N., Durazo, A., et al. (2009) Control of iron homeostasis by an iron-regulated ubiquitin ligase. Science 326, 718–721.

[19] Kawabata, H., Yang, R., Hirama, T., Vuong, P. T., Kawano, S., et al. (1999) Molecular cloning of transferrin receptor 2. A new member of the transferrin receptor-like family. J Biol Chem 274, 20826–20832.

[20] Fleming, R. E., Migas, M. C., Holden, C. C., Waheed, A., Britton, R. S., et al. (2000) Transferrin receptor 2: continued expression in mouse liver in the face of iron overload and in hereditary hemochromatosis. Proc Natl Acad Sci U S A 97, 2214–2219.

[21] Frazer, D. M. and Anderson, G. J. (2003) The orchestration of body iron intake: how and where do enterocytes receive their cues? Blood. Cells. Mol. Dis. 30, 288–297.

[22] Camaschella, C., Roetto, A., Cali, A., De Gobbi, M., Garozzo, G., et al. (2000) The gene TFR2 is mutated in a new type of haemochromatosis mapping to 7q22. Nat. Genet. 25, 14–15.

[23] Choi, J., Masaratana, P., Latunde-Dada, G. O., Arno, M., Simpson, R. J., et al. (2012) Duodenal reductase activity and spleen iron stores are reduced and erythropoiesis is abnormal in Dcytb knockout mice exposed to hypoxic conditions. J. Nutr. 142, 1929–1934.

[24] Ohgami, R. S., Campagna, D. R., McDonald, A., and Fleming, M. D. (2006) The Steap proteins are metalloreductases. Blood 108, 1388–1394.

[25] Bartnikas, T. B., Fleming, M. D., and Schmidt, P. J. (2012) Murine mutants in the study of systemic iron metabolism and its disorders: an update on recent advances. Biochim. Biophys. Acta 1823, 1444–1450.

[26] Mastrogiannaki, M., Matak, P., and Peyssonnaux, C. (2013) The gut in iron homeostasis: role of HIF-2 under normal and pathological conditions. Blood 122, 885–892.

[27] Ganz, T. (2012) Macrophages and Systemic Iron Homeostasis. J. Innate. Immun. 4, 446–453.

[28] Delaby, C., Rondeau, C., Pouzet, C., Willemetz, A., Pilard, N., et al. (2012) Subcellular localization of iron and heme metabolism related proteins at early stages of erythrophagocytosis. PLoS One 7, e42199.
[29] Shi, H., Bencze, K. Z., Stemmler, T. L., and Philpott, C. C. (2008) A cytosolic iron chaperone that delivers iron to ferritin. Science 320, 1207–1210.
[30] Leidgens, S., Bullough, K. Z., Shi, H., Li, F., Shakoury-Elizeh, M., et al. (2013) Each member of the poly-r(C)-binding protein 1 (PCBP) family exhibits iron chaperone activity toward ferritin. J. Biol. Chem. 288, 17791–17802.
[31] Le Gac, G., Ka, C., Joubrel, R., Gourlaouen, I., Lehn, P., et al. (2013) Structure-function analysis of the human ferroportin iron exporter (SLC40A1): effect of hemochromatosis type 4 disease mutations and identification of critical residues. Hum. Mutat. doi: 10.1002/humu.22369.
[32] Ward, D. M. and Kaplan, J. (2012) Ferroportin-mediated iron transport: expression and regulation. Biochim. Biophys. Acta 1823, 1426–1433.
[33] Jeong, S. Y. and David, S. (2003) Glycosylphosphatidylinositol-anchored ceruloplasmin is required for iron efflux from cells in the central nervous system. J. Biol. Chem. 278, 27144–27148.
[34] De Domenico, I., Ward, D. M., di Patti, M. C., Jeong, S. Y., David, S., et al. (2007) Ferroxidase activity is required for the stability of cell surface ferroportin in cells expressing GPI-ceruloplasmin. EMBO. J. 26, 2823–2831.
[35] Ha-Duong, N. T., Eid, C., Hemadi, M., and El Hage Chahine, J. M. (2010) In vitro interaction between ceruloplasmin and human serum transferrin. Biochemistry 49, 10261–10263.
[36] Vulpe, C. D., Kuo, Y. M., Murphy, T. L., Cowley, L., Askwith, C., et al. (1999) Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. Nat. Genet. 21, 195–199.
[37] Hadziahmetovic, M., Dentchev, T., Song, Y., Haddad, N., He, X., et al. (2008) Ceruloplasmin/hephaestin knockout mice model morphologic and molecular features of AMD. Invest. Ophthalmol. Vis. Sci. 49, 2728–2736.
[38] Cherukuri, S., Potla, R., Sarkar, J., Nurko, S., Harris, Z. L., et al. (2005) Unexpected role of ceruloplasmin in intestinal iron absorption. Cell Metab. 2, 309–319.
[39] Nemeth, E., Tuttle, M. S., Powelson, J., Vaughn, M. B., Donovan, A., et al. (2004) Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 306, 2090–2093.
[40] Ross, S. L., Tran, L., Winters, A., Lee, K. J., Plewa, C., et al. (2012) Molecular mechanism of hepcidin-mediated ferroportin internalization requires ferroportin lysines, not tyrosines or JAK-STAT. Cell Metab. 15, 905–917.
[41] Preza, G. C., Pinon, R., Ganz, T., and Nemeth, E. (2013) Cellular catabolism of the iron-regulatory peptide hormone hepcidin. PLoS One 8, e58934.
[42] Krause, A., Neitz, S., Magert, H. J., Schulz, A., Forssmann, W. G., et al. (2000) LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett. 480, 147–150.
[43] Meynard, D., Kautz, L., Darnaud, V., Canonne-Hergaux, F., Coppin, H., et al. (2009) Lack of the bone morphogenetic protein BMP6 induces massive iron overload. Nat. Genet. 41, 478–481.
[44] Andriopoulos, B., Jr., Corradini, E., Xia, Y., Faasse, S. A., Chen, S., et al. (2009) BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. Nat. Genet. 41, 482–487.
[45] Niederkofler, V., Salie, R., and Arber, S. (2005) Hemojuvelin is essential for dietary iron sensing, and its mutation leads to severe iron overload. J. Clin. Invest. 115, 2180–2186.
[46] Silvestri, L., Pagani, A., and Camaschella, C. (2008) Furin-mediated release of soluble hemojuvelin: a new link between hypoxia and iron homeostasis. Blood 111, 924–931.
[47] Meynard, D., Vaja, V., Sun, C. C., Corradini, E., Chen, S., et al. (2011) Regulation of TMPRSS6 by BMP6 and iron in human cells and mice. Blood 118, 747–756.
[48] Silvestri, L., Pagani, A., Nai, A., De Domenico, I., Kaplan, J., et al. (2008) The serine protease matriptase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemojuvelin. Cell Metab. 8, 502–511.
[49] Mleczko-Sanecka, K., Casanovas, G., Ragab, A., Breitkopf, K., Muller, A., et al. (2010) SMAD7 controls iron metabolism as a potent inhibitor of hepcidin expression. Blood 115, 2657–2665.
[50] Finch, C. (1994) Regulators of iron balance in humans. Blood 84, 1697–1702.
[51] Frazer, D. M., Wilkins, S. J., Darshan, D., Badrick, A. C., McLaren, G. D., et al. (2012) Stimulated erythropoiesis with secondary iron loading leads to a decrease in hepcidin despite an increase in bone morphogenetic protein 6 expression. Br. J. Haematol. 157, 615–626.
[52] Tanno, T., Bhanu, N. V., Oneal, P. A., Goh, S. H., Staker, P., et al. (2007) High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. Nat. Med. 13, 1096–1101.
[53] Tanno, T., Porayette, P., Sripichai, O., Noh, S. J., Byrnes, C., et al. (2009) Identification of TWSG1 as a second novel erythroid regulator of hepcidin expression in murine and human cells. Blood 114, 181–186.
[54] Casanovas, G., Spasic, M. V., Casu, C., Rivella, S., Strelau, J., et al. (2013) The murine growth differentiation factor 15 is not essential for systemic iron homeostasis in phlebotomized mice. Haematologica 98, 444–447.
[55] Corradini, E., Meynard, D., Wu, Q., Chen, S., Ventura, P., et al. (2011) Serum and liver iron differently regulate the bone morphogenetic protein 6 (BMP6)-SMAD signaling pathway in mice. Hepatology 54, 273–284.
[56] Corradini, E., Rozier, M., Meynard, D., Odhiambo, A., Lin, H. Y., et al. (2011) Iron regulation of hepcidin despite attenuated Smad1,5,8 signaling in mice without transferrin receptor 2 or Hfe. Gastroenterology 141, 1907–1914.
[57] Johnson, M. B. and Enns, C. A. (2004) Diferric transferrin regulates transferrin receptor 2 protein stability. Blood 104, 4287–4293.
[58] Robb, A. and Wessling-Resnick, M. (2004) Regulation of transferrin receptor 2 protein levels by transferrin. Blood 104, 4294–4299.
[59] Giannetti, A. M. and Bjorkman, P. J. (2004) HFE and transferrin directly compete for transferrin receptor in solution and at the cell surface. J. Biol. Chem. 279, 25866–25875.
[60] Ginzburg, Y. and Rivella, S. (2011) beta-thalassemia: a model for elucidating the dynamic regulation of ineffective erythropoiesis and iron metabolism. Blood 118, 4321–4330.
[61] Verga Falzacappa, M. V., Vujic Spasic, M., Kessler, R., Stolte, J., Hentze, M. W., et al. (2007) STAT3 mediates hepatic hepcidin expression and its inflammatory stimulation. Blood 109, 353–358.
[62] Wang, R. H., Li, C., Xu, X., Zheng, Y., Xiao, C., et al. (2005) A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression. Cell Metab. 2, 399–409.
[63] Besson-Fournier, C., Latour, C., Kautz, L., Bertrand, J., Ganz, T., et al. (2012) Induction of activin B by inflammatory stimuli up-regulates expression of the iron-regulatory peptide hepcidin through Smad1/5/8 signaling. Blood 120, 431–439.
[64] Millard, K. N., Frazer, D. M., Wilkins, S. J., and Anderson, G. J. (2004) Changes in the expression of intestinal iron transport and hepatic regulatory molecules explain the enhanced iron absorption associated with pregnancy in the rat. Gut 53, 655–660.
[65] van Santen, S., Kroot, J. J., Zijderveld, G., Wiegerinck, E. T., Spaanderman, M. E., et al. (2013) The iron regulatory hormone hepcidin is decreased in pregnancy: a prospective longitudinal study. Clin. Chem. Lab. Med. 51, 1395–1401.
[66] Yang, Q., Jian, J., Katz, S., Abramson, S. B., and Huang, X. (2012) 17beta-Estradiol inhibits iron hormone hepcidin through an estrogen responsive element half-site. Endocrinology 153, 3170–3178.
[67] Peyssonnaux, C., Zinkernagel, A. S., Schuepbach, R. A., Rankin, E., Vaulont, S., et al. (2007) Regulation of iron homeostasis by the hypoxia-inducible transcription factors (HIFs). J. Clin. Invest. 117, 1926–1932.
[68] Liu, Q., Davidoff, O., Niss, K., and Haase, V. H. (2012) Hypoxia-inducible factor regulates hepcidin via erythropoietin-induced erythropoiesis. J. Clin. Invest. 122, 4635–4644.
[69] Mastrogiannaki, M., Matak, P., Mathieu, J. R., Delga, S., Mayeux, P., et al. (2012) Hepatic hypoxia-inducible factor-2 down-regulates hepcidin expression in mice through an erythropoietin-mediated increase in erythropoiesis. Haematologica 97, 827–834.