Chapter 9
Molecular Regulation of Systemic Iron Metabolism

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Keywords BMP • Hemojuvelin • Hepcidin • HFE • Homeostasis • Iron • SMAD • STAT • TfR2 • TMPRSS6

1 Introduction

In humans, plasma iron concentrations are normally maintained in a relatively narrow range of 10–30 μM with transferrin saturation 20–40%. Prolonged decrease of iron concentrations causes cellular dysfunction and anemia (Chap. 15). Severe and prolonged increase of iron concentrations results in iron deposition in vital organs with consequent tissue injury (Chap. 18). With an average plasma volume of 3 l and iron concentration of 1 mg/l, plasma contains only about 3 mg of iron, a small fraction of the total body iron content of 3–4 g. Assuming free equilibration of iron–transferrin into 15 l of extracellular fluid, extracellular iron amounts to only 15 mg. Under normal circumstances, an average of 25 mg of iron enters this compartment every day, from macrophages recycling the iron of senescent erythrocytes, from hepatocyte storage, and from dietary iron intake. An equal amount of iron exits from the extracellular fluid, largely for hemoglobin synthesis but also for the synthesis of other iron-containing proteins, and to replenish iron stores in hepatocytes. Without regulation, the extracellular fluid compartment would be subject to large changes in iron influx from variable dietary iron content and erythrocyte destruction, and large changes in iron efflux due to variations in erythropoietic and other demand for iron. Mechanisms that adjust iron absorption and recycling to keep extracellular iron concentrations constant also effectively match iron supply to iron demand but would not assure stable iron reserves. Additional circuits must regulate iron absorption to maintain iron stores in the liver that help buffer surges in iron demand. Based on studies of iron absorption in animals and humans, it has long been suspected that a homeostatic system for the regulation of extracellular iron concentrations and iron stores must exist [1]. However, the specific molecular mechanisms, centered on the regulation of the iron efflux channel ferroportin by the hepatic peptide hormone hepcidin (Fig. 9.1), were identified only recently.

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G.J. Anderson and G. McLaren (eds.), *Iron Physiology and Pathophysiology in Humans*,
Nutrition and Health, DOI 10.1007/978-1-60327-485-2_9,

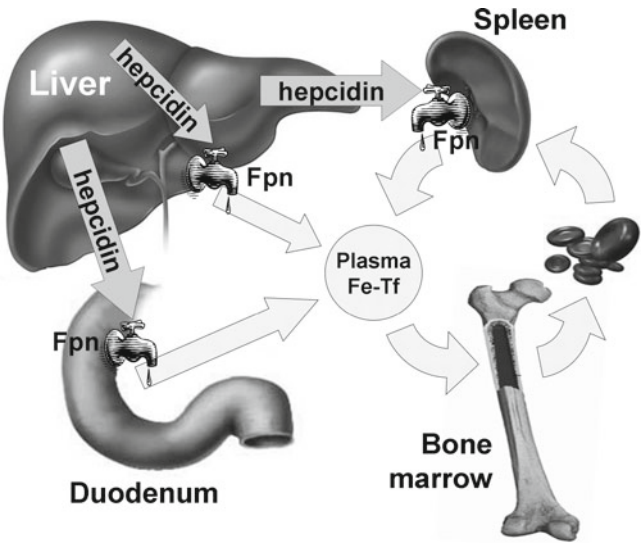

Fig. 9.1 The interaction of hepcidin with ferroportin controls the major flows of iron into extracellular fluid and plasma

2 The Interaction of the Hormone Hepcidin and Its Receptor Ferroportin Regulates Systemic Iron Metabolism

2.1 Hepcidin

Hepcidin is a 25 amino acid (2.7 kD) peptide containing four disulfide bridges [2–4] (Fig. 9.2). It is synthesized in vertebrate hepatocytes as an 84 amino acid prepropeptide that undergoes rapid intracellular processing to mature hepcidin followed by secretion [5]. Hepcidin is an amphipathic cationic peptide that structurally resembles antimicrobial peptides such as defensins and protegrins and displays weak antimicrobial activity in vitro [2, 3]. Although iron- and copper-associated forms of hepcidin have been detected in human urine [6], the biological role (if any) of metallated hepcidin is uncertain.

Hepcidin differs from antimicrobial peptides by greater evolutionary conservation [3] indicative of stricter functional constraints. The involvement of hepcidin in iron metabolism was suggested by its overexpression in the livers of iron-overloaded mice [4], and its essential iron-regulatory role was established by the rapidly progressive iron overload of mice and humans with genetic hepcidin deficiency [7, 8], and the development of severe iron deficiency refractory to oral iron in mice and human overexpressing hepcidin [9, 10]. The administration of synthetic hepcidin peptide to mice produced prolonged hypoferremia [11], demonstrating the in vivo iron-regulatory activity of the 25 amino acid hepcidin peptide.

2.2 Ferroportin

Vertebrate ferroportin [12–14] is also known as solute carrier family 40 (iron-regulated transporter), member 1 (SLC40A1); iron regulated gene 1 (IREG1); solute carrier family 11 (proton-coupled divalent metal ion transporters), member 3 (SLC11A3); and metal transport protein 1 (MTP1). Ferroportin is a 571 amino acid membrane protein with 10 or 12 transmembrane domains and cytoplasmic

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human      DTHFPICIFCCGCCHRSKCGMCCKT
chimp      DTHFPICIFCCGCCHRSKCGMCCKT
rhesus     DTHFPICIFCCGCCHRSKCGMCCRT
marmoset   DTHFPICIFCCGCCRQSNCGMCCKT
pig        DTHFPICIFCCGCCRKAICGMCCKT
dog*       DTHFPICIFCCGCCKTPKCGLCCKT
cow        DTHFPICIFCCGCCRKGTCGMCCRT
rat        DTNFPICLFCCKCCKNSSCGLCCIT
mouse      DTNFPICIFCCKCCNNSQCGICCKT
horse      DTHFPICTLCCGCCNKQKCGWCCKT
zebrafish  QSHLSLCRFCCKCCRNKGCGYCCKF
xenopus    QSHLSICVHCCNCCKYKGCGKCCLT
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Fig. 9.2 Conservation of hepcidin in vertebrates. *Dark gray shading* denotes the strong conservation of the six N-terminal amino acids that are essential for interaction with ferroportin. *Light shading* denotes the conserved cysteine framework and *the underlined sequence* indicates the highly variable loop region of hepcidin. *Asterisk* multiple dog sequences differing at the C-terminus (…LCCIT, …FCCKT, …LCCKT) are listed in NCBI Protein Database (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&id=51095241) and may represent polymorphic variants

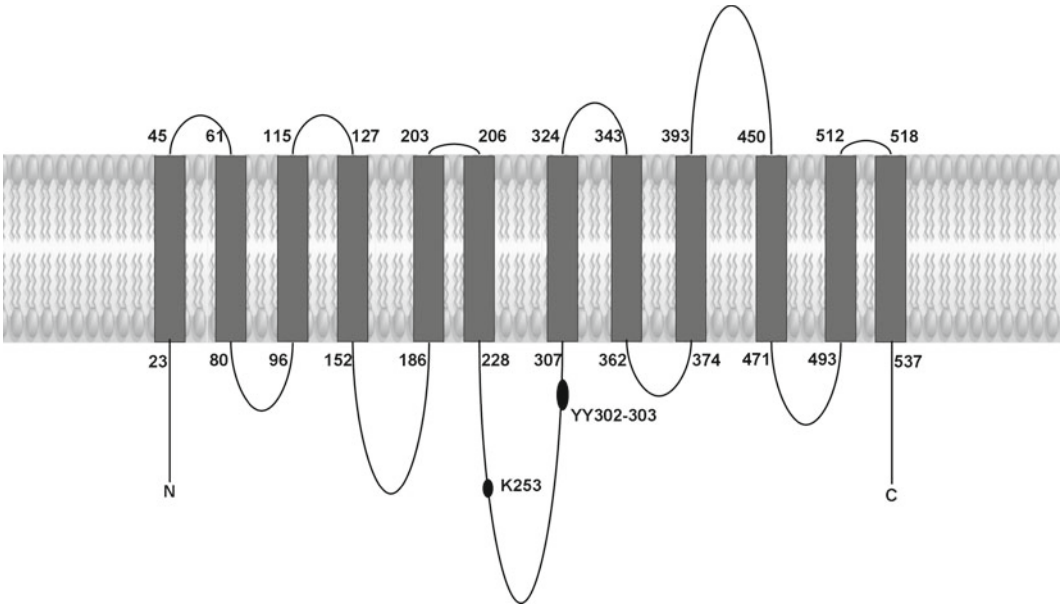

Fig. 9.3 A model of the topology of mammalian ferroportin. Extracellular loops are on the *top*, intracellular on the *bottom*. N- and C-termini and amino acids involved in internalization and degradation are *highlighted*

amino and carboxy termini [15, 16] (Fig. 9.3). Ferroportin constructs expressed in *Xenopus* oocytes or mammalian cells cause iron efflux [12, 13, 17]. Moreover, the mRNA and protein are found in all the cell types that export iron: duodenal enterocytes, placental syncytiotrophoblast, hepatocytes, and macrophages. The iron export function of ferroportin is essential as shown by the embryonic lethal systemic iron deficiency in mice and zebrafish that lack ferroportin [14, 17]. Mice with inactivation of ferroportin in the embryo but not the maternal–fetal interface survive to birth but rapidly become iron deficient and anemic, and manifest abnormal iron accumulation in iron-exporting tissues, including duodenal enterocytes, macrophages and hepatocytes [17]. These findings confirm the essential cellular iron export function of ferroportin.

2.3 Regulation of Ferroportin by Hepcidin

Hepcidin regulates cellular iron export by a surprisingly simple mechanism, dependent on hepcidin-induced ferroportin internalization and degradation [18]. This was shown by analyzing the fate of green fluorescent protein (GFP)-tagged ferroportin after exposure to hepcidin in model cell systems [18, 19]. Ferroportin preserves its iron-export function when marked with protein or peptide tags at its carboxy terminus. When expressed in mammalian cell lines, the ferroportin-GFP fusion protein generates membrane-associated fluorescence but upon exposure to 0.1–5 μg/ml hepcidin, the fusion protein is taken up into lysosomes within 1 h and degraded. The molecular mechanism of ferroportin internalization is similar to that of other receptors internalized by their ligands, and involves hepcidin-induced serial phosphorylation and ubiquitination of ferroportin [20]. The large cytoplasmic loop containing residues 229–306 contains the motifs that undergo ligand-induced modification. Tyrosine phosphorylation is required for ferroportin internalization, as revealed by the resistance of Y302F, Y303F double mutants to tyrosine phosphorylation and hepcidin-induced internalization. Subsequent lysine ubiquitination on K253 is required for efficient ferroportin degradation, as the K253A mutant (but not other lysine mutants) is not ubiquitinated and its degradation is very slow. The effect of all these mutations is specific for internalization as they do not impair the iron-exporting function of ferroportin.

In ex vivo macrophages or macrophage-like cell lines, endogenous ferroportin also undergoes internalization and degradation [21, 22]. Conversely, macrophages subjected to iron loading or erythrophagocytosis not only induce hepcidin synthesis but also translocate intracellular ferroportin to the membrane [21]. In duodenal enterocytes, ferroportin is located on basolateral membranes and it is also degraded by a systemic signal, most likely hepcidin [23], as indicated by dramatically increased duodenal ferroportin in states of hepcidin deficiency [24, 25]. Although ferroportin appears to be controlled by hepcidin in both macrophages and enterocytes, the specific mechanisms that determine the subcellular location of ferroportin may differ [22, 23].

2.4 Genetics of Ferroportin-Related Disorders

A number of genetically dominant missense (but not nonsense) mutations of ferroportin have been identified. They are manifested either as loss of ferroportin function [26], leading to iron accumulation in macrophages due to decreased cellular iron export, or less commonly, as gain of function [27], leading to hyperabsorption of dietary iron presumably from inappropriately high iron flow through enterocyte ferroportin. In cellular models, loss of function mutations cause defective trafficking of ferroportin to the cell membrane [16, 28], while gain of function mutations cause resistance to hepcidin-induced internalization [16, 28]. A mouse model of heterozygous ferroportin ablation does not manifest disease [17] but a recently identified missense mutation [29] causes iron accumulation in macrophages with iron-restricted erythropoiesis. Based on the genetics of ferroportin-related disease, on biochemical studies, and the dominant-negative effect of clinically significant ferroportin mutations, ferroportin is dimeric or multimeric [30], but this interpretation is not universally accepted [31–33].

2.5 Structure–Function Analysis of Hepcidin

Evolutionary analysis of mammalian hepcidin sequences indicates a strong conservation of the disulfide bridge structure and the N-terminal six amino acids (Fig. 9.2). The N-terminus differs in fish and amphibian hepcidins (Fig. 9.2) but the substitutions are quite conservative. Deletion of amino

acids from the N-terminus causes progressive loss of activity [34] so that the 20 amino acid form of hepcidin, also naturally found in urine and serum [2, 3], is inactive in cellular ferroportin internalization assays [18, 34] and in vivo [11]. In contrast, the hepcidin structure retains bioactivity in cellular assays when amino acids in the rest of the structure, including the disulfide bonds, are altered [34] although the stability of the molecule may be decreased.

2.6 Hepcidin Catabolism

Fluorescently tagged hepcidin is taken up by ferroportin-expressing cells in culture and colocalizes in lysosomes with GFP-ferroportin (G. Preza et al., in preparation). When radiolabeled hepcidin is given to mice, the tracer is predominantly found in urine but is also taken up by ferroportin-rich tissues [11], indicating that both renal excretion and uptake and degradation in ferroportin-rich tissues could contribute to hepcidin clearance from plasma.

2.7 Cellular Regulation of Ferroportin

Hepcidin is not the only signal that regulates ferroportin. Each macrophage that ingests senescent erythrocytes faces a large load of iron that must be stored or exported, depending on systemic requirements for iron. Whereas the systemic regulation of macrophage iron export is mediated by plasma hepcidin, which internalizes and degrades membrane-associated ferroportin [19], ferroportin is also subject to independent cellular regulation by macrophage heme and iron levels [21, 35, 36]. Cellular iron and heme increase ferroportin mRNA and protein, and induce the translocation of ferroportin from intracellular vesicles to the cellular membrane. The increase in ferroportin is mediated by both transcriptional and translational mechanisms, the latter involving the 5 iron regulatory element (IRE) located in the ferroportin mRNA [37, 38]. The amount of ferroportin on the membrane, and therefore the ability of macrophages to export iron, is thus closely linked to the iron and heme content of each macrophage. In combination, the cellular and systemic regulators deliver iron to extracellular fluid and plasma when iron is required for systemic needs, obtaining it from those macrophages that contain abundant iron and heme.

3 Regulation of Hepcidin Synthesis by Iron

3.1 Hereditary Hemochromatosis Proteins Are Hepcidin Regulators

Analysis of hepcidin expression in patients with hereditary hemochromatosis [8, 39–42] and in mouse models [7, 25, 40, 43–47] revealed that the major forms of juvenile and adult hereditary hemochromatosis are due to deficiency of hepcidin that allows excessive iron absorption and reticuloendothelial iron recycling. The rate of development of iron overload is most severe in the juvenile form of hemochromatosis, which manifest the lowest hepcidin expression, usually caused by homozygous disruption of hepcidin (HAMP) or hemojuvelin (HJV) genes. The adult forms are less hepcidin deficient and are usually caused by homozygous disruption of the genes encoding transferrin receptor 2 (TfR2) or the hemochromatosis gene HFE. Two siblings with phenotypically juvenile form of hereditary hemochromatosis were found to have both homozygous Q317X TfR2 mutations

and compound heterozygous C282Y/H63D HFE mutations, suggesting an additive effect of HFE and TfR2 disruption [48]. The simplest explanation of the genotype–phenotype relationships is that HFE, TfR2, and hemojuvelin are regulators of hepcidin, and that HFE and TfR2 are partially redundant and perhaps operating on parallel pathways that converge on hepcidin or hemojuvelin.

3.2 Hemojuvelin

Hemojuvelin (also known as HFE2 and RgmC) was identified as a hepcidin regulator through positional cloning of the gene mutated in most cases of juvenile hemochromatosis [41]. The gene encodes a GPI-linked membrane protein with homology to repulsive guidance molecules (RgmA and RgmB) involved in the development of the central nervous system. Suppression of hemojuvelin by siRNAs proportionally decreased hepcidin mRNA in hepatic cell lines, indicating that hemojuvelin, unlike other Rgm [49], directly regulates hepcidin synthesis and is not principally a developmental mediator. In addition to the membrane-associated GPI-linked form, hemojuvelin also exists as a soluble protein that acts as a suppressor of hepcidin synthesis by hepatocytes in cell culture [49] and in vivo in the mouse [50]. The prohormone convertase furin is responsible for the release of soluble hemojuvelin and may act in the Golgi or on the membrane [51, 52]. The shedding or secretion of hemojuvelin is suppressed by iron [49, 53] through an as yet undefined mechanism and stimulated by hypoxia and iron deficiency in part through increased synthesis of the prohormone convertase furin [51]. Other Rgms act, at least in part, through their interactions with the receptor neogenin, and indeed, such an interaction may modulate the effect of hemojuvelin as well [53, 54], probably through the regulation of hemojuvelin shedding. However, the predominant effect of hemojuvelin on hepcidin synthesis is mediated by its interactions with the BMP pathway [55]. The BMP-dependent effect of hemojuvelin is required for the regulation of hepcidin synthesis by iron-transferrin in primary hepatocytes [56].

3.3 The Bone Morphogenetic Protein (BMP) Pathway in Hepcidin Regulation

With the exception of hepcidin itself, all the genes disrupted in hereditary hemochromatosis encoded proteins whose function was not known. An important insight into how these proteins may fit together came from the phenotype of a liver-specific SMAD4 knockout mouse [57] which manifested systemic iron overload and nearly complete deficiency of hepcidin. SMAD4 is a transcription factor used by the BMP and TGFβ pathways and the phenotype of the SMAD4 knockout implicated both pathways in iron regulation. In unrelated studies [58], RgmB (Dragon) was shown to act as a coreceptor for the BMP receptor and to enhance its signaling, and subsequently, hemojuvelin (RgmC) was also found to act as a coreceptor for the BMP receptor [55] and BMP2, 4 and 9 were shown to be strong inducers of hepcidin synthesis [50, 55, 59]. Based on the comparative potency of their respective ligands, the role of the BMP pathway in hepcidin regulation appears to be much greater that of the TGFβ pathway [50].

The BMP signaling pathway is activated by dimeric ligands that bring together type I and type II receptor serine/threonine kinases on the cell membrane. The constitutively active type II receptor kinase phosphorylates and activates the kinase activity of type I receptor, which in turn phosphorylates the receptor-regulated Smads, Smad 1, 5, and 8. Upon phosphorylation, these Smad proteins form a complex with the common mediator Smad-4. The activated Smad complex translocates into the nucleus and regulates transcription of its target genes. The BMP receptor heterodimers are formed by combining one of three type II receptors (BMPRII, ActRIIA, and ActRIIB) with one of

three type I receptors (ALK3, ALK6, and ALK2). Specific combinations of type I and type II receptors are preferentially utilized by different BMP ligands, and additional coreceptors can modify this preference and the intensity of signaling [60]. Multiple BMPs and other potential ligands are expressed in the liver [50] but only a few appear to be involved in the regulation of hepcidin by iron. Although BMP2 and BMP4 interact with hemojuvelin and appear to function in the pathway by which iron regulates hepcidin [56], BMP9 does not interact with hemojuvelin and uses a different BMP receptor heterodimer not involved in iron-related signaling [50, 56]. The dramatic effect of the BMP pathway on hepcidin transcription and the strong phenotype of the hemojuvelin-deficient mice and humans puts them at the center of current models of hepcidin and iron regulation. However, neither hemojuvelin nor the BMPs and their receptors are iron-binding molecules and so they must interact with other molecules that bind iron and can "sense" iron concentrations.

3.4 Transferrin and Transferrin Receptors 1 and 2

3.4.1 TfR1

Transferrin receptors 1 and 2 bind holotransferrin (diferric transferrin) as well as monoferric transferrin and both can mediate the cellular uptake of iron (see Chap. 7). The affinity constants for TfR1 and TfR2 binding of holotransferrin are 1.1 and 29 nM, respectively [61], indicating that both receptors would be saturated at physiologic extracellular holotransferrin concentrations which are thousand times higher than the affinity constants. TfR1 is expressed abundantly on erythropoietic precursors and present in most other cell types while TfR2 is hepatocyte-specific. Homozygous ablation of TfR1 is embryonic lethal, producing severe anemia and malformation of the central nervous system [62]. TfR1+/− heterozygotes have iron-deficient erythropoiesis despite iron reserves in macrophages, indicating a defect in iron uptake by erythrocyte precursors [62]. Despite the lack of direct involvement of TfR1 in intestinal iron absorption, iron stores in TfR1 +/− mice are diminished, indicating that the receptor could play a role in iron regulation.

3.4.2 TfR2

The effects of TfR1 deficiency on iron regulation contrast with the effects of TfR2 or transferrin deficiency. Deficiency of TfR2 in mice or humans causes systemic iron overload [63–65] and liver-specific deficiency of TfR2 is sufficient for iron overload, clearly indicating TfR2 involvement in iron regulation [66]. Hepcidin is very low in TfR2-deficient humans [42] and mice [46, 65], indicating that TfR2 defects cause iron overload through the lack of hepcidin.

3.4.3 Holotransferrin

Genetic deficiency of transferrin is associated with a very severe form of iron overload in humans (summarized in [67]) and in mice [68], indicating that transferrin is required for systemic homeostatic regulation of iron. After a test dose of iron, transient increases in transferrin saturation elicit proportional changes in urinary hepcidin concentrations [56]. Holotransferrin, but not elemental iron, induces hepcidin mRNA in freshly isolated hepatocytes [56]. These observations suggest that holotransferrin is an important (although perhaps not the only) form of iron sensed by the systemic homeostatic mechanisms.

3.4.4 Iron Sensing

In the aggregate, genetic studies of transferrin and its receptors suggest that all three are involved in iron regulation, acting by regulating hepcidin synthesis. Based on their reported affinity constants, both transferrin receptors would be saturated with iron-transferrin at physiologic concentrations. Current models of the involvement of TfR1 and TfR2 in iron sensing propose complexes of TfRs with other molecules that effectively lower the affinity of TfRs for holotransferrin so that such complexes could sense changes in holotransferrin concentration in its physiologic range.

3.5 HFE

3.5.1 HFE Regulates Hepcidin

Mutations in the gene HFE are responsible for most cases of hereditary hemochromatosis in patients of European descent. Patients with HFE hemochromatosis carry homozygous or compound heterozygous mutations, and manifest hepcidin mRNA and protein levels that are either lower than normal or normal but inadequate for the high iron load and transferrin saturation [40, 69]. Moreover, patients with HFE hemochromatosis lack the acute hepcidin response to iron ingestion but the chronic response to iron loading is at least partially preserved [69]. Both humans and mice with HFE hemochromatosis are deficient in hepcidin mRNA [40, 43, 45], suggesting decreased hepcidin gene transcription (or less likely, mRNA instability). Transplantation of normal livers into HFE recipients is not followed by reaccumulation of iron, suggesting that HFE expression in the liver is sufficient for iron homeostasis [70, 71] but enterocyte-specific ablation of HFE in mice does not cause an iron disorder [72]. These studies support the idea that HFE acts primarily in the liver by regulating hepcidin. Transplantation of HFE mice with wt bone marrow [73] ameliorated the iron overload and increased hepatic hepcidin mRNA compared to HFE mice transplanted with HFE bone marrow, indicating that HFE in myeloid cells could also contribute to hepcidin regulation.

3.5.2 HFE Interacts with Transferrin Receptors

The HFE gene encodes a membrane protein that forms a heterodimer with β2-microglobulin, and is similar to proteins of the type I major histocompatibility complex. HFE lowers the affinity of TfR1 for holotransferrin [74]. The HFE ectodomain competes with holotransferrin for binding to the TfR1 ectodomain, and the binding sites of HFE and holotransferrin on TfR1 overlap [75, 76]. Moreover, complete TfR1 and holotransferrin compete for HFE in cellular models [77]. Although the TfR2 ectodomain was reported not to bind to the HFE ectodomain, in cellular models with overexpressed whole proteins, TfR2 competed with TfR1 for binding to HFE [78] and TfR2 was stabilized by its interaction with HFE [79]. Unlike the interaction of TfR1 with HFE, the interaction of TfR2 with HFE was not diminished by holotransferrin. In fact, holotransferrin stabilized TfR2 [80, 81], enabling it to compete more effectively for HFE. Based on the phenotypes of mice with a deficiency of transferrin or TfR1 or TfR2, both transferrin receptors could act as sensors for holotransferrin, acting synergistically, and using HFE as a signaling intermediary. Holotransferrin would release HFE from its association with TfR1 and make more HFE available to bind to the holotransferrin-stabilized TfR2. The TfR2–HFE complex would then stimulate hepcidin synthesis.

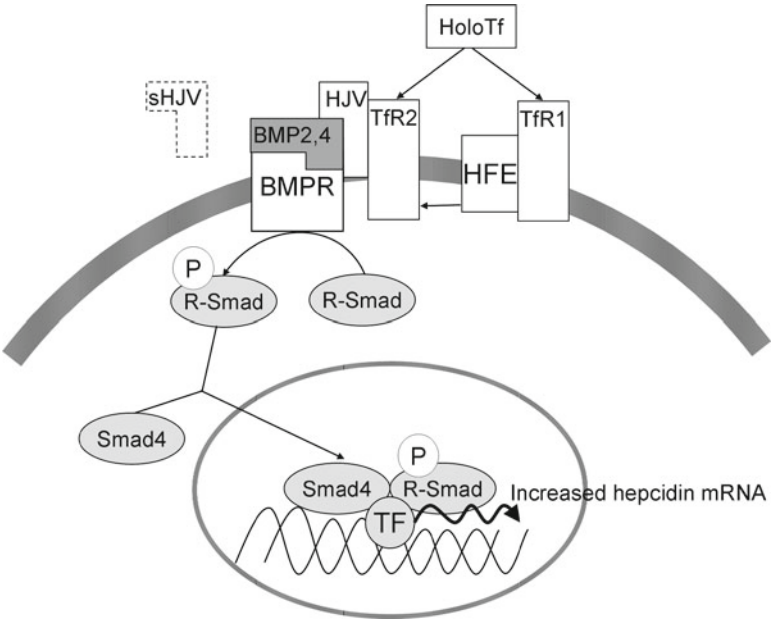

Fig. 9.4 A model of hepcidin regulation by holotransferrin (*holoTf*). Signaling by the receptor complex consisting of BMPR, its ligand BMP2/4, and the coreceptor HJV is stimulated by the binding of holoTf to both TfRs. The binding of holoTf to TfR1 releases HFE and the binding of holoTf to TfR2 stabilizes TfR2, so both HFE and TfR2 can associate with the receptor complex which then phosphorylates R-Smad. R-Smad binds Smad4, they translocate to the nucleus and help form a transcription complex to increase the synthesis of hepcidin mRNA. Soluble HJV (*sHJV*) represses hepcidin expression by selectively inhibiting BMP signaling

3.6 A Model of Hepcidin Regulation

A comprehensive model of hepcidin regulation must account for the effects of known genetic lesions on iron metabolism, and must provide a plausible scheme by which iron is sensed and its concentrations affect the synthesis of the iron-regulatory hormone. A current model of hepcidin regulation (Fig. 9.4) is built around the regulatory complex of hemojuvelin, BMP2/4, and the BMP receptor regulating the transcription of hepcidin via the SMAD pathway. The activity or assembly of this complex is regulated by its association with TfR2 and HFE, with their effects synergistic. The availability of TfR2 and HFE is in turn determined by the concentration of holotransferrin which stabilizes TfR2 and releases HFE from TfR1.

7 Regulation of Systemic Iron Metabolism and Hepcidin Synthesis by Hypoxia-Inducible Transcription Factors

7.1 Gene Regulation by Hypoxia

Hypoxia is a potent regulator of cellular and systemic processes, and has a particularly strong effect on erythropoiesis where it acts as a dominant inducer of the production of erythropoietin. Hypoxia regulates the transcription of erythropoietin and dozens of other hypoxia-regulated genes through heterodimeric hypoxia-inducible transcription factors (HIF) that bind to hypoxia-responsive elements (HRE) in the promoters of target genes [82]. HIF consist of one of three cytoplasmic

HIF1α, HIF2α, or HIF3α that can combine with the constitutive HIF1β subunit. During hypoxia, HIFα subunits accumulate, translocate into the nucleus, and interact with HIF1β and other transcription factors. When oxygen is abundant, HIFα subunits are subjected to hydroxylation on one or two of their prolines, and this modification targets the HIFα for interaction with the von Hippel–Lindau tumor-suppressor protein (VHL) and for degradation. Another oxygen-sensing hydroxylase, FIH-1 (factor inhibiting HIF-1) hydroxylates a specific Asn on HIFα, thereby inhibiting the interaction of HIF with other transcription factors. The hydroxylases contain an essential iron, and their activity is inhibited by iron chelators.

7.2 Iron and Hepcidin Regulation by Hypoxia

Mice and rats subjected to hypoxia respond by increased production of erythropoietin, enhanced erythropoiesis, and increased iron absorption. The increase in iron absorption is, at least in part, independent of the effect of increased erythropoiesis, as revealed by early experiments in which the erythropoietic response is suppressed by nephrectomy or by a combination of irradiation of the bone marrow by radioactive strontium and splenectomy [83, 84]. More recent studies suggest that the increased iron absorption is due to suppression of hepcidin by hypoxia. Mice made hypoxic by exposure to oxygen pressures found at 5,500 m, manifested gradually decreasing hepcidin mRNA but the effect appears to be relatively slow, peaking at 4 days [85], suggesting that the effect on hepcidin included indirect, erythropoiesis-mediated effects of hypoxia. Direct effects of hypoxia on hepatocytes were deduced from hypoxia-exposed hepatocyte cell lines that showed a decrease in hepcidin mRNA within 24–48 h [85, 86]. In the aggregate, these studies suggest that hepcidin is subject to direct regulation by hepatic hypoxia but the relative contribution of direct and indirect effects remains to be demonstrated.

7.3 HIF Involvement in Hepcidin Suppression During Iron Deficiency

Systemic iron deficiency was shown to suppress hepcidin in humans [87, 88]. In mice, the suppression of hepcidin by iron deprivation appears to be partially dependent on HIF1α because mice with ablation of hepatic HIF1α suppress hepcidin mRNA less than do wt mice [89]. Nevertheless, hepcidin suppression still takes place even in HIF1α-deficient mice, indicating that other pathways may also regulate hepcidin during iron deficiency. Although the specific HREs in the hepcidin promoter differ in number, and location between mice and humans, both promoters were found to bind HIF [89]. The HIF-dependent mechanism of hepcidin regulation would be expected to be responsive to hepatocyte iron stores rather than holotransferrin concentration, and thus could complement the effect of the BMP/hemojuvelin/TfR pathway that senses holotransferrin.

8 Regulation of Plasma Iron and of Hepcidin Synthesis by Inflammation

8.1 Hepcidin and the Acute Hypoferremia of Inflammation

Hypoferremia develops within hours of acute infections and persists in states of chronic infection or inflammation (Chaps. 11 and 15). Recent studies indicate that hepcidin is the key mediator of this hypoferremic response. In humans, hepcidin is induced by inflammation within hours and increased

hepcidin is followed by hypoferremia a few hours later [90]. The hypoferremic response to turpentine-induced inflammation is lost in hepcidin-deficient mice, indicating that the increased hepcidin concentrations mediate the hypoferremia of inflammation [86]. Injection of hepcidin produces hypoferremia within 1 h [11], in agreement with the time course of the development of hypoferremia in acute infections in humans and in mice. Thus, the role of circulating hepcidin in acute hypoferremia of inflammation is well supported by evidence. Additional mechanisms may contribute to local or systemic hypoferremia. Autocrine secretion of hepcidin by macrophages [91] may act locally to reduce macrophage ferroportin, cause macrophage iron retention, and decrease the local extracellular iron concentrations. In addition, transcriptional suppression of ferroportin by inflammatory stimuli may also contribute to iron retention in macrophages [92, 93].

8.2 Hepcidin and Anemia of Inflammation

Mice and humans chronically overexpressing hepcidin develop not only hypoferremia but also an iron-restricted anemia [9, 10, 94, 95]. Urinary hepcidin is increased in patients with anemia of inflammation [88], suggesting that the overproduction of hepcidin accounts for the defining features of anemia of inflammation: iron-restricted anemia with hypoferremia.

8.3 Regulation of Hepcidin During Infection and Inflammation

The induction of hepcidin by infection or microbial products has been demonstrated in humans [87, 90], in mice [4, 85], and in fish [96]. Infection or microbial products induce hepcidin mRNA directly in primary hepatocytes [4] and in monocytes and macrophages [91, 93, 97–101]. Moreover, media conditioned by lipopolysaccharide-treated blood monocytes induce hepcidin synthesis in isolated hepatocytes and hepatocyte-derived cell lines [87] and this effect is neutralized by anti-IL-6 antibody, indicating that IL-6 is an important macrophage-derived mediator of hepcidin regulation. Unlike wild-type C57Bl6 mice, IL-6-deficient mice injected with turpentine do not acutely increase their hepcidin mRNA, and do not develop hypoferremia, supporting the role of IL-6 in acute hypoferremia of inflammation [101]. Moreover, human volunteers infused with moderate amounts of IL-6 develop increased hepcidin and hypoferremia within hours of infusion [101]. Patients with multicentric Castleman's disease that causes overproduction of IL-6 develop a microcytic anemia with increased serum hepcidin levels but treatment with tocilizumab (anti-IL-6 receptor antibody) decreases hepcidin and reverses the anemia [102]. The transcriptional induction of hepcidin by IL-6 is dependent on the STAT3 pathway [103–105], but may also require the Smad pathway since in liver-specific Smad4 KO mice, IL-6-mediated hepcidin induction is blunted [57]. Other inflammatory mediators and the direct effect of bacterial substances on hepatocytes and macrophages can also induce hepcidin [4, 90, 93, 106, 107] and IL-6-independent pathways may be especially important in the chronic setting. Moreover, the production of increased amounts of hepcidin by macrophages and adipocytes could contribute to hypoferremia in obesity and perhaps other inflammatory disorders [108].

8.4 Regulation of Hemojuvelin by Inflammation

Inflammation not only exerts a stimulatory effect on hepcidin synthesis but also affects the iron-regulatory pathway by suppressing hemojuvelin mRNA [47, 109–111]. Although this remains to be demonstrated experimentally, the suppression of hemojuvelin would be expected to uncouple the

iron-regulatory pathway so that the effect of the inflammatory stimuli on hepcidin synthesis would not be outweighed by the opposing effects of iron restriction.

9 Regulation of Hepcidin Synthesis by Erythropoietic Activity

9.1 Erythropoiesis Suppresses Hepcidin Synthesis

It has been known for at least 50 years that increased erythropoietic activity leads to increased intestinal iron absorption [83, 112]. The predicted factor that controls intestinal iron absorption is hepcidin, and indeed hemolytic anemia or hemorrhage both suppress hepcidin synthesis [85, 113, 114]. However, as anticipated by earlier iron absorption studies, anemia does not suppress hepcidin synthesis in the absence of active erythropoiesis [113, 114]. The nature of the factor that signals from the bone marrow to the liver to modulate hepcidin is not yet known.

9.2 Hepcidin Suppression in Expanded but Ineffective Erythropoiesis

β-Thalassemia major and intermedia represent an extreme example of increased erythropoiesis accompanied by premature death of erythroid precursors. Hepcidin is very low in untransfused patients with β-thalassemia intermedia [115, 116] and in the mouse models of β-thalassemia [117–119]. Hepcidin is higher in transfused patients with β-thalassemia but still inappropriately low for the degree of iron overload [115, 116, 120, 121]. A hepcidin-regulating humoral factor present in β-thalassemia was implicated by the observation that sera from patients with β-thalassemia suppressed hepcidin production in hepatocyte cell lines [122]. GDF15, a member of the TGFβ/BMP family of ligands, is produced by erythrocyte precursors and is present at very high concentrations in the plasma of patients with β-thalassemia [123]. At these high concentrations, GDF15 suppressed hepcidin production by primary human hepatocytes and by hepatocyte cell lines and emerged as a strong candidate for bone marrow-derived humoral factor in β-thalassemia. It is not yet clear whether GDF15 contributes to hepcidin regulation in situations where erythroid precursor expansion and apoptosis are less extreme.

10 Summary

Systemic iron homeostasis is mediated predominantly by the interaction of the iron-regulatory hormone hepcidin with the cellular iron efflux channel, ferroportin. In the efferent arm of iron homeostasis, hepcidin binds to ferroportin, causing its internalization and degradation, and thereby inhibiting cellular iron export into extracellular fluid. In the afferent arm of the homeostatic arc, extracellular iron, hepatic iron stores, and inflammation stimulate hepcidin synthesis, and hypoxia and erythropoietic activity suppress it. Direct effects of many of these stimuli on ferroportin synthesis have also been observed and may contribute to iron regulation.

Notes

Since the writing of this article, several important developments updated our understanding of iron homeostasis:

1. The bone morphogenetic protein BMP6 has been shown to be an iron-related ligand of the BMP receptor, necessary for normal hepcidin expression and iron regulation, at least in the mouse [1, 2].
2. The membrane serine protease matriptase 2 (MT-2, also abbreviated TMPRSS6) was found to be a negative regulator of hepcidin [3]. MT-2 acts by cleaving membrane hemojuvelin at an external site [4]. Homozygous or compound heterozygous mutations in MT-2 are a major cause of the rare autosomal recessive disorder iron-refractory iron-deficiency anemia [5] wherein increased circulating hepcidin inhibits iron absorption and recycling without any evidence of inflammation.
3. Accumulating evidence [6, 7] is pointing to two distinct pathways in hepcidin regulation by iron. The first is responsive to extracellular iron, likely sensed as holotransferrin concentration [8]. The extracellular iron pathway modulates signaling by the BMP receptor complex, and requires HFE, TfR2, hemojuvelin and BMP6. The second pathway, historically called the "stores" regulator, responds to intracellular iron and regulates BMP6 concentrations [9] even in the absence of HFE, TfR2 or hemojuvelin. The intracellular regulator exerts its effect on hepcidin even in the absence of BMP6 indicating that other ligands may also be involved.
4. The proposed mechanism of ferroportin internalization by its ligand hepcidin, and the specific roles of hepcidin-induced ferroportin phosphorylation and ubiquitination, have been contested [10] and remain unresolved.
5. Several types of human serum hepcidin assays have been developed and crosscorrelated [11–17] for use in disease-related research and drug development. Assay standardization is underway [14]. Measurements of human serum hepcidin in various disease conditions generally confirmed the regulatory schema presented in this review.

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