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Flatiron Mice and Ferroportin Disease

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Abstract

Flatiron mice provide the first genetic model that fully recapitulates the iron-loading disorder ferroportin disease. Unlike the other known genetic causes of hemochromatosis, missense mutations in the ferroportin gene are autosomal dominant. These new findings show that ferroportin disease results from dominant negative effects rather than haplo-insufficiency.

Keywords

iron transport; ferroportin; hemochromatosis

REGULATION OF IRON EFFLUX THROUGH FERROPORTIN AND HEPcidIN

The maintenance of iron homeostasis involves regulatory feedback mechanisms that govern intestinal iron absorption and macrophage iron release. In healthy adults, dietary iron uptake by duodenal enterocytes corresponds to the rate of obligatory iron loss due to sloughing of epithelial cells, bile secretion, and extravasation of erythrocytes. This results in a relatively small daily exchange of iron between the body and the environment (1–2 mg/d). Although steady-state iron levels are maintained by intestinal absorption, the majority of iron used for cellular demands such as hemoglobin synthesis (24 mg/d) is derived from iron recycled by macrophages of the reticuloendothelial system.¹

It is well-established that enterocytes of the intestinal mucosa and macrophages of the reticuloendothelial system acquire iron through different mechanisms. Dietary iron is transported across the apical surface of duodenal enterocytes by the import protein divalent metal transporter-1 (DMT1). In the reticuloendothelial system, macrophages acquire iron through phagocytosis and degradation of damaged or senescent red blood cells. Following uptake, iron can be stored in ferritin, used for metabolic purposes, or released. Despite different mechanisms of iron acquisition (Figure 1), enterocytes and macrophages release iron into plasma via a common export protein known as ferroportin (Fpn). To date, Fpn (IREG1/SLC40A1/MTP1) is the only known iron transport protein that functions to release iron into the circulation.^{2–5}

Iron efflux is regulated systemically through the interaction of Fpn with hepcidin, a 25-amino acid, cysteine-rich, liver-derived peptide. Although originally identified in urine and plasma during a search for novel antimicrobial peptides, hepcidin is now regarded as the fundamental hormone involved in maintenance of iron homeostasis.⁶ Hepcidin synthesis is up-regulated in response to iron loading, lipopolysaccharide, and inflammation, and is suppressed by anemia

and hypoxia.⁷⁻⁹ Elucidation of the molecular mechanism of hepcidin's role in iron metabolism occurred when it was discovered that circulating hepcidin binds to Fpn and leads to its internalization and degradation through endocytic trafficking to the lysosome¹⁰ (Figure 2).

TYPE IV HEREDITARY HEMOCHROMATOSIS OR FERROPORTIN DISEASE

Hereditary hemochromatosis (HH) is the most frequent form of inherited iron overload in Caucasians, afflicting approximately one in every 200 to 400 individuals. The Online Mendelian Inheritance in Man (OMIM) database currently divides HH into four categories based on inheritance, genetic mutations, and clinical manifestations of the disease. Types I, II, and III are autosomal recessive disorders caused by mutations in the HFE, hemojuvelin (HJV) [subtype a] and hepcidin (HAMP) [subtype b], and the transferrin receptor 2 (TfR2) genes, respectively. These three forms of HH are phenotypically similar. Patients present with a progressive increase in transferrin saturation accompanied by inappropriately low hepcidin levels. This can lead to systemic iron accumulation in parenchymal cells, particularly hepatocytes, cardiomyocytes, adrenal glands, and pancreatic β -cells. The current standard of treatment for these types of HH requires phlebotomy and/or iron chelation therapy to prevent organ damage due to iron overload.

Unlike the other known genetic causes of HH, missense mutations in the Fpn gene are autosomal dominant. To date, there have been no frameshift or nonsense mutations described in the characterization of FPN variants. The clinical manifestations of type IV hemochromatosis or “ferroportin disease” are variable but have been generally categorized into two groups. In the first, patients present with high ferritin levels, low-to-normal transferrin saturation, normal-to-high hepcidin, and macrophage iron loading. This is referred to as “classic” ferroportin disease. In the second form of this ironloading disorder, patients exhibit high transferrin saturation typically associated with other inherited forms of HH, along with iron accumulation predominantly in hepatocytes rather than macrophages. The latter patients respond well to the conventional treatments of phlebotomy and iron chelation; however, patients with the classic disease become anemic in response to phlebotomy and there is currently no known effective treatment for this type of HH.^{11, 12}

Functional consequences of several human FPN mutations have been analyzed in vitro. Most of the variants appear to be hepcidin resistant. However, these variants can be further separated into two classes (Figure 2). The first demonstrate impaired iron export activity and resistance to hepcidin due to an intracellular distribution. This results in low transferrin saturation and macrophage iron loading characteristic of classical ferroportin disease. The second form of FPN variants localize to the cell surface and exhibit normal iron export activity but have impaired hepcidin-induced degradation. These mutations confer a gain-of-function due to the loss of hepcidin regulatory control resulting in a phenotype similar to types I, II, and III HH.^{11, 12}

GENETIC MODELS OF TYPES I, II, AND III HEREDITARY HEMOCHROMATOSIS

Targeted deletions of HFE, HJV, HAMP, and TfR2 in the mouse all recapitulate the classical HH phenotype seen in humans. This has allowed for advancement in understanding the molecular basis for disease. As in human HH, hepcidin expression is significantly impaired in HFE, HJV, and TfR2 knockout mice. Based on the phenotypes of these mice, a unifying pathogenic model for HH has been proposed in which HFE, HJV, and TfR2 are independent but complementary regulators of hepcidin synthesis. Normally, these proteins serve as sensors that stimulate hepcidin synthesis and subsequent reduction of Fpn and iron efflux from enterocytes and macrophages. When one or more of these genes is lost, the Fpn-hepcidin

regulatory axis is disrupted, allowing for uncontrolled iron release and iron overload in organs. Current research in this area using these genetic models is aimed at defining the specific roles of HFE, HJV, and Tfr2 in sensing iron status and regulating hepcidin synthesis.¹³

GENETIC MODELS OF FERROPORTIN DISEASE (TYPE IV HEREDITARY HEMOCHROMATOSIS)

In vivo analysis of ferroportin disease began in the zebrafish. In 2000, Donovan et al.² used positional cloning to identify FPN as the gene responsible for hypochromic anemia in the *weissherbst* (*whe*) zebrafish mutant.² At the time, only two known alleles of the *whe* phenotype existed, and both exhibited a homozygous recessive pattern of inheritance. The *whe*^{Th238} allele carried a nonsense mutation and was thought to be a null allele. In contrast, the *whe*^{Trp85c} encoded a missense mutation in a conserved region of the Fpn gene later found to harbor missense mutations described in humans with ferroportin disease. Complete characterization of *whe*^{Trp85c/-} zebrafish revealed impairment in iron export from enterocytes and macrophages, but, unlike other models of HH, normal Fpn function was not required for regulation of hepcidin synthesis.^{2,14}

Although the zebrafish model provided significant insight into the function of Fpn, it did not recapitulate the human phenotype. Further modeling in the mouse demonstrated that the targeted deletion of FPN is lethal to the embryo. Donovan et al.⁴ determined that this lethality resulted from the inability of the embryos to transfer iron from the extra-embryonic visceral endoderm prior to placental formation.⁴ Although the homozygous FPNnull mice were not viable, the heterozygous animals fully developed. Interestingly, at 3 months, the heterozygous animals were phenotypically identical to controls. By 6 months, there was some evidence that iron transport was disrupted in these animals. Although not anemic, the animals presented with decreased cell volume and hemoglobin in erythrocytes, suggestive of iron-restricted erythropoiesis. As in the human disease, the heterozygous mice also displayed significant iron loading in macrophages of the spleen. However, liver iron stores were unexpectedly depleted throughout development up to one year in the heterozygous animals compared with controls. This is inconsistent with the progression of human ferroportin disease and led Donovan et al.⁴ to conclude that the FPN heterozygous-null mice did not provide a genetic model faithful to the human disease. However, the heterozygous-null mouse model does present strong evidence that the human disease cannot be explained simply by a loss-of-function in the mutant allele.

In order to preclude embryonic lethality and to study the role of Fpn in adult tissues, Donovan et al.⁴ also created a conditional FPN-knockout mouse using the Cre-Lox recombinate system. Postnatal conditional knockout resulted in hypochromic anemia and severe iron deficiency in erythrocytes. Examination of tissues known to express Fpn demonstrated iron overload in duodenal enterocytes, indicating a failure to release absorbed dietary iron. In the conditional knockout, splenic and liver macrophages (Kupffer cells) and hepatocytes accumulated much more iron than controls 12 days after birth, consistent with progression of the human disease. However, over time, the liver iron content of the conditional knockouts decreased similar to the phenotype demonstrated in the heterozygous-null animals. Donovan et al.⁴ suggest that this phenotype results from the inability of the conditional knockout mice to further deposit iron in the liver as they are growing, which causes a decrease in iron liver stores.

Donovan et al.⁴ then generated a mouse model with intestinal-specific knockout of FPN. These mice presented with severe iron-deficient anemia with marked accumulation of iron in duodenal enterocytes. The nonheme iron content in the spleen and liver was significantly reduced in these animals. Intestinal-specific knockout of FPN impaired dietary iron absorption. However, the spleen and liver did not retain iron, which is indicative of an intact response mechanism to iron depletion. Parenteral treatment of the intestinal knockout mice with iron

dextran reversed the iron-deficient anemia demonstrating the significant role of Fpn in intestinal iron absorption.⁴

The careful analysis of FPN knockout, FPN heterozygous-null, and tissue-specific FPN knockout mice unequivocally demonstrated that Fpn is the protein responsible for iron export in the extra-embryonic visceral endoderm, the intestine, and macrophages. These studies also provided a biological mechanism of how hepcidin deficiency seen in other types of HH leads to systemic iron overload. In the absence of hepcidin, iron egress from duodenal enterocytes and macrophages via Fpn is unregulated, resulting in progressive iron overload.

FLATIRON MICE FULLY RECAPITULATE FERROPORTIN DISEASE

Although none of the mouse models discussed above fully recapitulated human ferroportin disease, the data suggest that human mutations act through a mechanism other than loss-of-function or haploinsufficiency, such as disruptions of protein-protein interactions required for iron export. A recent report by Zohn et al.¹⁵ has now finally confirmed this hypothesis by characterizing a missense mutation that recapitulates human ferroportin disease in a mouse model. This “flatiron” mutation was identified in a screen for ethylnitrosurea-induced mutations that affected embryonic formation and was mapped to chromosome 1. Further sequencing determined a single nucleotide point mutation in the Fpn gene that leads to a H32R substitution in the first putative transmembrane domain of the iron exporter. Embryos homozygous for this mutation showed severe anemia and midgestational lethality. However, heterozygous animals are viable and develop the phenotypic characteristics of human ferroportin disease. At 6 months of age, serum ferritin levels in the heterozygous animals were increased by as much as 10-fold compared with controls. There was also a concomitant decrease in transferrin saturation. The heterozygotes display some hypochromic erythrocytes and target cells consistent with a mild anemic state. Tissue analysis of aged mice revealed accumulation of iron in Kupffer cells of the liver, which was not achieved in previous knockout models.¹⁵

Characterization of the mechanism by which the animals develop the disease was initiated by in vitro analysis of the flatiron mutation. Wild-type and Fpn(H32R), both tagged with green fluorescent protein (GFP), were exogenously expressed in HEK293T cells. Using fluorescence microscopy, the wild-type protein was localized to the plasma membrane, while the H32R mutant remained intracellular. Incubation of these cells with ferric ammonium citrate revealed that the ability of the mutant to export iron was significantly impaired, most likely due to its mislocalization. Importantly, coexpression of the H32R mutant with wild-type Fpn resulted in the intracellular localization of both proteins, suggesting a dominant negative effect of the mutant allele. This feature of ferroportin disease was confirmed using macrophages isolated from flatiron mice. Following iron treatment, Fpn is upregulated and localized to the cell surface of macrophages isolated from wild-type animals. In contrast, Fpn remains intracellular in the flatiron macrophages. Although there is debate as to whether Fpn exists as a monomer or in a multimeric state, this study clearly shows that the presence of the flatiron allele reduces the cell surface localization of the wild-type protein in a dominant negative fashion. Using RNAi, Zohn et al.¹⁵ also demonstrated that there is no iron transport deficiency in macrophages until over 70% of Fpn is lost, further supporting their observations and those of Donovan et al.⁴ that ferroportin disease is the result of a dominant negative effect rather than haploinsufficiency.

The work by Zohn et al.¹⁵ has provided the first definitive mouse model of classical ferroportin disease. This model has provided much needed insight into the mechanism of disease progression describing the dominant negative impairment of Fpn localization. Given that there are limited treatment options for humans afflicted with this disease, the flatiron mouse provides the first tool to explore the complete pathophysiology of the disease as well as potential

treatment options. Continuing research with the flatiron, HFE, HJV, and TfR2 transgenic mice is the first step in developing a comprehensive nutritional model in which iron sensing, assimilation, and regulation via the ferroportin-hepcidin axis can be completely understood.

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Figure 1.

Iron efflux through ferroportin. Dietary iron is transported across duodenal enterocytes after it is imported by divalent metal transporter-1 (DMT1) at the apical surface. Macrophages of the reticuloendothelial system acquire iron through phagocytosis of senescent red cells. Despite different mechanisms of iron acquisition, both enterocytes and macrophages release iron through the common exporter, ferroportin.

Figure 2.

Regulation of ferroportin by hepcidin. Hepcidin, a circulating peptide induced by iron loading, infection, or inflammation, binds to ferroportin. Interaction with hepcidin triggers the internalization and lysosomal degradation of the exporter. In ferroportin disease, genetic variants of ferroportin appear to be hepcidin resistant and fall into two classes. The first have impaired export activity due to intracellular mislocalization, resulting in low circulating iron levels and macrophage iron-loading (classic ferroportin disease). The second group localize to the cell surface but fail to bind hepcidin or become internalized such that iron is constitutively released. These gain-of-function mutations result in a phenotype similar to types I, II, and III hereditary hemochromatosis.