New and old players in the hepcidin pathway

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The identification of the liver peptide hepcidin at the beginning of the new millennium opened a new era in our understanding of iron metabolism.[1-3] Hepcidin is the main regulator of intestinal iron absorption and macrophage iron release, thus ultimately of the iron available for erythropoiesis. The evidence of the fundamental role of hepcidin is provided by both animal models and human disorders. Mice in which the hepcidin gene was inadvertently inactivated develop severe iron overload,[4] while transgenic mice overexpressing hepcidin have severe iron deficiency anemia at birth.[5] In humans, genetic inactivation of hepcidin causes a rare form of juvenile hemochromatosis,[6] whereas hepcidin overexpression in inflammation causes anemia of chronic diseases (ACD), which has features of iron restricted erythropoiesis.[7] The effect of hepcidin is clearly explained by its interaction with the cellular iron exporter ferroportin[8] that leads to ferroportin internalization and lysosomal degradation, a process now known in detail.[9]

In 2004, hemojuvelin (*HJV*) was identified as the gene mutated in the common form of juvenile hemochromatosis.[10] *HJV* was the last hemochromatosis gene cloned after *HFE, TFR2, HAMP* encoding hepcidin and *SLC40A1* encoding ferroportin.[11] Patients with *HJV* mutations have extremely low hepcidin levels indicating that HJV is the most important hepcidin modulator.[10] HJV was then demonstrated to be a multifaceted protein with membrane and soluble isoforms. The main role of the GPI-anchored form (m-HJV) was clarified when it was discovered that it is a coreceptor for Bone Morphogenetic Proteins (BMPs),[12] cytokines that stimulate hepcidin expression both *in vitro*[12-14] and *in vivo* in mice.[15] m-HJV amplifies the activation stimulus provided by BMP recruiting their receptors and is required for full hepcidin activation. Soluble HJV (s-HJV) released both in culture media and in sera (likely by muscle cells) is an inhibitory component[16] of the hepcidin pathway. s-HJV is cleaved by furin and is released in iron deficiency and hypoxia.[17] Large amounts of s-HJV administered to mice induce hepcidin downregulation and a hemochromatosis phenotype.[15] These observations suggest that s-HJV plays a role in iron deficiency, although a real physiological function remains unproven.

Very recently a novel protein with a relevant function in iron metabolism was recognized, encoded by *TMPRSS6* and named matriptases-2.[18] We predict that also this discovery will become a milestone in the story of iron regulation since mutations of *TMPRSS6* cause a form of inherited iron deficiency.[19] In this issue of the Journal, three papers provide new insights into the hepcidin pathway, its regulation and its disorders. Melis *et al*. report a Sardinian inbred family with 5 members affected with IRIDA due to a novel *TMPRSS6* mutation,[20] Pagani *et al*. studied the processing of HJV mutants and their ability to activate hepcidin *in vitro*,[21] while Kanda *et al*. investigated the relationship of hepcidin with markers of erythropoiesis in the stem cell transplantation (SCT) setting.[22] This review summarizes current knowledge of the hepcidin pathway and of the regulation of the hepcidin expression.

Molecular mechanisms of hepcidin activation

Hepcidin response occurs in inflammation and in iron overload. In inflammation, IL-6 triggers hepcidin activation binding to IL-6 and activating the Signal Transduction and Activator of Transcription 3 (*STAT3*), that binds to a consensus sequence in the hepcidin promoter.[14,23,24] Signaling through BMP/SMAD is another powerful mechanism to activate hepcidin transcription, through BMP responsive elements in the hepcidin promoter.[14] HJV works in the BMP/SMAD pathway amplifying BMP stimulation. It is likely that the BMP-HJV complex on the cell surface communicates with the HFE-TFR1 or HFE-TFR2 complex, that sense iron in the circulation.[25] The study of *HJV* mutations found in juvenile hemochromatosis patients may be a useful tool to understand the molecular pathogenesis of the disease and to gain insights into the complex physiological processing of the protein. Most *HJV* mutants are unable to reach the plasma membrane[26] and thus to mediate the hepcidin activation triggered by BMPs. However, as shown by Pagani *et al*., several mutants at the N-terminal of the protein reach the plasma membrane, although their intracellular processing appears somehow altered.[21] Their delayed exposure to the cell surface and likely the lack of interaction with essential components of the pathway, e.g. BMPs or BMP receptors, might explain their inability to up-regulate hepcidin. Indeed, hepcidin activation is greatly reduced both in the case of proteins that reach the plasma membrane, and with cell surface defective proteins.

A new genetic iron-deficient anemia

An unusual mouse model of genetic iron deficiency, the *Mask* mouse, was first reported by B. Beutler at the ASH Meeting in Atlanta in December 2007. Mask mice are smaller than their littermates, show gradual loss of body hair with preservation of the face hair, females are infertile and both sexes have microcytic anemia with low serum and total body iron. Mask mice are unable to increase intestinal iron absorption and have inappropriately high liver hepcidin, which blocks iron absorption and is the cause of anemia.[18] The phenotype was the result of a homozygous mutation induced by N-ethyl-N-nitrosourea (ENU). Positional cloning identified the *Mask* locus on chromosome 15. Sequencing of all positional candidates led to the identification of an A→G homozygous mutation of *TMPRSS6*, which encodes a type II plasma membrane serine protease, matriptase-

2,[27] a member of a cell surface proteolytic enzyme (TTPS) family. Matriptase-2 is highly conserved in humans, mouse and rat, is highly expressed in the liver and olfactory epithelium in mice,[18] and is homologous to the ubiquitous matriptase-1. The protein has 811 amino acids forming an N-terminal cytoplasmic tail, a transmembrane domain, two CUB and three LDLR domains and a C-terminal trypsin-like serine-protease domain (Figure 1). The *Mask* mutation abrogates a splice acceptor site of intron 14 and originates a truncated protein that lacks the serine protease domain (Figure 1). That the *Mask* mutation was responsible for the phenotype was conclusively shown by the correction of the phenotype obtained in transgenic animals by microinjection of a BAC clone bearing the wild type *Tmprss6* gene into the fertilized Mask/Mask oocytes.[18]

Functional studies demonstrated that the proteolytic activity of the serine protease domain was essential for hepcidin activation. Cotransfection of human hepatoma HepG2 cells with the human hepcidin promoter and matriptase-2 caused a strong reduction of the hepcidin activation in basal conditions and also blocked reporter induction by several activators such as HJV, BMPs, IL-6 and even SMAD1.[18] The Mask protein without the serine protease domain and the protease-dead *Tmprss6* mutant was unable to suppress hepcidin expression. The Authors went on to demonstrate that the matriptase-2 intracellular domain, although less conserved among species and without clear signaling motifs, is integral to signal transduction and autonomously drives a *Hamp* suppression signal. They hypothesized that matriptase-2 participates in a novel transmembrane signaling pathway that senses iron deficiency independently of the known pathways.[18]

The function of matriptase-2 is maintained in humans, since its mutations cause iron-refractory iron-deficiency anemia (IRIDA) The first study reported 5 familial and 2 sporadic cases of IRIDA with different types of *TMPRSS6* mutations: *non sense*/frameshift/splicing mutations leading to truncated variants reminiscent of Mask were prevalent, whereas missense mutations in the protease domain or in other conserved aminoacids were rare[19] (Figure 1). Urinary hepcidin, measured in a proportion of patients, was normal/high, in spite of the severe iron deficiency, which is usually associated with undetectable values. This study led to the conclusions that the serine protease matriptase-2 is required to sense iron deficiency in humans. Melis *et al.* show that matriptase-2 is responsible for the inherited microcytic anemia found in 5 members of a large six generation Sardinian pedigree that was well-known to the scientific community as the best example of iron-deficient anemia of genetic origin.[20] A homozygous G→C mutation at the 5' donor splice site of intron 6 (IVS6 +1G→C), present in all patients, is predicted to produce a truncated protein lacking two CUBs, three LDLRs and the protease domains. A homozygous haplotype in the region encompassing *TMPRSS6*, present in all patients, confirmed the single origin of the mutation from a common ancestor.

Melis *et al*. observed a variable age of IRIDA presentation.[20] Three patients presented in the first two years of age. Some were followed since infancy, when they required parenteral iron to improve their Hb levels. Two adult males had unremarkable anemia, even if they had microcytic red cells, low serum iron and low transferrin saturation. This study emphasizes that the severity of the disease is greater during infancy than during adult life, with the implication that matriptase-2 has a prevalent role when iron requests are higher. Serum hepcidin levels were normal/increased in all patients, and urinary hepcidin levels, measured in 4 cases, were even higher. A preliminary analysis of the urinary hepcidin levels measured in IRIDA patients shows that those with null mutations have extremely high hepcidin levels.[19,20] The elevated hepcidin levels explain not only the lack of absorption of oral iron, but also the partial response to parenteral iron treatment, since the infused iron is captured by macrophages before utilization. This translates into the atypical condition of high serum ferritin in spite of persistently low transferrin saturation after parenteral iron treatment. The iron may be released through ferroportin irrespective of the high hepcidin levels likely because of the IRE-IRP mediated transcriptional upregulation of ferroportin occurring when the iron content of macrophages is acutely increased. A similar post-transcriptional regulation of ferroportin might also explain

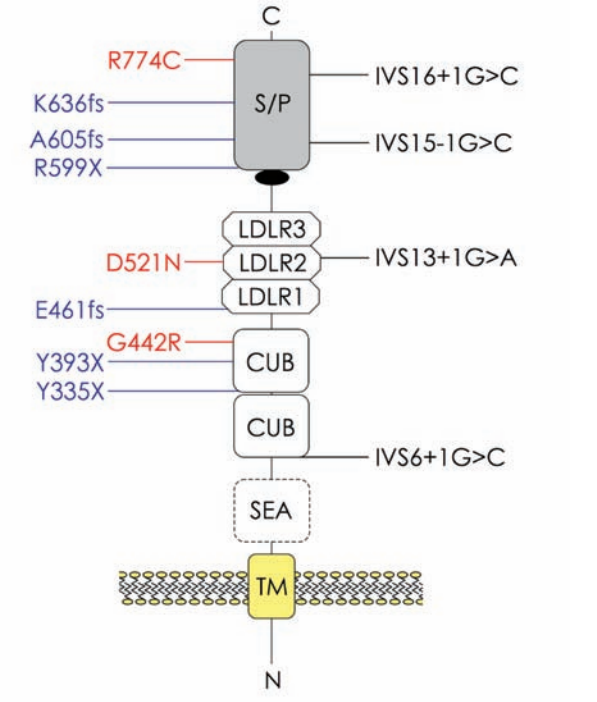

Figure 1. Schematic representation of the TMPRSS6 protein and of all the mutations described in patients.[19,20,28] N: amino-terminus, C: carboxy-terminus. TM: transmembrane domain. SEA: sea urchin sperm protein, enteropeptidase agrin. CUB: complement protein subcomponents C1r/C1s, urchin embryonic growth factor and bone morphogenetic protein 1 domain. LDLR: low density lipoprotein receptor class A domain. S/P: serine protease domain. Black oval: cleavage activation site. Missense mutations are shown in red, frameshifts and nonsense are in blue, splicing mutations are in black.

why a high iron diet reversed the phenotype of Mask mice.[18] The IRPs in the gut control the expression of both the luminal iron importer divalent metal transporter 1 (DMT1) and the basolateral iron exporter ferroportin.[29] Thus, a high intracellular iron level might in part counteract the effect of hepcidin on ferroportin degradation. However, it remains unclear why even parenteral iron cannot be efficiently utilized by IRIDA patients, while anemia and hair loss resolve upon iron treatment in both murine *Tmprss6*-deficient models.[18,30]

Molecular mechanisms of hepcidin inhibition

Hepcidin is inhibited by hypoxia, anemia, iron deficiency and erythropoietic expansion, all conditions that require iron for hemoglobin synthesis. The molecular mechanisms of hepcidin downregulation are not completely understood. HIF-1α has been shown to inhibit hepcidin in hypoxia.[31] s-HJV is an inhibitor of hepcidin that *in vitro* competes with m-HJV[12,16] and *in vivo* leads to increased iron absorption and iron overload.[15] Now the identification of *TMPRSS6* makes this picture even more complex. The effect of matriptase-2 within this scenario remains to be clarified. It can be hypothesized that matriptase-2 cleaves an activator or increases the expression of an inhibitor (Figure 2). It has been suggested that its mechanism of action may be downstream of SMAD;[18] studies are in progress to clarify this point. Growth differentiation factor 15 (GDF15), a member of the BMP/TGF-β superfamily is another candidate hepcidin inhibitor, since at a very high concentration it may partially suppress hepcidin transcription in primary hepatocyte cultures and in hepatoma cell lines.[32] GDF15, released in large amounts by the erythroblasts in patient sera, is presumed to contribute to hepcidin inhibition in thalassemic patients but it is still not clear whether it is the physiological erythroid regulator of hepcidin. J. Kanda *et al.* performed an *in vivo* physiological study of the relationship between serum hepcidin and erythropoiesis in the clinical setting of stem cell transplantation (SCT).[22] They monitored the pre- and post-SCT serum hepcidin levels together with other factors potentially affecting hepcidin expression. Four weeks after SCT, at a time when hepcidin levels are no longer related to the inflammatory cytokine IL-6, they found that serum hepcidin levels show a significant inverse correlation with markers of erythropoietic activity, such as the soluble transferrin receptor and the reticulocyte counts, but not with GDF15 levels. Although the sample size was relatively small and the analysis was performed at a single time point, these results exclude the possibility that GDF15 suppresses hepcidin expression in the SCT setting and suggest the existence of an unknown factor that negatively regulates hepcidin expression to allow iron absorption in physiological expansion of erythropoiesis.

Whether matriptase-2 could play a role during erythropoietic expansion is unknown. Further studies will clarify this point and its relationship to the other play-

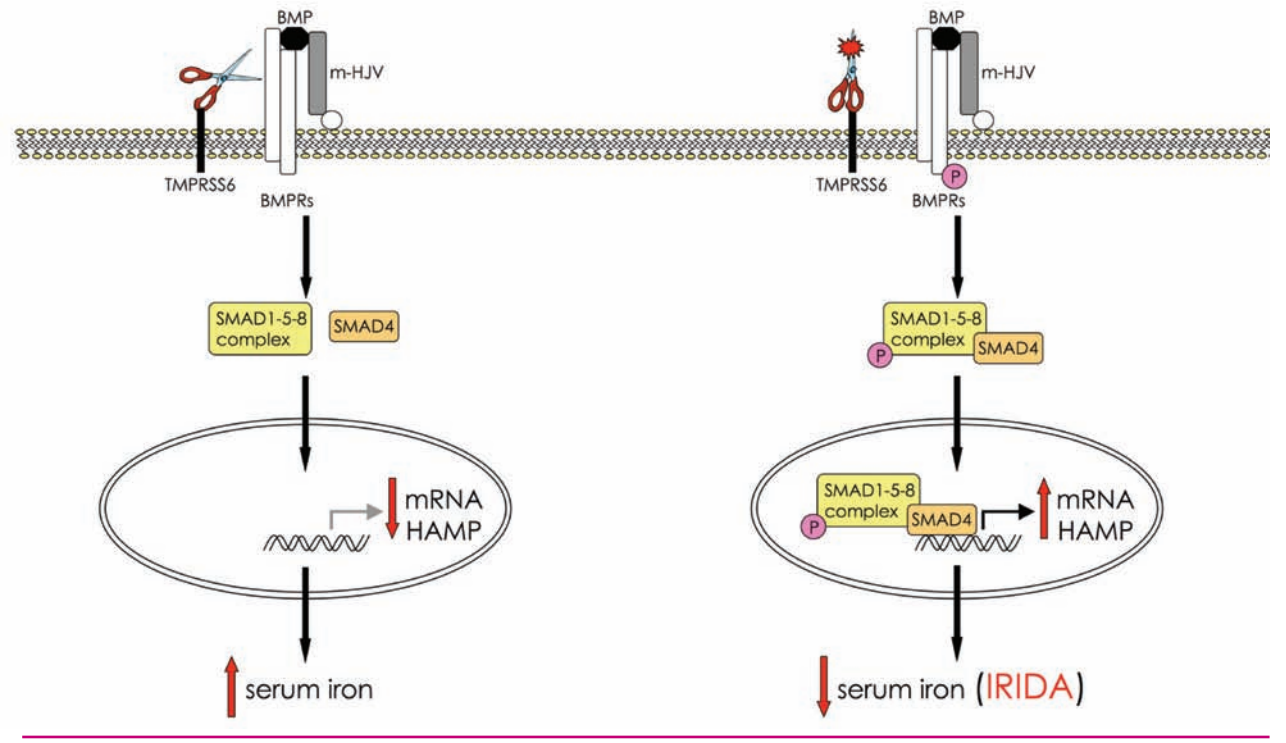

Figure 2. Hypothetical mechanism of *TMPRSS6* function related to the main hepcidin activation pathway represented by BMP, BPM receptors (BMPR) and HJV. Wild type *TMPRSS6* location on the plasma membrane is indicated by the scissors (open in iron deficiency) on the left. Mutant *TMPRSS6* in IRIDA is indicated by the closed scissors on the right. P indicates phosphorylation.

ers in the hepcidin pathway. In the meantime, clinicians have a new diagnostic option for patients that do not absorb iron, who are usually considered to be affected by acquired disorders of the gastrointestinal tract. Patients with matriptase-2 mutations will likely prove to be rare. However, common polymorphic changes in the gene might modulate *TMPRSS6* expression, with implications for hepcidin modulation and variations in iron absorption.

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