Into the matrix: regulation of the iron regulatory hormone hepcidin by matriptase-2

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Matriptase-2 is a recently identified membrane-bound, cell-surface serine protease expressed primarily in liver. Mutations in matriptase-2 in mice and humans cause iron-deficiency anemia that responds poorly to iron therapy. The poor response results from an inability to decrease hepcidin production during iron deficiency. Cell culture studies reveal that matriptase-2 inhibits hepcidin induction by cleaving membrane hemojuvelin, a potent activator of hepcidin transcription. As a novel suppressor of hepcidin expression, matriptase-2 emerges as a possible candidate for therapeutic interventions aimed at treating disorders of iron metabolism.

INTRODUCTION

Systemic iron balance is regulated chiefly by hepcidin, a 25-amino acid peptide produced by the liver and secreted into the circulation. Hepcidin controls the flux of iron into the plasma by binding to the cell-surface iron export protein, ferroportin, causing its rapid internalization and degradation in lysosomes.[1] The two main sites of hepcidin action are the duodenum and macrophages of the liver, spleen, and bone marrow (collectively known as the reticuloendothelial system, RES). In the duodenum, hepcidin induces the degradation of ferroportin located on the basolateral membrane of enterocytes, limiting the transfer of dietary iron into the portal blood. In RES macrophages, the hepcidin-mediated ferroportin down-regulation blocks cellular export of iron recycled from phagocytosis and degradation of old erythrocytes.[2] The overall net effect of hepcidin action is a decrease in plasma iron levels. If circulating iron concentrations remain low over time, an insufficient amount of the metal reaches the bone marrow, resulting in impaired hemoglobin synthesis and, ultimately, iron-deficiency anemia. In an effort to correct the anemia, the body normally responds by decreasing hepcidin synthesis. Low plasma hepcidin concentrations promote an increase in the cell-surface expression of ferroportin, which enhances dietary iron absorption and promotes efficient recycling of eryth-

rocyte iron by macrophages. However, in some types of anemia, hepcidin levels do not decrease. In the anemia of inflammation, for example, serum hepcidin concentrations are elevated because inflammatory cytokines, such as IL-6, increase hepcidin transcription.[3] A number of studies in the past year describe the identification and characterization of another form of anemia that manifests with normal to high hepcidin levels without inflammation. These independent studies, ranging from clinical reports, genetic analyses, knockout animals, and cell culture models, have converged to reveal a cell-surface protease known as matriptase-2 as a novel suppressor of hepcidin expression. This review will focus on the discovery of matriptase-2 as an essential regulator of iron homeostasis and how this protein participates in the signaling network that governs hepcidin production.

MATRIPTASE-2

Matriptase-2 was identified in 2002 by Velasco et al.,[4] who screened the human genome database for novel nucleotide sequences similar to those conserved in members of the TTSP protein family (type II transmembrane serine proteases). The protein was originally named TMPRSS6 (transmembrane protease, serine 6), but is now designated matriptase-2 based on its sequence homology and structural similarity to matriptase.[5] Although 20 distinct

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TTSPs have been described, few have been functionally characterized *in vivo*.[6] The most well-known TTSP family member, enteropeptidase, plays an essential role in digestion by cleaving the pancreatic zymogen trypsinogen into the digestive enzyme trypsin. TTSP proteins all share several structural features: a short N-terminal cytoplasmic tail, a transmembrane domain, a stem region consisting of one or more protein-interacting domains, an activation motif, and a catalytic serine protease domain. Matriptase-2, like many TTSP proteins, displays a very restricted tissue expression, suggesting tissue-specific functions. Northern blot analysis of 16 different human tissues found matriptase-2 transcripts only in liver.[4] In a survey of 12 mouse tissues, RT-PCR analysis identified most abundant matriptase-2 expression in liver, as well as detectable transcripts in kidney, uterus, and nasal cavity.[7] At the cellular level, matriptase-2 localizes to the plasma membrane with the proteolytic domain in the extracellular space.[7] The first functional analyses of matriptase-2 demonstrated that its catalytic domain could degrade several extracellular matrix proteins, including fibronectin, fibrinogen, and type I collagen.[4] These observations raised the possibility that matriptase-2, analogous to matriptase,[8] plays a role in matrix-degrading processes that occur in both normal and pathological states.

UNMASKING THE FUNCTION OF MATRIPTASE-2

A physiological function of matriptase-2 was established in 2008 by Du et al.[9] in studies of a mouse mutant that was generated from a screen of random mutations induced by N-ethyl-N-nitrosourea. The mutant displayed gradual hair loss that led to almost complete nudity except for hair on the face and head. The mutant strain, aptly named *mask*, was also found to be iron deficient, anemic, and infertile. The *mask* phenotype is dependent on iron deficiency, because intraperitoneal iron injection or dietary iron loading fully reversed the mutant's hair loss, infertility, and anemia. Radiotracer studies using intragastrically administered ^{59}Fe demonstrated that iron-deficient *mask* mice absorbed less iron than did iron-deficient control animals. Importantly, control animals reduced their hepcidin levels in response to iron depletion, whereas *mask* mice did not. These observations suggested that the anemia of *mask* mice resulted from an inability to downregulate hepcidin production in response to iron deficiency.

To identify the mutation responsible for the *mask* phenotype, mice were crossbred for linkage analysis, which mapped the mutation to a 1.3 Mbp region in chromosome 15. Positional cloning subsequently revealed a mutation in *matriptase-2*. The mutation, an A → G transversion, changes the wild-type sequence at the end of intron 14 from AG to GG, preventing normal splicing of

intron 14 and giving rise to a premature stop codon. The resultant matriptase-2 mutant protein lacks the serine protease domain. Du et al.[9] confirmed that the point mutation in matriptase-2 causes the *mask* phenotype by showing rescue of hair loss, infertility, and iron-deficiency anemia after transgenesis experiments in which the wild-type matriptase-2 gene was introduced into homozygous *mask* embryos. Additional confirmation was provided by the generation of *matriptase-2* knockout mice, which recapitulate the *mask* phenotype.[10]

IRON-REFRACTORY IRON-DEFICIENCY ANEMIA

In 2008, three papers reported genetic defects in human patients with an atypical form of iron-deficiency anemia.[11–13] This type of anemia, termed iron-refractory iron-deficiency anemia (IRIDA),[11] could not be corrected by oral iron supplementation or administration of parenteral iron. The individuals with IRIDA were of various ancestries (Turkish, Northern European, Sardinian, Nigerian, and African American) and most were first diagnosed in infancy or childhood. All affected subjects were found to have mutations in *Matriptase-2*. The mutant *Matriptase-2* alleles harbored missense, frameshift, or splice junction mutations. Most of these alterations affected or deleted the matriptase-2 serine protease domain. Two individuals had mutations exclusively in the matriptase-2 stem region,[11] implicating the involvement of the protein-interacting domains in normal matriptase-2 function. Similar to *mask* mice, patients with *Matriptase-2* mutations displayed normal to high levels of urinary hepcidin despite having iron-deficiency anemia. These studies therefore confirm the suppressive effect of matriptase-2 on hepcidin expression in humans.

REGULATION OF HEPCIDIN EXPRESSION AND THE ROLE OF MATRIPTASE-2

Serum hepcidin levels can change dramatically in response to a variety of stimuli: inflammation, iron status, erythropoiesis, and hypoxia. Normal serum hepcidin levels range between 30 and 300 ng/mL, whereas patients with inflammation typically have values around 600 ng/mL, with some as high as 4000 ng/mL.[14] In iron-deficiency anemia, serum hepcidin levels drop precipitously and become virtually undetectable. Much research now focuses on elucidating the molecular events that control hepcidin production, with a particular emphasis on transcriptional regulation.

The number of proteins that regulate hepcidin expression continues to grow.[15] The list includes key proteins involved in human disorders of iron overload: HFE, the hereditary hemochromatosis protein; hemojuvelin

(HJV), the juvenile hemochromatosis protein; and transferrin receptor 2, a putative iron sensor. Mutations in these proteins, or their genetic ablation, result in diminished hepcidin expression, indicating that they positively regulate hepcidin production. Signaling through the bone morphogenic protein (BMP) pathway has been shown to be a central axis for hepcidin regulation. BMPs (such as BMP2, 4, 6, or 9) are secreted soluble factors that interact with cell-surface BMP receptors, initiating an intracellular signaling cascade that activates hepcidin transcription.[16] In vivo, BMP6 seems especially important for iron homeostasis, because Bmp6-null mice display reduced hepcidin expression and iron overload.[17] Efficient BMP signaling through BMP receptor requires HJV, a 50-kDa protein with a glycosylphosphatidylinositol (GPI) anchor that tethers the protein to the extracellular surface of the plasma membrane. This membrane-bound hemojuvelin (m-HJV) is capable of binding BMPs, facilitating their association with the BMP receptor.[18] As such, m-HJV is often referred to as a BMP co-receptor. The potent contribution of m-HJV to BMP-mediated hepcidin activation is illustrated by mutations in HJV that abrogate cell-surface expression. Individuals with such mutations develop juvenile hemochromatosis, characterized by exceedingly low serum hepcidin concentrations (<5 ng/mL)[14] and severe hepatic iron overload. Cleavage of the GPI-anchor of m-HJV gives rise to a 42-kDa soluble form of HJV (s-HJV).[19] Unexpectedly, cell culture studies indicate that s-HJV originates not from cell-surface m-HJV, but from secretion of a cleaved form of intracellular HJV anchored to the endoplasmic reticulum.[20] S-HJV levels increase in iron deficiency due to an increase in furin, the enzyme that cleaves endoplasmic reticulum m-HJV. Studies in hepatocytes show that m-HJV on the plasma membrane and s-HJV exert opposite effects: m-HJV increases hepcidin expression, whereas s-HJV decreases it.[19] The reciprocal regulation arises from the fact that both m-HJV and s-HJV can bind BMPs. S-HJV inhibits BMP signaling by acting as a "decoy receptor", binding BMPs and limiting their interaction with m-HJV. Consistent with this model, injection of mice with s-HJV decreases BMP signaling and suppresses hepcidin expression.[21]

The discovery of matriptase-2 as a modulator of iron homeostasis adds yet another protein to the regulatory network of hepcidin expression. As noted above, studies of mask mice and humans with mutations in Matriptase-2 implicated the protease as a suppressor of hepcidin induction. To examine the effect of matriptase-2 on hepcidin transcription, Du et al.[9] performed luciferase reporter gene assays using a plasmid construct containing the hepcidin-promoter. In HepG2 cells, a human hepatoma cell line, transcription from the hepcidin promoter increased in response to various known hepcidin

activators (HJV, BMP2, BMP4, BMP9, or IL-6). When cells were transfected to express matriptase-2, the activators failed to elicit transcription. This observation is consistent with the hypothesis that matriptase-2 suppresses hepcidin transcription. No inhibition of transcription occurred in experiments using either mutant mask matriptase-2 or a wild-type matriptase-2 in which the catalytic serine in the protease domain was inactivated by point mutation. Thus, the suppression of hepcidin promoter activity depends on the proteolytic function of matriptase-2.

MATRIPTASE-2 AND HEMOJUVELIN

A key breakthrough in our understanding of matriptase-2 function came from detailed studies by Silvestri et al.,[22] who also used hepcidin promoter reporter constructs to interrogate the effect of hepcidin activators on hepcidin promoter activity. Similar to studies by Du et al.,[9] Silvestri et al.[22] found that transfection of a human hepatoma cell line (in this case HepB3 cells) with matriptase-2 prevented hepcidin reporter gene activation in response to HJV. Subsequent experiments examined the effect of matriptase-2 on the amount of cell-surface m-HJV as well as the amount of s-HJV released into the cell culture medium. Expression of matriptase-2 not only reduced the amount of m-HJV and s-HJV, but also resulted in the production of three soluble HJV fragments ranging in size from 25 to 30 kDa. Investigation of matriptase-2 proteolytic activity against a set of HJV variants and exogenous s-HJV indicated that the soluble HJV fragments originated from cleavage of m-HJV from the plasma membrane and not s-HJV. Whether the soluble fragments have any physiological function remains to be determined. The fragments arise from the proteolytic activity of matriptase-2, because concomitant expression of mask matriptase-2 had no effect on m-HJV or s-HJV expression. Concomitant immunoprecipitation experiments further demonstrated that HJV interacts with matriptase-2, suggesting that HJV and matriptase-2 form a complex. The cleavage of HJV by matriptase-2 may therefore involve direct binding of the two proteins. The interaction does not require the serine protease domain because HJV interacts with mask matriptase-2 as well. Collectively, these studies indicate that matriptase-2 cleaves cell-surface m-HJV, supporting a model in which matriptase-2 suppresses hepcidin expression by interfering with the hepcidin-activating pathway involving HJV and BMP signaling (Figure 1).

CONCLUSION

The identification of matriptase-2 as a suppressor of hepcidin expression and the demonstration of its interaction
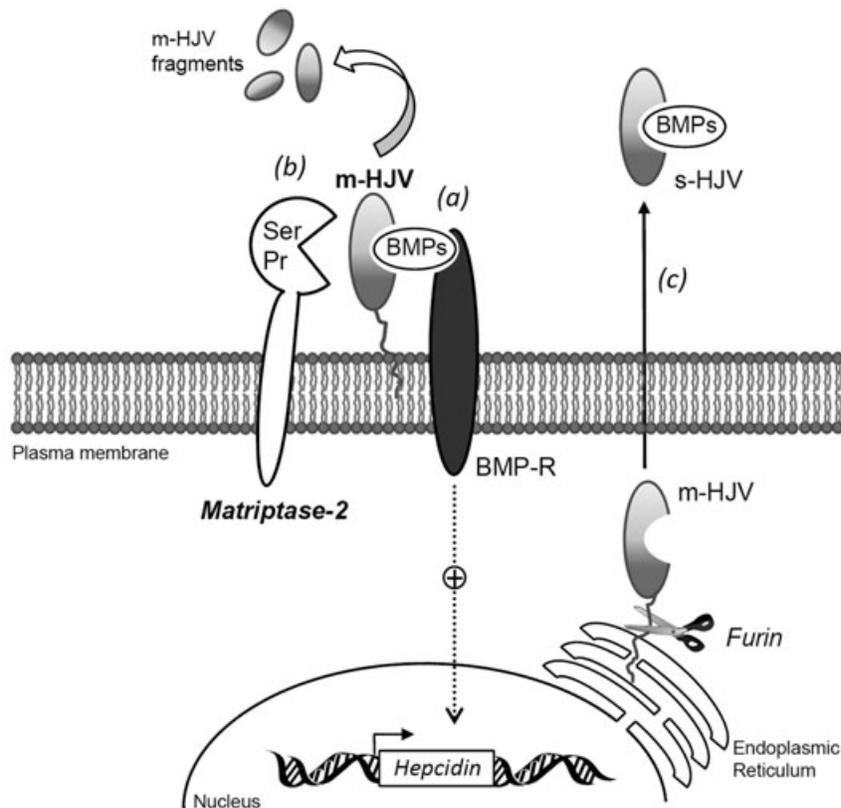
Figure 1 **Model of matriptase-2 suppression of HJV-associated BMP signaling and hepcidin expression.** (a) Membrane-bound HJV (m-HJV) at the cell surface is a potent activator of hepcidin expression. M-HJV functions as a co-receptor for bone morphogenic proteins (BMPs), positioning them to interact with their receptor (BMP-R). The association of mHJV, BMP, and BMP-R triggers an intracellular signaling cascade that increases hepcidin transcription. (b) Matriptase-2, a plasma-membrane protein with a serine protease (Ser Pr) domain, can suppress BMP signaling and hepcidin expression by cleaving m-HJV into small fragments. Matriptase-2 mutant proteins that lack the Ser Pr domain or proteolytic activity cannot cleave m-HJV, resulting in an inability to downregulate hepcidin. (c) Hepcidin expression can also be suppressed by soluble HJV (s-HJV), which originates from furin-mediated cleavage of intracellular m-HJV. S-HJV decreases BMP signaling by acting as a "decoy receptor" for BMPs, competing with m-HJV.

with HJV represent significant advances in our understanding of the regulation of iron metabolism. As with all important discoveries, these findings raise many questions. Do matriptase-2 levels change with iron status? What regulates matriptase-2 activity in response to iron deficiency? Does matriptase-2 cleave only HJV or does it have additional proteolytic substrates? What other proteins interact with matriptase-2? Can selective inhibition of matriptase-2 activity be used to treat disorders of iron overload? Answers to these questions and others are sure to provide exciting new insights into the dynamic interplay of proteins that control body iron homeostasis.

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