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Hepcidin: Discovery to Destiny

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Abstract

Iron plays an important role in many essential biochemical processes of living creatures, so tight regulation of iron metabolism is needed. In recent years many new players were introduced in this field of iron homeostasis, among them liver derived anti-microbial peptide named as hepcidin plays a vital role. It is produced as propeptide and processed by furin into active peptide of 25 amino acid. Ferroportin is the key ligand for hepcidin bioactivity. It causes degradation and internalization of ferroportin molecule, thus prevents the export of iron from the key iron regulatory cells like enterocyte, hepatocyte, macrophages, etc. Main pathways responsible for expression of hepcidin molecule are BMP/HJV/SMAD and IL6/STAT3, they respond to iron status and inflammation respectively. Other conditions which can alter the hepcidin expression are anaemia, hypoxia and ineffective erythropoiesis. In different iron related clinical conditions, hepcidin production alters the pathogenesis of the diseases. So, hepcidin is a promising and unexploited therapeutic target for the development of newer drugs for iron metabolism related diseases.

Keywords: Ion homeostasis, ferroportin, hepcidin, therapeutic target

1. Introduction

Iron is the important micro-mineral involved in the broad range of biological activity. It is an important biocatalyst that exists in two different forms i.e., oxidised insoluble (Fe^{3+}) and reduced soluble form (Fe^{2+}). In mammals majority of the iron molecule is used for the synthesis of haemoglobin, DNA, myoglobin and iron-sulphur containing proteins [1]. Iron plays a vital role in the innate immune response of animal system by the generation of toxic oxygen and nitrogen intermediates to destroy the invading pathogens [2]. Even though iron is an important mineral for maintaining the normal homeostasis of the animal body, its bioavailability has to be tightly regulated because a mere excess or deficit of iron can lead to severe pathological conditions. The dark side of the iron were it plays important role in Haber-Weis-Fenton's reactions that produces reactive oxygen species (ROS) which can cause damage to nucleic acids, proteins and lipids [3]. Deficiency of iron leads to anemic condition in animals. In mammals, daily iron uptake (1-2mg) equals the daily loss from body. Major iron consumer of body is the bone marrow (approximately 20mg/day) for the synthesis of erythrocytes. This high iron requirement is met out by the recycling of iron from the senescent erythrocytes in macrophages [4]. Mammalian body system does not contain any active iron excretory mechanism, so iron homeostasis must be finely manipulated. In recent years there are many new players are introduced in this field of iron homeostasis, among them a unique liver derived anti-microbial peptide, hepcidin has a central role in this aspect. Expression of hepcidin molecule from liver varies according to the systemic iron status and other factors. It controls the export of iron from various cells into circulation. So, the study of hepcidin biology will provide a new diagnostic and therapeutic approaches in iron related disorders.

2. Body iron distribution and storage

Approximately 60-70% of body iron is incorporated in haemoglobin of erythrocytes and 20-30% is in the form of hemosiderin and ferritin. A small amount of iron is present in the form of muscle pigment called myoglobin and also it is bounded with the transport protein called transferrin [5]. Iron is stored in the form of ferritin and hemosiderin in hepatocytes and kuffer cells of liver to prevent excess intracellular iron concentration in the body system which can cause cellular damage [6]. Ferritin contains two subunits H and L, ferroxidase activity is needed for incorporation of iron into ferritin that is attributed to H and L subunits, which plays an

important role in mineralization [7]. Mature ferritin can able to accumulate 4500 iron atoms in it [3]. Majority of the ferritin molecule will be located in the cytoplasm of the cell but sometimes small quantity can be located in nucleus. Ferritin stored in the nucleus could deliver iron to iron-dependent enzymes or transcription factor activities and also it can have a role in protecting DNA from oxidative damage by scavenging of free iron [8, 9]. Another important form of ferritin is mitochondrial ferritin, which controls the iron homeostasis inside the mitochondria [10]. Second form of storage iron is hemosiderin molecule. It is formed by incomplete lysosomal degradation of ferritin and it is an insoluble form. Hemosiderin becomes a predominant iron storage protein during iron overloaded conditions. Under normal physiological status it is not an effective donor of iron but during inflammation and hypoxia it readily donates iron molecule, that favours oxidative cellular damages [11].

3. Physiology of iron metabolism

Dietary iron is present in two forms – as inorganic and heme form. Inorganic form of iron will be in higher concentration (90%) in food stuffs when compare to heme form (10%) [12]. The bioavailability of heme is high because various dietary factors will affect the absorption of inorganic form [13]. Absorption of iron occurs in duodenum and upper jejunum of the intestine with the help of different types of proteins present in apical and basolateral membrane of enterocytes. Inorganic iron is reduced to ferrous from ferric form by the help of duodenal cytochrome B (DcytB) [14] and members of six-transmembrane epithelial antigen of the prostate (STEAP) family located in the brush border of enterocytes [15]. Reduced ferrous form is transported across the apical membrane into the cytoplasm by the help of divalent metal transporter 1 (DMT1) [16]. Some researchers found out that ferric form uses integrin-mobilferrin pathway (IMT). This pathway contains several proteins like beta-3-integrin, mobilferrin and flavin-monooxygenase [13, 17]. Heme iron is transported across the apical membrane of enterocytes via heme-carrier protein 1 (HCP1) [18, 19]. Once the heme enters the cell, iron molecules from heme is released by the help of heme oxygenase 1 (HO-1) and joins with the cytosolic iron pool. After this, both forms of iron shares common pathway. Fate of the iron entered into the enterocyte is either they should be transported into circulation or stored as ferritin. Only known exporter of iron from the cells into the plasma is ferroportin [20]. Ferroportin will be present in all the cells which export iron into plasma. Exportation of iron into plasma involves conversion of intracellular Fe (II) to extracellular Fe(III) by the help of hephaestin protein present in basolateral membrane [21]. When the iron molecule enters the plasma it binds reversibly with a serum glycoprotein called transferrin. Under physiological pH transferrin has high affinity to iron so, all non heme iron will be present in the bounded form in plasma. In conditions like iron over loaded disorders, nontransferrin bound iron (NTBI) will be present in plasma, which can enter freely into the cells. So NTBI is considered as a marker of iron toxicity [22, 23]. Uptake of transferrin into the cells is mediated by transferrin cycle. Binding of transferrin to the cells occurs through two type of transferrin receptors (TfR1 and TfR2). TfR1 is expressed in the surface of all iron requiring cells [24]. Diferric transferrin has more affinity for TfR1 than monoferric form and iron-free apotransferrin. Cells of liver, haematopoietic cells, duodenal crypt predominantly expresses the TfR2. When transferrin binds with TfR1, initiation of transferrin cycle occurs by the

formation of clathrin-mediated endosome. Acidification of endosomal contents occurs by the action of proton pump on its membrane which leads to change in the conformation of transferrin that causes iron release [25]. Released iron is reduced by STEAP and transported into the cytoplasm by DMT1. Now the transferrin molecule is released into the circulation for binding with new iron molecule [26]. After entering into the cytoplasm, iron joins with the liable iron pool (LIP). LIP is the dynamic iron compartment which supplies iron to mitochondria and for the synthesis of storage form of iron (e.g. Ferritin) [27]. Utilization of iron is high in mitochondria because it is the site of synthesis of heme and iron-sulfur cluster. Overall Physiology of iron metabolism is shown in figure 1.

4. Regulation of iron metabolism

Iron-regulatory proteins (IRPs) and hepcidin controls the iron metabolism at cellular and systemic levels respectively [28]. At cellular level two type of IRPs are recognised, IRP1 and IRP2. They coordinate the expression of TfR, DMT1, ferritin and ferroportin molecules. Molecular action of IRPs is by binding to a iron responsive element (IREs) of mRNA coded for iron metabolism proteins. Iron deficiency and hypoxia upregulates the binding capacity of IRPs and *vice-versa*. At systemic level, hepcidin controls the iron homeostasis by making internalization and degradation of iron exporter molecule ferroportin. By which hepcidin controls the release of iron into plasma from different cells of the body. Up-regulation of hepcidin leads to decreased plasma iron level and down-regulation leads to increased plasma iron level [29].

5. Biology of hepcidin

5.1. History of hepcidin discovery

In the years 2000-2001, hepcidin was discovered by various independent groups of researchers. Krause *et al.* (2000) discovered a peptide with antimicrobial activity in the human blood and named it as LEAP-1 (liver-expressed antimicrobial peptide 1) [30]. Park *et al.* (2001) characterized a cysteine-rich peptide from human urine and named it as hepcidin (hepatic bactericidal protein) because of its site of synthesis in liver and biological activity [31]. The homologous cDNAs in liver of various species from fish to human was also found out by them. In 2004, canine hepcidin gene expression was studied and it was found that the canine hepcidin was homologous to other species hepcidin in the order of 74%, 73%, 61% and 59% of human, pig, rat and mouse respectively [32]. In the subsequent years many researcher found out the important role of hepcidin in iron metabolism [33-37].

5.2. Structure of hepcidin

Hepcidin (Hepc) is a 25-amino-acid protein which contains eight cysteine residues and four disulfide bonds that forms hairpin like structure (an amphipathic beta sheet) [38]. 19th chromosome of humans has hepcidin (HAMP) gene which codes for precursor prepropeptide of 84 amino acids that is processed by two sequential cleavages by help of furin enzyme. End product of the sequential cleavage of prepropeptide is matured bioactive hepcidin of 25 amino acids [39]. Three forms of hepcidin are recognised, Hepc-25, Hepc-22 and Hepc-20 respectively. Hepc-25 and Hepc-20 are processed in golgi apparatus intracellularly and released into blood. Hepc-22 is only present in urine [31].

5.3. Synthesis of hepcidin

Liver is the major site of hepcidin synthesis [30, 31]. Recent studies have demonstrated that hepcidin is not only expressed in hepatocytes but also by the cells of kidney tubule, heart, retina, monocytes, neutrophils, adipose tissue, lungs and pancreas in lower concentrations [40-47]. Biological action of these locally secreted hepcidin is still unclear.

5.4. Kinetics of hepcidin

Hepcidin when it enters into blood circulation it binds with α 2-macroglobulin. A small fraction of hepcidin was found to be bound with albumin. Under normal physiological conditions, 11% of hepcidin was found to be freely circulating in the plasma [48]. But the physiological significance of plasma protein bound hepcidin is still unmasked. Kidney takes the role of hepcidin clearance via cellular codegradation with ferroportin. As the hepcidin molecule has low molecular weight and small radius it can be filtered freely into the glomerular filtrate. Finding of some human studies shows that final percentage of hepcidin excreted in the urine is as low as 0% to 5%, this may be due to either by absorptive process in renal tubules or may be due to not freely filtered because of binding with plasma proteins [49, 50].

5.5. Functions of hepcidin

Hepcidin was first identified as an antimicrobial peptide and classified as a member of defensin family. But the antimicrobial activity of hepcidin requires higher concentration than those found in blood [30, 31]. Of note, isomer of hepcidin isolated from urine has same antimicrobial activity similar to circulating active hepcidin, but it is unable to degrade ferroportin *in vitro* and *in vivo* [51]. This shows antimicrobial and iron homeostasis maintaining functions of hepcidin are not overlapping. Hepcidin binds in the hepcidin binding domain (HBD) of ferroportin which leads to JAK2-dependent tyrosines phosphorylation. Once internalized, proteasomal degradation occurs after ubiquitination [52, 53, 54]. Previously there was a controversy that whether ferroportin is a dimer or monomer but researchers found that ferroportin is dimer [54]. Ferroportin is a sole iron exporter protein present in macrophages, hepatocytes and basolateral aspect of enterocytes in duodenum. Once hepcidin causes internalisation and degradation of ferroportin it prevents efflux of iron from the cells into circulating blood. So, the main function of hepcidin is the systemic regulation of iron homeostasis. Hepcidin is found to be produced from many other cells of body apart from hepatocytes, there they exert autocrine role in maintaining iron homeostasis. In the aspect of blood circulation brain is separated from rest of the body by blood brain barrier (BBB). Blood brain barrier was formed by ependymal cells, which has the hepcidin expression regulating proteins. So, the intracranial iron level will be changed accordingly to the systemic iron content through altering the hepcidin expression. This phenomenon plays an important role in neurodegenerative disorders [55].

6. Regulation of hepcidin expression

6.1. Crosstalk between iron and hepcidin

Iron mediates the expression of hepcidin through bone morphogenetic proteins (BMPs). In both *in vivo* and *in vitro* conditions upregulation of BMP signaling pathway leads to increased hepcidin expression. There are 20 different members of BMP family are identified of which BMP6 is the important regulator of hepcidin expression [56, 57]. Hemojuvelin (HJV) is the member of the repulsive guidance

molecule (RGM) family, which is a co-receptor of BMP [58]. HJV is produced in liver, heart and skeletal muscle. Degradation of HJV is done by liver-specific serine protease Matriptase-2 (MT2/TMPRSS6) [59] it is a negative regulator of hepcidin expression. When BMP6 binds with BMP receptor along with HJV it activates the SMAD transcriptional system (SMAD1/5/8). Once SMAD members get activated they bind with SMAD4 a key regulator of BMP pathway to form the SMAD complex. These complexes bind the responsive elements of the hepcidin genes and causes upregulation of hepcidin expression [60]. HFE is the gene that causes hemochromatosis (HH). HFE can interact with Tfr, mostly with Tfr2. The HFE-Tfr2 complex acts through the MAPK/ERK pathway to increase the hepcidin expression [61]. Neogenin is a transmembrane protein that can interact with HJV. Neogenin can able to stimulate or suppress the hepcidin production by favouring either BMP pathway or stimulating the activity of MT2 [62].

6.2. Hepcidin and inflammation

Various studies on hepcidin provided a new mechanism for understanding the anaemia of inflammation. Hepcidin is an acute phase protein which is increased during both acute and chronic inflammatory conditions [63]. During inflammatory conditions different type of cytokines are produced, among those cytokines IL6 and IL1 are the most potent activators of hepcidin expression. They act through janus kinase (JAK)/signal transducer and activator of transcription-3 (STAT3) signaling pathway. After JAK2 phosphorylates the STAT3, it binds to cognate motifs in the hepcidin gene and increases hepcidin expression [64]. Activin-B a member of TGF-beta family, which appears during inflammatory conditions can up regulate the hepcidin expression by activating the BMP/SMAD pathway [65]. Oxidative or endoplasmic stress can also increase the hepcidin expression by activating the transcription factor cAMP-response-element-binding-protein-H or by the stress-inducible transcription factors CCAAT-enhancer-binding protein (C/EBP α) and C/EBP-homologous protein [66, 67]. During inflammatory conditions, myeloid cells also produce hepcidin by the activation of TLR4 receptor located in the neutrophils and macrophages [68]. So the hepcidin production is increased during the inflammatory conditions that can lead to hypoferrremia and anaemia due to iron restricted erythropoiesis.

6.3. Relationship among hepcidin, erythropoiesis and hypoxia

Increased erythropoietic activity decreases the hepcidin production because erythropoiesis requires high amount of iron (approximately 25 mg/daily), so the down-regulation of hepcidin expression is of greater physiological importance. Erythropoietic signal leads to production of two proteins called growth differentiation factor 15 (GDF 15) and twisted gastrulation I (TWSGI) from erythroblasts. These molecules act through BMP/SMAD pathway [69-71]. During hypoxic condition erythropoietin (EPO) is produced from the kidney. EPO can down-regulate the hepcidin synthesis by two pathways, one is directly through erythropoietin receptor (EPOR) mediated regulation of the transcription factor C/EBP α and another is by indirectly suppressing the STAT3 and SMAD4 signaling pathway [72, 73]. Liver-specific stabilization of hypoxia-inducible factor (HIF)-1 is also produced during hypoxic conditions, which causes decreased hepcidin production and also have direct influence on iron

absorption from enterocytes by increasing the expression of both ferroportin and divalent metal transporter 1 [74].

6.4. Hepcidin and heme

Heme, a potent pro-inflammatory iron-containing molecule and it is one of the end products of senile erythrocyte catabolism in macrophages. Heme binds to toll like receptor (TLR)-4 and mediates the up-regulation of hepcidin expression in macrophages through extracellular signal-regulated kinases (ERK) pathway [75]. Heme can also cause harmful pro-oxidant and cytotoxic effects to the cells by producing reactive oxygen species [76, 77]. So high level of heme produces oxidative stress, which can further increase the hepcidin production. In certain bacterial infections and haemolytic conditions there will be excessive amount of heme production. This heme can further accelerate the pathology by hepcidin mediated iron deficient erythropoiesis, leading to anaemia. Overview of mechanism for hepcidin expression was shown in figure 2.

7. Hepcidin assay

Developing an assay for hepcidin quantification is a real challenge to the researchers because it is small, evolutionarily conserved and has the properties like aggregating and sticking to laboratory plastics. Due to its small and compact structure development of antihepcidin antibody is very difficult [78]. Both circadian rhythm and day-to-day variations of hepcidin concentration is also reported [79]. There was a decrease in hepcidin-25 values within 1 to 2 days of storage at room temperature but are stable at 4 °C for 1 week, -20 °C for 4 weeks and -80 °C for ~2years [80, 81]. In spite of these constraints scientist developed two groups of assays; they are mass spectrometry (MS)-based and classical immunoassays. Mass spectrometry (MS)-based assays can able to differentiate up to the level of hepcidin isoforms but classical immunoassays will measure only the total hepcidin level [82]. Mass spectrometry (MS)-based assays includes liquid chromatography/selected reaction monitoring mass spectrometry, HPLC-tandem mass spectrometry, liquid chromatography tandem mass spectrometry, ultra-high-pressure liquid chromatography and a linear ion trap mass spectrometer, SELDI-TOF MS, quadrupole - time-of-flight mass spectrometry and MALDI-TOF MS. Classical immunoassays includes c-ELISA, c-RIA and Sandwich ELISA [83]. Even though there are various blood parameters which can indicate the anaemic and iron status of animal, measurement of hepcidin will provide sufficient information to understand the pathology of underlying condition.

8. Hepcidin in different clinical disorders

Disorders associated with hepcidin activity alterations can be classified into three categories; disorders with increased hepcidin, decreased hepcidin and hepcidin resistance. Disorders associated with decreased hepcidin activity include hereditary hemochromatosis, anaemia of ineffective erythropoiesis, hypotransferrinemia, iron deficiency anaemia and chronic liver diseases. Ferroportin mutation comes under disorder associated with hepcidin resistance. Anaemia of inflammation and infections, iron refractory iron deficiency anaemia, renal diseases, obesity-related diseases, anaemia of chronic heart failure and cancer comes under the disorders associated with increased hepcidin activity [83, 84]. Recent studies show that concentration of hepcidin is increased in liver fibrosis due to Cholestasis [85] and in neurodegenerative

diseases [86]. Xu *et al.*, (2017) found out that the regulation of iron metabolism by hepcidin contributes to unloading-induced bone loss [87]. Clinical application of hepcidin takes upper hand when compare to other iron markers like ferritin, even though their changes are similar in iron related disorders alteration in hepcidin concentration occurs in matter of hours but ferritin takes longer time. Evaluation of hepcidin and other iron related parameters in different pathological conditions will help in identifying the actual cause of anaemia. Hepcidin estimation will helpful in differentiating the iron deficiency anaemia and anaemia of inflammation because in actual iron deficiency anaemia both hepcidin and iron levels will be decreased but in anaemia of inflammation hepcidin level will be increased but iron level will be normal, so it is considered as functional iron deficiency [84]. Levels of hepcidin and other iron related parameters are showed in table 1.

9. Hepcidin; as diagnostic tool and therapeutic target

Hepcidin plays a central role in iron disorders so estimation of hepcidin in such conditions helps in therapeutic planning. Measuring hepcidin is anticipated to be a helpful tool for screening patients and in monitoring the effects of novel targeted therapies. Hepcidin discovery has opened a new field in pharmacology with a number of drugs in the pipeline; including hepcidin agonists for iron overloaded disorders and hepcidin antagonists for iron deficiency disorders. Substances which modulate the hepcidin level can be classified into hepcidin agonist and hepcidin antagonist. Hepcidin agonists includes synthetic hepcidin-25, small hepcidin peptides, BMP agonists and HIF stabilizers they can increase the hepcidin level in iron overloaded conditions leads to decrease in the circulating iron level. Hepcidin agonist finds lesser role in veterinary field because iron overloaded disorders are rare in veterinary subjects. Another group of substances which can decrease the level of circulating hepcidin are classified based upon their action as anti-hepcidin agents, inhibitors of BMPs/BMPr complex, inhibitors of IL6/STAT3 axis and agents altering the hepcidin-ferroportin interaction. Their mechanism actions are briefed in table 2 [88]. Recent studies show that ursodeoxycholic acid [85] and estrogen [89] can inhibit the hepcidin expression. Hepcidin antagonists finds role in the treatment of iron deficiency disorders. Various hepcidin antagonists and their mode of actions were given in table 2. Even though the treatments with hepcidin modulators are promising, they also tend to cause risk. In related veterinary subjects presently only two studies has been published which shows that hepcidin can also be used as a prognostic marker of sepsis in canine parvo virus [90] and as an indicator of iron metabolism alterations in cats with chronic kidney diseases [91].

Table 1: Levels of hepcidin and other parameters in different types of anaemia.

Type Of Anemia	Hepcidin Level	Ferritin	Transferrin Saturation	Soluble Transferrin Receptor
Iron Deficiency Anaemia (Ida)	Low	Low		Increased
Iron-Refractory Iron Deficiency Anaemia (Irida)	High	Low	Low	Variable
Anemia With Iron Overload	Low	High	High	Variable
Anemia Of Chronic Disease/Inflammation (Ackd)	High	Normal Or High	Low	Normal
Mixed Anaemia		Normal Or High	Low	Normal Or High
Anemia In Chronic Kidney Disease (Ackd)	Normal	High	Variable	Variable
Resistance To Erythropoietin	High	Variable	Variable	Variable

Table 2: Hepcidin antagonists and their mode of action.

Hepcidin Antagonists	Mode Of Action
Acting on BMPs/BMPr complex	
sHJV-Fc	Inhibits the BMPs/SMAD pathway
LDN-193189	Inhibitor of phosphorylation of BMPs receptor type I
siHJV, siTFR2 Anti-BMP6	Degradation of HJV or TFR2 mRNA
Anti-BMP6 antibody	Sequestration of BMP6
Heparin	Inhibitors of BMPs/SMAD pathway
Acting on IL6/STAT3 axis	
Anti-IL6r (Tocilizumab)	Sequestration of IL6 receptor
Anti-IL6 (Siltuximab)	Sequestration of IL6
AG490	Inhibitor of STAT3 phosphorylation
PpYTK	Disruptor of STAT3 dimerization
Anti-hepcidin agents	
siHep	Degradation of hepcidin mRNA
Anti-hepcidin antibody	Sequestration of hepcidin protein
Anticalin	Sequestration of hepcidin protein
Spiegelmers	Sequestration of hepcidin protein
Agents altering the hepcidin-ferroportin interaction	
Anti-ferroportin antibodies	Interfering with hepcidin binding to ferroportin
Fursultiamine	Sequestration of Cys326-HS on ferroportin heparin binding site

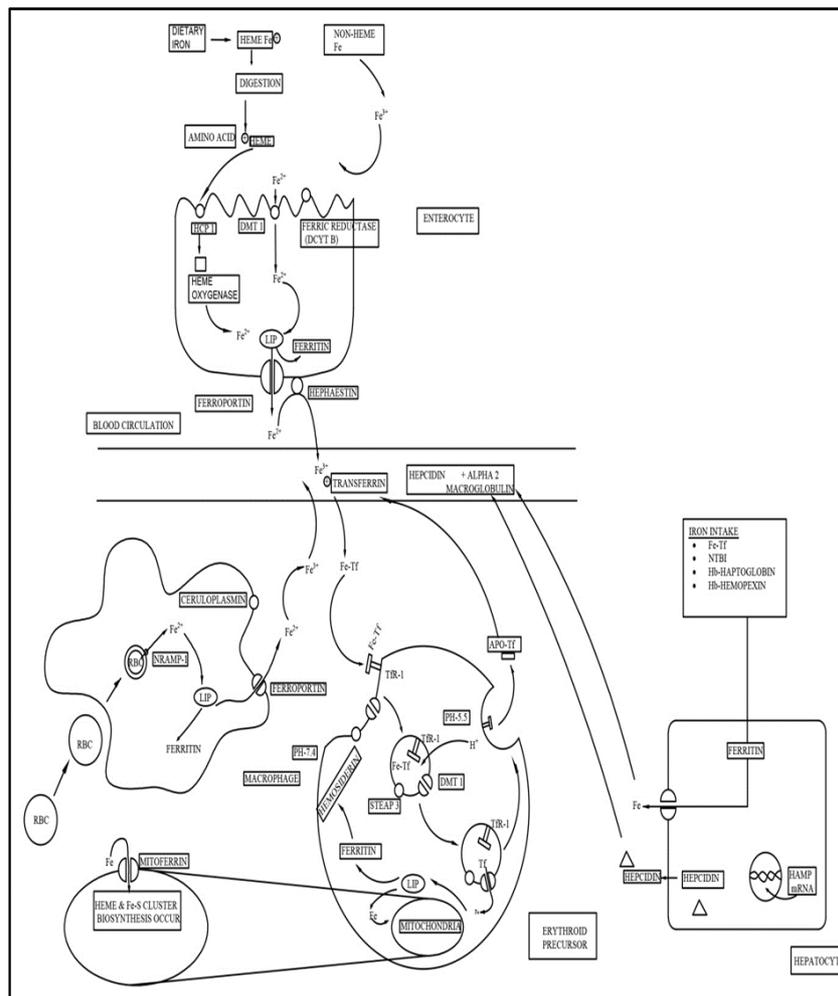


Fig 1: Physiology of iron metabolism.

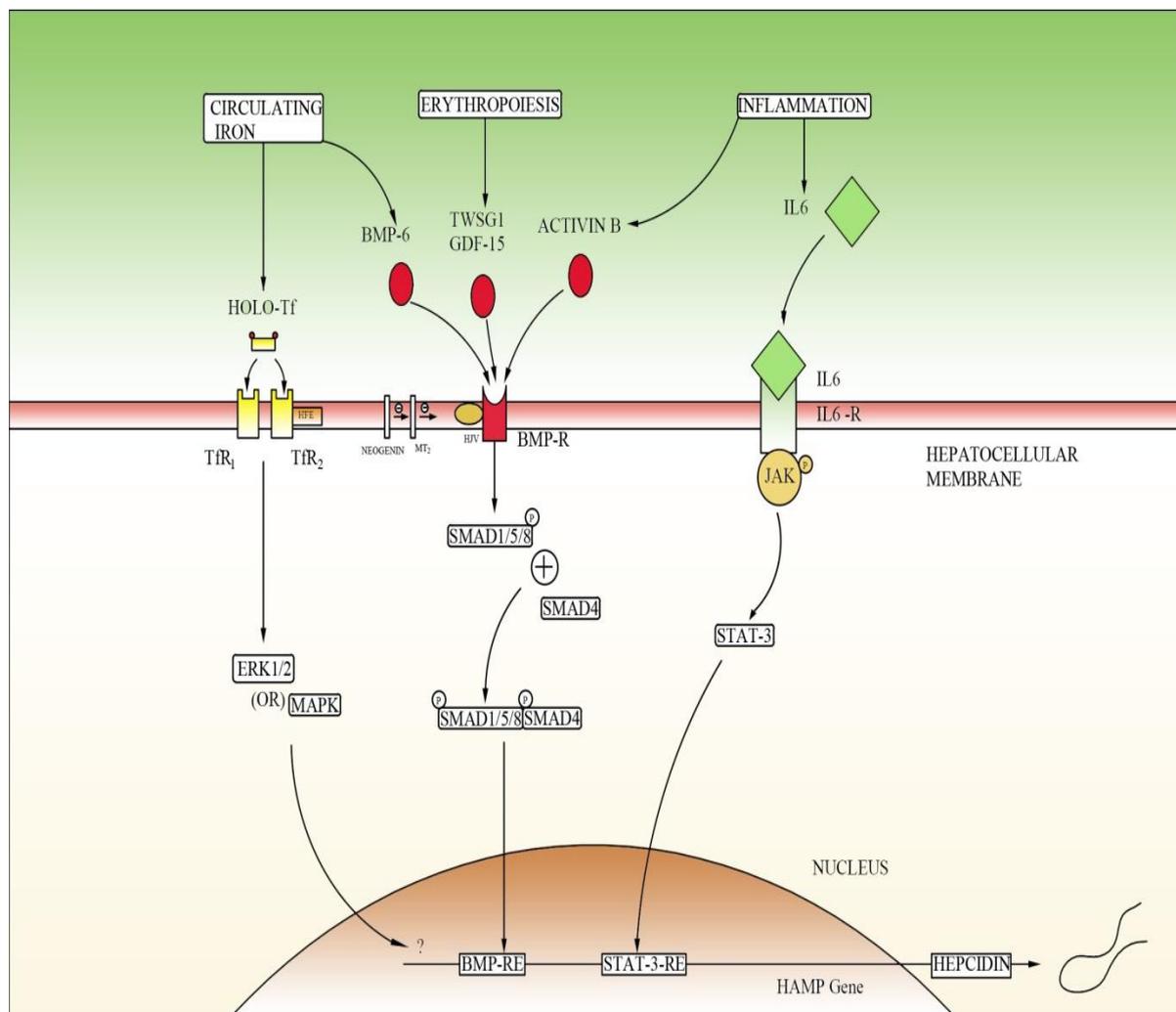


Fig 2: Mechanism of hepcidin expression

10. Conclusion

Discovery of hepcidin paved a new path in the field of iron physiology. This small peptide proved to be a central regulator of iron homeostasis and liver is the major controller of iron metabolism. Hepcidin is an important tool that can be added to the present battery of diagnostic tests for iron related disorders. Researches related to hepcidin in the veterinary field should be increased to validate the potential use of this hormone as a novel diagnostic and therapeutic tool in various disorders.

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