Hepcidin: A Promising Therapeutic Target for Iron Disorders

A Systematic Review

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Abstract: Iron is required for most forms of organisms, and it is the most essential element for the functions of many iron-containing proteins involved in oxygen transport, cellular respiration, DNA replication, and so on. Disorders of iron metabolism are associated with diverse diseases, including anemias (e.g., iron-deficiency anemia and anemia of chronic diseases) and iron overload diseases, such as hereditary hemochromatosis and β-thalassemia. Hepcidin (encoded by HAMP gene) is a peptide hormone synthesized by hepatocytes, and it plays an important role in regulating the systematic iron homeostasis. As the systemic iron regulator, hepcidin, not only controls dietary iron absorption but also regulates iron storage in various organs. Deregulated hepcidin is often seen in a variety of iron-related diseases including anemias and iron overload disorders. In the case of iron overload disorders (e.g., hereditary hemochromatosis and β-thalassemia), hepatic hepcidin concentration is significantly reduced.

Since hepcidin deregulation is responsible for iron disorder-associated diseases, the purpose of this review is to summarize the recent findings on therapeutics targeting hepcidin. Continuous efforts have been made to search for hepcidin mimics and chemical compounds that could be used to increase hepcidin level. Here, a literature search was conducted in PubMed, and research papers relevant to hepcidin regulation or hepcidin-centered therapeutic work were reviewed. On the basis of literature search, we recapitulated recent findings on therapeutic studies targeting hepcidin, including agonists and antagonists to modulate hepcidin expression or its downstream signaling. We also discussed the molecular mechanisms by which hepcidin level and iron metabolism are modulated. Elevating hepcidin concentration is an optimal strategy toameliorate iron overload diseases, and also to relieve β-thalassemia phenotypes by improving ineffective erythropoiesis. Relative to the current conventional therapies, such as phlebotomy and blood transfusion, therapeutics targeting hepcidin would open a new avenue for treatment of iron-related diseases.

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INTRODUCTION

Iron, as a necessary element, plays an important role in several physiological processes including oxygen carrier, electron transfer in mitochondrial, DNA replication, DNA repair, cell signaling, and free radical production. Iron balance is necessary for normal physiology; however, iron disorder is associated with many types of diseases including hereditary hemochromatosis (HH), β-thalassemia, anemia of inflammation, and iron-refractory iron deficiency anemia (IRIDA). In the real world, more than 1 billion people are suffering from iron deficiency. Thalassemia major, a representative iron overload disease, is still very popular in the world. There are estimated 56,000 thalassemia major cases annually, and 30,000 of them require regular transfusion to survive. These huge numbers of patients present an urgent need to improve their survival and life quality. Nowadays, iron chelation, phlebotomy, splenectomy, bone marrow transplantation, and iron administration are widely accepted therapies; however, serious toxic and side
effects (such as secondary iron overload and anemia) are associated with these therapies, which are not satisfactory to all patients.4,5

Previous pathology studies revealed that iron disorder is due to the dysregulation of hepcidin–ferroportin (FPN) axis. Thus, correcting hepcidin–FPN axis would be potential therapeutic strategy for iron disorders. Hepcidin (encoded by \textit{Hamp} gene) is a 25-amino acid peptide hormone and synthesized in hepatocytes (Figure 1).6 It binds to FPN to promote the latter’s degradation, and thus controls iron release from spleen and hepatocytes, and also dietary iron uptake from enterocytes.7,8 Since hepcidin deregulation is closely associated with iron overload or deficiency, fine-tuning \textit{Hamp} expression would be an efficient strategy to ameliorate iron disorder diseases. In this review, we summarized the iron disorders due to deregulated hepcidin and the development of hepcidin agonists and antagonists for hepcidin regulation.

\section*{METHOD}

In this systemic review, we performed literature search in PubMed (http://www.ncbi.nlm.nih.gov/pubmed/). The key words used in searching are as follows: hepcidin, iron overload, hereditary hemochromatosis, anemia of inflammation, and hepcidin regulation. The criterion for exclusions is that the studies are irrelevant to hepcidin regulation or hepcidin-centered therapeutic work. Since no animals or humans were used in the current review paper, ethics statement does not apply here.

\section*{MOLECULAR BASIS OF SYSTEMIC IRON HOMEOSTASIS}

Hepcidin is a hormone secreted by hepatocytes which plays a crucial role in regulating iron homeostasis.6 \textit{Hamp} deficiency mice (\textit{Hamp} \textsuperscript{-/-} mice) exhibit severe iron overload; in contrast, \textit{Hamp} overexpression reversely results in iron deficiency.9 Studies demonstrated that the treatment with hepcidin or its analogs caused dose-dependent hypoferremia.10 In fact, hepcidin regulates iron by binding to its receptor, FPN, and inducing FPN internalization and degradation.11,12 FPN is a multimembrane protein, and it is highly expressed in duodenal enterocytes, liver Kupffer cells, portal hepatocytes, splenic macrophages, and placental syncytiotrophoblasts.8 FPN is the only known iron exporter in mammals, and its deficiency causes loss of iron absorption from duodenum and reduction of iron egress from liver and spleen macrophages, as evidenced in FPN null mice (\textit{Fpn1} \textsuperscript{-/-} mice).8,13,14 Thus far, the hepcidin–FPN axis has been considered as the central player in regulating iron homeostasis. We here reviewed the main upstream-signaling pathways responsible for hepcidin regulation, including iron concentration, inflammation, erythropoiesis, and so on (Figure 2).

\section*{Hepcidin is Regulated by Iron Concentration}

Iron concentration can regulate \textit{Hamp} expression in the liver. Under an iron deficiency condition, holo-transferrin will first bind to transferrin receptor 1 (TIR1), and then interact with
FIGURE 2. The mechanisms responsible for Hamp transcription. Hamp transcription is regulated by diverse signaling pathways, including iron concentration, inflammation, erythropoiesis, and sex hormone. (1), Iron levels contribute to Hamp transcription through the Smad signaling. In an iron-replete condition, iron binds to TIR2, and then forms a complex with HFE, HJV, and BMPs to promote Smad1/5/8 activation. In contrast, iron binds to TIR1 and fails to stimulate Hamp transcription in iron deficiency. (2), BMPs are involved in hepcidin regulation. BMPs bind to BMPRs, and thus form BMPs, BMPRs, and HJV complex to activate Smad phosphorylation and promote Hamp transcription. (3), The preinflammatory factors (including IL-6, IL-22, and IL-1β) and activin B incur Hamp up-regulation. IL-6 and IL-22 can bind to its receptor to increase hepcidin expression via Stat3, but IL-1β and activin B (a member of the TGF-β protein family) increase Hamp transcription through the BMP-Smad signaling. (4), Cytokine factors produced by erythroblasts are also implicated in hepcidin regulation. GDF15, TWSG1, and ERE are recognized to be the hepcidin regulators. GDF15 could modulate hepcidin expression through BMP inhibition, whereas the detailed mechanisms underlying the action of TWSG1 and ERE in Hamp repression remain unclear. (5) Endogenous hormones (such as estrogen) negatively regulate Hamp transcription by binding to the ERE of Hamp promoter. BMP = bone morphogenetic protein, BMPR = bone morphogenetic protein receptor, ERE = erythroferrone, GDF = growth differentiation factor, HFE = hemochromatosis protein, HJV = hemojuvelin, IL = interleukin.

Hemochromatosis protein (HFE). However, the molecular events downstream of this complex are not thoroughly understood. Holo-transferrin will shuttle to transferrin receptor 2 (TIR2) under iron repletion condition, and the holo-transferrin, TIR2, and HFE complex interacts with hemojuvelin (HJV). HJV is a glycosylphosphatidylinositol-linked membrane protein; it promotes Hamp transcription through Smad1/5/8 phosphorylation. As demonstrated in previous studies, TIR2, HFe, or HJv deficiency mice (TIR2−/− mice, HFe−/− mice, or HJv−/− mice) displayed iron overload.\textsuperscript{15,16} Wu et al’s study further demonstrated that HJv−/− mice exhibited more severe iron overload, compared with HFe−/− mice, but similar to HFe−/− HJv−/− mice.\textsuperscript{17,18} Wu et al’s study also uncovered that mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase does not play a prominent role in this process.\textsuperscript{19} Overall, these results indicate that HFE is involved in hepcidin regulation in an HJV-dependent manner.

Hepcidin is Regulated by Bone Morphogenetic Protein Signaling

There are nearly 20 bone morphogenetic proteins (BMPs) expressed in mammals, and among these, BMP2, BMP4, BMP5, BMP6, BMP7, and BMP9 can induce Hamp expression.\textsuperscript{19,20} Studies demonstrated that BMP6 plays a key role in Hamp induction.\textsuperscript{19,21} It binds to BMP receptor (BMPR) to activate Smad1/5/8 phosphorylation, and the latter together with Smad4 translocates to nucleus and binds to Hamp promoter to induce Hamp transcription.\textsuperscript{22} This signaling pathway requires HJV, a BMPs coreceptor. Since HJv−/− mice have severe low hepcidin concentration, iron overload and a low level of p-Smad1/5/8 in mice.\textsuperscript{26} HJV can bind onto the membrane proximal region of neogenin, and this interaction is necessary for BMP4-promoted Hamp transcription.\textsuperscript{27,28} As the coreceptor of HJV, it is also involved in HJV regulation; however, this process is more predominantly regulated by Tmprss6.\textsuperscript{29} Tmprss6 is a type of plasma membrane serine protease and it has been recently recognized to have a novel role in regulating Hamp expression and iron homeostasis in both human and mice.\textsuperscript{30–32} Tmprss6-mediated hepcidin down-
regulation is closely dependent on interaction with HJV that conducts HJV cleavage, and this process needs neogenin to form a trimer complex with HJV and TMPRSS6. HJV is also necessary to keep the stability of TMPRSS6, as being demonstrated on liver Timprss6 mutant mice. TMPRSS6 is regulated by hypoxia, BMP6, iron, and inflammation. Meynard et al demonstrated that BMP6 and chronic iron treatment induced the induction of not only Hamp but also Timprss6. Further research by Zhao et al revealed that iron participated in the regulation of TMPRSS6 through protein degradation rather than mRNA regulation.

Meanwhile, activin B, as a member of the TGF-β protein superfamily, induces hepatic hepcidin synthesis through the BMP-Smad signaling. Additionally, under inflammation conditions, interleukin (IL)-1β was also involved in hepcidin regulation via Smad signaling. Recently, homocysteine was identified to play an important role in regulating Hamp expression. Homocysteine could up-regulate Hamp via BMP-Smad signaling pathway, and the HAMP regulation was compromised upon siRNA-mediated gene knockdown treatment against BMP6 and its receptors in Hep G2 cells.

Stat3 Signaling Pathway
Stat3 signaling pathway is another hepcidin regulator, mainly through inflammation. Early study found that treatment of hepatocytes with lipopolysaccharide (LPS) resulted in high hepcidin levels. It is contributed to IL-6 and IL-22 productions, but not IL-1β and tumor necrosis factor-α (TNF-α). Further research confirmed that Hamp promoter contains Stat3-binding site, and siRNA-mediated Stat3 knockdown significantly decreased hepcidin transcription. Stat3 signaling pathway is necessary not only under inflammation conditions, but also baseline hepcidin expression. IL-6-mediated hepcidin induction is dependent on IL-6-Stat3 signaling pathway. IL-6 binds to IL-6 receptor (IL-6R), thereafter to glycoprotein 130 (gp130), to activate Stat3 phosphorylation, which promotes Stat3 translocation to nucleus and provokes Hamp transcription.

Erythropoiesis-related Regulators Involved in Hepsin Regulation
Recent studies demonstrated that the erythroid-derived proteins including growth differentiation factor 15 (GDF15), twisted gastrulation BMP signaling modulator (TWSG1), and erythroferrone (ERFE) (produced by erythroblasts) are involved in Hamp suppression. GDF15 is a member of transforming growth factor-beta superfamily, and it is secreted by late-stage erythroblasts, revealing a role in Hamp suppression in β-thalassemia patients. GDF15 is correlated with soluble transferrin receptor, erythropoietin, and ferritin. Sera from these patients can also inhibit hepatic Hamp expression ex vivo, and another research group confirmed this result in humans as well. However, Fertrin et al demonstrated that the high concentration of GDF15 is not necessary for Hamp repression. TWSG1 is a bone marrow-binding protein, secreted by early erythroblasts, and it was considered to be the potential erythroid regulator of hepcidin. In thalassemic mice, its expression was up-regulated significantly in bone marrow, spleen, and liver. Following studies revealed that TWSG1-mediated Hamp reduction is through the Smad1/5/8 signaling. ERFE is another erythroid regulator produced by erythroid precursors that participate in hepcidin regulation. It belongs to the necrosis factor related family. ERFE was highly expressed to suppress Hamp in β-thalassemia intermedia mice in Epo-ERFE-Stat5 dependent manner. Further studies demonstrated that Erfe ablation in thalassemia mice fully restored hepcidin level, suggesting that ERFE contributes to iron overload in β-thalassemic mice. Nonetheless, the signaling downstream of Stat5 underlying Hamp regulation is still unclear.

Other Signaling Pathways That Regulate Hamp Expression
As a sex hormone, estrogen was found to down-regulate Hamp expression through an estrogen receptor element (ERE) in Hamp promoter. As the negative regulator of Hamp, 17β-estradiol (E2) treatment decreased Hamp expression in HuH7 and Hep G2 cell lines, which could be blocked by ICI 182780, an antagonist of estrogen receptor. This negative regulation of E2 was confirmed in both wild-type and Hfe−/− mice. In support of this finding, our study revealed that the ERE-binding site of estrogen on Hamp promoter is responsible for the repression of Hamp transcription upon E2 treatment. Moreover, estrogen was also delineated to regulate hepcidin in GPR30-BMP6-dependent manner. A recent study suggested that progesterone receptor membrane component-1 (PGRMC1) is a membrane-bound progesterone receptor, also contributed to hepcidin regulation through Src-family tyrosine kinases (SFKs). However, the signaling downstream of SFKs responsible for Hamp expression modulation is still elusive, and warrants further detailed investigation.

IRON DISORDERS DUE TO Deregulation of Hepcidin
Several diseases exhibited abnormal HAMP and iron metabolism, such as smoking-caused low hepcidin level in pregnant women, non-alcoholic fatty liver disease, sideroblastic anemia and chronic renal failure. Meanwhile, β-thalassemia and HH are the most typical low HAMP diseases, and anemia of inflammation and IRIDA are the most representative high HAMP diseases.
mutations, and type 4 is due to genetic mutations in Hfe or other genes (including Hamp, Hja Tfr2, Fpn1), characterized by normal erythropoiesis and iron accumulation in liver, heart, and endocrine glands. There are mainly 4 types of HH. Type 1 harbors genetic mutations in Hfe. Type 2A carries Hja mutations, whereas type 2B has Hamp mutations. Type 3 is caused by Tfr2 mutations, and type 4 is due to Fpn1 mutation, known as FPN disease. HFE, TIR2, and HJV intertwinedly control Hamp transcription, and mutations of these genes are associated with low hepcidin and iron overload in organs. Excessive iron results in organ damage and dysfunction. In clinical practice, phlebotomy is the main therapeutic strategy; however, it may cause secondary low hepcidin and excess iron absorption and storage. In addition, this method is not suitable for patients with poor vascular access.

Hereditary Hemochromatosis

HH carries genetic mutations in Hfe or other genes (including Hamp, Hja Tfr2, Fpn1), characterized by normal erythropoiesis and iron accumulation in liver, heart, and endocrine glands. There are mainly 4 types of HH. Type 1 harbors genetic mutations in Hfe. Type 2A carries Hja mutations, whereas type 2B has Hamp mutations. Type 3 is caused by Tfr2 mutations, and type 4 is due to Fpn1 mutation, known as FPN disease. HFE, TIR2, and HJV intertwinedly control Hamp transcription, and mutations of these genes are associated with low hepcidin and iron overload in organs. Excessive iron results in organ damage and dysfunction. In clinical practice, phlebotomy is the main therapeutic strategy; however, it may cause secondary low hepcidin and excess iron absorption and storage. In addition, this method is not suitable for patients with poor vascular access.

Anemia of Inflammation

Anemia of inflammation is a common disease that exhibits normocytic and normochromic anemia, sometimes with microcytic and hypochromic anemia. Anemia of inflammation is caused by inflammatory diseases, such as infections, rheumatology disorders, inflammatory bowel diseases, chronic kidney disease, and malignance. Anemia of inflammation manifests a canonical phenotype of hypoferremia, which is due to high hepcidin levels caused by chronic inflammation. Inflammation promotes hepcidin induction through the IL-6–Stat3 signaling and activin B-Smad1/5/8 signaling. In addition to IL-6, IL-1β and IL-22 could also stimulate Hamp expression in cultured cells and mice. However, the mechanisms underlying Hamp induction by IL-6 and IL-1β are different. IL-6 binds to IL-6R and thereafter to gp130, which activates phosphorylation of Stat3 that docks onto Hamp promoter to increase hepcidin expression. In contrast, the induction of hepcidin by IL-1β is due to the increase of BMP2 expression and activin B from hepatocytes. IL-22 is another positive regulator of hepcidin via the Stat3 signaling pathway, and a significant increase of hepatic hepcidin was observed after the administration of IL-22 receptor agonist. Based on these previous data, the contribution of ILs to hepcidin modulation under anemia of inflammation still warrants further investigation. Considering the characteristic of anemia of inflammation, blood transfusion, erythropoietin, and iron supplementation are the main strategies to ameliorate the diseases in clinical practice; however, these interventions may be accompanied by the risk of secondary iron overload.

Iron-refractory Iron Deficiency Anemia

Iron-refractory iron deficiency anemia is an autosomal recessive disorder caused by mutations of Tmprss6, and a few mutation sites have been identified recently. TMPRSS6 is a negative regulator of hepcidin by cleaving HJV. Tmprss6 deficiency is incapable to promote HV shedding, leading to excessive Hamp transcription. The canonical phenotypes of IRIDA are hypochromic, microcytic anemia, low transferrin saturation, and serum ferritin; however, serum iron is overmuch relative to the degree of anemia. This disease is refractory to oral iron administration.

To better depict the role of hepcidin in directing systemic iron homeostasis, we summarized the iron-related diseases due to hepcidin deregulation in Table 1.

| Diseases Related to Disordered Hepcidin Levels |
|-----------------------------------------------|
| Diseases                                      | Phenotypes                | Hepcidin Changes | Current Therapeutics | References |
| Hereditary hemochromatosis                    | Iron overload             | Low hepcidin     | Splenectomy, Phlebotomy, Iron chelation | 81         |
| β-thalassemia                                | Iron overload, Anemia     | Low hepcidin     | Iron chelation, Blood transfusion | 82         |
| Anemia of inflammation                       | Anemia                    | High hepcidin    | Iron supplement, Erythropoiesis-stimulating agents, Erythrocyte transusions | 72         |
| Refractory iron-deficiency anemia             | Iron deficiency, Anemia   | High hepcidin    | Intravenous iron      | 83         |

These deregulated signaling pathways together lead to ineffective erythropoiesis and iron overload in a vicious cycle. β-thalassemia patients with stroke showed higher HAMP levels and iron concentrations. These results indicate that β-thalassemia patients with higher HAMP levels and other complications should be paid attention to. Blood transfusion and iron chelating are the main therapeutic strategies for β-thalassemia. However, iron chelators have severe side effects, and blood transfusion would cause secondary iron overload.

### THERAPEUTICS TARGETING HEPcidIN AND RELEVANT MOLECULES

Based on the molecular basis of iron overload diseases, different strategies are being developed to modulate hepcidin expression.

### Heparin Agonist

**Mimi-hepcidin and Derivatives**

Hamp mutation would cause severe iron overload, and Hamp overexpression and hepcidin administration both diminished serum iron accumulation in mice, suggesting that increasing hepcidin level should be able to ameliorate iron overload in HH and β-thalassemia. However, due to a short half-life of natural hepcidin, hepcidin mimics or other drugs that could stimulate hepcidin level are in urgent need. On the basis of structural and functional analysis of hepcidin–FPN axis, the aromatic and hydrophobic residues were identified as the binding site between hepcidin and FPN. Thus, a series of mini-hepcidin were designed through computer modeling. Detailed molecular mechanisms are delineated in Figure 3. Serum iron was reduced after chronic administration of retro-inverso mini-hepcidin in Hamp–/– mice. Furthermore, an optimized mini-hepcidin named PR65 was chosen to
examine the benefits and side effects in *Hamp*−/− mice. PR65 could effectively redistribute tissue iron after the administration for 2 weeks. The mini-hepcidin has free sulfhydryl group in position 7, which may cause dermatological side effects. Thus, a new type of mini-hepcidin analog, PR73, is synthesized through S-protected strategy. The analog PR73SH reveals a significant ability in FPN degradation both in vitro and in vivo. To limit unusual and expensive amino acid in mini-hepcidin, new cyclic mimics of hepcidin are designed, for example, mHS17. Unfortunately, this new cyclic compound is found to be less active in inducing FPN degradation in vitro, and it even increases serum iron in mice.

**Small Chemical Compounds**

Compared with peptide or RNA-based technology, chemical compounds are more cost-effective. Through high-throughput screening, several compounds including genistein and progesterone are identified because they show excellent efficacy in hepcidin induction.

Genistein is an isoflavone compound isolated from plants. The capability of hepcidin induction is screened through zebrafish embryos. Its potential to induce hepcidin expression is confirmed in HepG2 cells through *Hamp* promoter luciferase activity and endogenous mRNA analyses. Further studies demonstrated that its hepcidin induction activity is mediated through Smad and Stat3 signaling pathways. Unfortunately, whether this compound can ameliorate iron overload in mouse models is still unknown. Nonetheless, these results suggest possible direction for the follow-up studies, in which the natural compounds would be an optional solution to search for more active groups for hepcidin induction.

With the application of high-throughput screening, epitostanol was identified to degrade FPN from 3120 small chemicals. Afterwards, several steroid hormones (e.g., progesterone and mifepristone) were found to have similar capability to diminish FPN concentration through PGRMC1–SFK signaling pathway. Ferristatin II, identified as an iron transport inhibitor, induces the internalization and degradation of TfR1, and reduces intestinal iron uptake and serum iron level. A recent study further demonstrated that ferristatin II increased hepcidin through phosphorylation of Stat3 without affecting Smad 1/5 phosphorylation.

Gaun et al constructed a screening study using a firefly reporter plasmid containing the human *Hamp* promoter for a library of 10,169 chemicals. Only 16 of them were found to induce *Hamp* expression in endogenous HepG2 cells. These chemicals can induce the BMP-Smad and/or Stat3-dependent gene expression; however, none of them enhanced phosphorylation of Smad1/5/8 or Stat3. In addition, some of these
chemicals are inhibitors of histone deacetylase and serotonin receptors, highlighting a new strategy for chemical screening or synthesis. However, the detailed molecular mechanisms warrant further investigation.

Additionally, our recent results demonstrated that icariin and its analogs had a robust ability to elevate hepatic hepcidin level and then regulate systemic iron homeostasis. This study signifies the potential application of certain natural compounds in treating iron disorders through regulating hepcidin expression.94

TMPRSS6 Antagonist

As previously described, TMPRSS6 takes part in hepcidin regulation through HJV cleavage.33,93 To induce Hamp expression, TMPRSS6 inhibition would be an alternative strategy. Nai et al96 demonstrated that homozygous loss of Tmprss6 in thalassemia mice could improve ineffective erythropoiesis and reduce splenomegaly and iron load. This study suggested that TMPRSS6 would be a potential target to reduce iron overload and improve ineffective erythropoiesis. Based on this study, oligonucleotides and siRNA are designed.97,98 The molecular mechanism for TMPRSS6 and the potential therapeutic significance are described in Figure 4. antisense oligonucleotides (ASO), an antisense oligonucleotide, is synthesized to against Tmprss6 mRNA.97 Sera and liver iron levels are significantly reduced after ASO administration in Hfe−/− mice.97 In addition, it also improved ineffective erythropoiesis and decreased splenomegaly, with resultant increase of total hemoglobin levels in β-thalassemia mice after ASO treatment for 4 weeks. Another RNA-based technology, namely double-stranded nucleic acid, also showed an excellent effect.98 siRNA molecules are capsuled in a lipid nanoparticle (LNP-RNAi), and the assembled complex displayed a dose-dependent TMPRSS6 inhibition after 24 hours administration.98 It greatly induced Hamp expression and ameliorated HH phenotypes in Hfe−/− mice after the administration for 2 or 6 weeks.98 Furthermore, LNP-RNAi treatment diminished secondary iron overload, and even reduced α-globin expression.98 To achieve a better result, this group treated β-thalassemia mice with LNP-RNAi in combination with oral deferiprone. This combination therapy reached better results than any of the individual ones.98 Recently, a new type of matriptase-2 inhibitors was designed. Kunitz-type inhibitor hepatocyte growth factor activator inhibitor (HAI-1) and HAI-2 were demonstrated to inhibit the function of matriptase-2, and HAI-2 was shown to have better capability in matriptase-2 inhibition.100 These kunitz-type inhibitors indicated a new strategy in negative hepcidin regulation.

FIGURE 4. The mechanism of antisense oligonucleotide for hepcidin induction. TMPRSS6 is a transmembrane protease serine 6, and it cleavages HJV to inactivate the Smad signaling. Antisense oligonucleotide molecules could silence the mRNA of Tmprss6, and then down-regulate the expression of Tmprss6 that functions to increases the stability of HJV. HJV is a part of the complex formed by BMPRs to induce Hamp transcription. BMPR = bone morphogenetic protein receptor, HJV = hemojuvelin.
BMP Protein Administration

The study by Meynard et al21 unearthed that lack of BMP6 caused massive iron overload and undetectable hepcidin. Corradini et al101 concluded that exogenous BMP6 treatment could relieve the HH phenotypes caused by \( Hfe \) deficiency. BMP6 administration enhanced hepcidin expression, and thus reduced iron overload in \( Hfe^{-/-} \) mice, suggesting that BMP6-like agonists may represent a promising route to ameliorate iron overload disease.101 However, there are still some concerns on the use of BMP6. For example, BMP6 administration could lead to peritoneal calcifications.101 Not only BMP6, but also BMP2, 4, 5, 7, and 9, can induce \( Hamp \) transcription as well. Nonetheless, BMP6 elicits the most robust induction of \( Hamp \) expression than others.19,20

Hepcidin Antagonists

Hepcidin Blockade by Antibody, Antisense Oligonucleotides, or siRNA

Considering the pathologies of anemia of inflammation and IRIDA, a high level of hepcidin and inflammatory cytokines are the main causes of iron deficiency. Thus, targeting hepcidin mRNA, protein, and its upstream regulators such as IL-6 and IL-6R would be optional strategies.

Targeting hepcidin mRNA or protein would be the direct manner to reduce hepcidin concentration. Thus, \( Hamp \) siRNA molecules and antibody were designed.102,103 NOX-H94 (also called Lexapteripid) is an L-oligoribonucleotide with a strong affinity to hepcidin mRNA. The pharmacological study on cynomolgus monkeys showed a reduction of serum hepcidin concentration, and increase of hemoglobin level after NOX-H94 administration under IL-6-induced anemia.104 NOX-H94 also exhibited a great capability to increase serum iron and transferrin saturation in a dose-dependent manner during the phase I clinical trial in humans.105 It also showed a significant effect on blocking the inflammation-associated low iron in volunteers with systemic inflammation.106 NOX-H94 also reveals an excellent efficacy with satisfactory tolerance in a phase II human trial study.107 Anticalin PRS-080 is an efficient peptide that can specifically bind hepcidin.103 Cynomolgus monkeys showed significant mobilization of iron and hyperferremia after PRS-080 administration.103 Additionally, hepcidin antibodies were also developed in animal studies on the model of inflammation of anemia, with promising results for modulating iron mobilization.108

Hepcidin Repression Through Acting on BMP and BMPR Complex

As described above, the BMP–Smad signaling pathway fundamentally regulates hepcidin. Thus, inhibition on BMPs or BMPRs would be able to diminish \( Hamp \) expression. sHJV is a
soluble fragment of HJV that binds to BMPs to prevent the latter’s association with BMPRs (as illustrated in Figure 5). sHJV shows a significant inhibition on BMP6 and BMP2, but to a less extent on other types of BMPs in Hep3B cells. Afterwards, a soluble HJV–Fc fusion protein (sHJV.Fc) was synthesized, and it manifested a greater ability to ameliorate anemia of inflammation in rats after peptidoglycan-polysaccharide (PG-APS) injection. As a result, the rats exhibited elevated serum iron and hemoglobin concentrations.

Not limited to BMPs, BMPRs are also the target to reduce hepcidin level. A small molecule inhibitor, LDN-193189, selectively antagonizes activin-like kinase type I receptors (ALK2 and ALK3), and increases hemoglobin level in mice. However, it cannot decrease Hamp expression and increase hemoglobin concentration in a rat model. HJV, as a cofactor of BMPRs, is a potential target to down-regulate Hamp expression as well. Two monoclonal antibodies, namely ABT-207 and h5F9-AM8, were developed to target HJV/repulsive guidance molecule C. Hepatic and serum hepcidin were reduced in rats after a single administration of ABT-207 or h5F9-AM8, and the serum iron concentration was consequently increased several weeks later. Furthermore, TNF-α elicits the down-regulation of hepcidin through suppressing HJV transcription in human hepatoma cells. But anti-TNF-α antibody greatly suppressed Hamp expression in patients with rheumatoid arthritis.

Repression of Hepcidin through IL-6 Signaling

Inflammation caused by IL-6 and other cytokines stimulates Hamp expression via the IL-6–Stat3 or other possible signaling pathways. Thus far, a few strategies have been developed to target IL-6 or IL-6R, or to inhibit the phosphorylation of Stat3. Tocilizumab is a humanized anti-IL-6R antibody, and it works to inactivate the phosphorylation of Stat3. IL = interleukin.

FIGURE 6. The mechanism of IL-6 Ab in hepcidin suppression. IL-6-provoked proinflammation effects activate Hamp expression through the Stat3 signaling. Blocking IL-6-activated Stat3 signaling would be an optimal strategy to reduce hepcidin level under inflammation. Tocilizumab is a humanized anti-IL-6R antibody, and it works to inactivate the phosphorylation of Stat3. IL = interleukin.

Chemical Compounds

Heparin, a type of glycosaminoglycan, is a well-recognized hepcidin inhibitor. Heparin functions to sequester
BMP6 and to repress Smad activation, leading to suppression of Hempp expression. In addition to reducing Hempp expression, heparin is also known for its anticoagulant activity. Thus, it would be optimal to maintain the hepcidin-inhibitory activity with low anticoagulant activity. For this purpose, a new generation of heparin, named glycol-split heparins (gs-heparins), are designed. Four gs-heparins, termed as RO-82, RO-68, NAc-91, and NAcRO-00, have been synthesized, and all of them repressed Hempp expression in HepG2 cells and primary hepatocytes. RO-82 and RO-68 were chosen for animal studies, and both of them were proved to reduce hepatic Hempp expression and serum hepcidin concentration in mice.

Another research group found that vitamin D could decrease Hempp expression. Prohormone 25-hydroxyvitamin D or active 1,25-dihydroxyvitamin D repressed Hempp expression by 50% in hepatocytes and monocytes. Further experiments in healthy humans confirmed this reduction of hepcidin by vitamin D. Vitamin D is thus a potential drug to ameliorate diseases with hepcidin overproduction. K-7174 is a synthesized compound that was identified to improve anemia induced by inflammatory cytokines in mice. K-7174 was verified to reduce hepcidin expression in human hematoma cells and in mice as well. Further mechanistic studies indicated that the signaling of hepatic iron inhibition by K-7174 was through GDF15. Ganz et al developed a high-throughput platform to screen hepcidin antagonists, and they identified 14 chemicals out of 7000 that efficiently antagonized hepcidin function. Fursultiamine, a FDA-approved thiamine can bind to C-326 thiol residue of FPN, which blocks hepcidin binding to FPN. However, it fails to interfere with the action of hepcidin in vivo due to its quick coevolution to inactive metabolites, and thus more efforts are warranted.

Other Options for Heparin Repression

Not limited to synthesized chemical compounds, Chinese medicinal plant extracts are another promising source for hepcidin modulation. Guan et al's study found that Caulis spatulolobi (CS, also named Jixueteng) exerted a potent inhibiting effect on Hempp expression through suppression of Smad 1/5/8 phosphorylation. It incurred significant hepcidin suppression after CS supplementation for 5 days in animals. In addition to chemical compounds, hormones are also able to modulate Hempp expression. For example, testosterone can down-regulate hepatic Hempp expression through disrupting the signaling of Samd1/4-mediated hepcidin induction. Meanwhile, it can increase hemoglobin and tissue iron in mice subjected to the inhibition of hepcidin after testosterone administration. 17-estradiol represents another sex hormone that could suppress hepcidin, as discussed above. Therefore, hormones would be additional considerations for developing therapeutics for iron disorders.

CONCLUSIONS

Thus far, strategies targeting hepcidin–FPN axis are being developed to correct iron disorders for diverse diseases. We reviewed the pathogenesis of iron disorders and according molecular mechanisms with relevance to hepcidin. We also recapitulated the current progress on hepcidin modulation including siRNAs, antibodies, chemical compounds, and plant extracts. Compared with conventional therapies (namely phlebotomy and blood transfusion), strategies targeting the hepcidin–FPN axis may open a new avenue for hepcidin regulation through an endogenous physiological way by avoiding secondary iron overload and other implications.

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