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The use of hypotransferrinemic mice in studies of iron biology

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Abstract

The hypotransferrinemic (hpx) mouse is a model of inherited transferrin deficiency that originated several decades ago in the BALB/cJ mouse strain. Also known as the hpx mouse, this line is almost completely devoid of transferrin, an abundant serum iron-binding protein. Two of the most prominent phenotypes of the hpx mouse are severe anemia and tissue iron overload. These phenotypes reflect the essential role of transferrin in iron delivery to bone marrow and regulation of iron homeostasis. Over the years, the hpx mouse has been utilized in studies on the role of transferrin, iron and other metals in a variety of organ systems and biological processes. This review summarizes the lessons learned from these studies and suggests possible areas of future exploration using this versatile yet complex mouse model.

Keywords

Transferrin; Mouse; Hypotransferrinemia; Iron; Metal; Hpx

Intro

The hypotransferrinemic (hpx) mouse is a genetic model of extreme pathophysiology. Nearly devoid of endogenous transferrin, these mice require treatment with exogenous transferrin prior to weaning to ensure their survival to adulthood. In those mice that do survive, profound transferrin deficiency results in severe chronic anemia and progressive iron loading in non-erythropoietic tissues. Over the past several decades, this mouse line has been used in a multitude of studies. While some have focused on aberrant uptake or distribution of metals such as manganese, aluminum, gallium, cadmium, copper and zinc (Dickinson et al. 1996; Radunovi et al. 1997; Takeda et al. 1998; Raja et al. 2006; Herrera et al. 2014), most studies have employed this mouse model to study transferrin-dependent and -independent pathways of iron metabolism. As such, we will focus on transferrin and iron in this review. We first discuss transferrin and the initial establishment and characterization of the mouse line. We then describe more recent studies dissecting the mechanisms underlying aberrant iron homeostasis in hpx mice, and conclude with a

description of findings in various tissues and organs and possible avenues for future research.

Transferrin

Transferrin is an abundant serum protein of roughly 80 kDa synthesized mainly by the liver (Mizutani et al. 2012). It consists of an N- and C-lobe connected by a short loop (Fig. 1). Each lobe consists of two domains (N1 and N2; C1 and C2), with one atom of ferric iron (Fe^{3+}) coordinated by four protein ligands and one carbonate anion in the cleft between the two domains in each lobe. Transferrin binds iron with high affinity at physiologic pH and lower affinity under acidic conditions. Binding of ferric iron to transferrin maintains ferric iron in a soluble form and minimizes its redox activity. Transferrin can exist in monoferric and diferric forms.

Cellular uptake of transferrin is mediated by transferrin receptor, a membrane protein expressed on many cell types including erythroid precursors (Gkouvatsos et al. 2012). Diferric transferrin has a higher affinity for transferrin receptor than do monoferric transferrin or apo (iron-free) transferrin. Binding of transferrin to transferrin receptor is followed by internalization of the transferrin–transferrin receptor complex, endosomal acidification, release of iron from transferrin and transfer of iron into the cell. Most transferrin-bound iron is delivered to the bone marrow. In contrast, nontransferrin-bound iron (NTBI), a redox active form of iron, is cleared largely by the liver by a mechanism that is most likely transferrin receptor-independent.

Initial characterization of the hypotransferrinemic mouse

First described by Bernstein in 1987, the hypotransferrinemic mouse line, also known as hpx, originated during routine breeding of the BALB/cJ laboratory mouse strain. Affected mice are distinguishable at birth by pallor and runted growth and have very low circulating levels of serum transferrin electrophoretically indistinct from wild-type transferrin (Bernstein 1987). Mutant mice invariably die before weaning unless they are treated with a source of exogenous transferrin or red blood cells. Effective sources include red blood cells from wild-type mice, serum from healthy mice, rabbits and humans and purified transferrin. Alleviation of disease severity correlates with dosages of specific treatments. Heterozygous mice do not require treatment to survive. Hpx mice that do survive past weaning age exhibit a severe microcytic, hypochromic anemia with pronounced reticulocytosis. The profound anemia highlights the essential role for transferrin in iron delivery to the bone marrow.

Although transferrin injections are essential in mice before they are weaned, treatment of mice with exogenous transferrin after they are weaned is not essential for their survival—survival up to 9 months has been reported (Trenor et al. 2000). This is a key point to consider when interpreting studies on hpx mice, given that most but not all research groups administer low doses of transferrin to weaned mice throughout the respective study periods. While the source of transferrin used to correct the inherent deficiency in these mice differs from study to study, most investigators treat hpx mice with some amount of transferrin throughout the life of the mice, while others treat only prior to weaning. In this manner, mice in the former studies may be best described as transferrininsufficient, while mice in the

latter studies are best described as nearly transferrin-deficient. Whether or not this difference impacts the interpretations of various studies remains at the discretion of the reader. Another issue to consider is the difference in mouse chow used from study to study, which may modify the observed phenotype of affected mice (Malecki et al. 2000).

The profound anemia observed in untreated mutant mice is accompanied by severe tissue iron overload, the extent of which is unmatched by most other mouse models of inherited iron overload. Tissue iron overload is attributed to hyperabsorption of dietary iron, detectable as early as 1 week, which can be reversed by correction of anemia by interventions such as red blood cell transfusions (Kaplan et al. 1988; Buys et al. 1991; Raja et al. 1994). In heterozygotes, iron deposits in similar tissues as in mutants, though at later age points (Bernstein 1987). Tissue iron stores in hpx mice exist in a variety of ultrastructural forms: the multi-protein subunit complex known as ferritin, ferritin degradation aggregates known as hemosiderin or membrane-enveloped collections of hemosiderin known as siderosomes (Iancu et al. 1995).

Identified early on as an autosomal recessive mutation (Bernstein 1987), the underlying, spontaneously arisen mutation in hpx mice was eventually identified as a point mutation in a splice donor site in the transferrin gene, resulting in aberrant transcript splicing (Huggenvik et al. 1989; Trenor et al. 2000). While the mature transferrin transcript is 2.5 kb, missplicing from cryptic donor splice sites produces a 5 kb transcript detectable in both heterozygous and homozygous mutant mice. At steady state, hpx mice express 5 % mature transferrin mRNA and <1 % serum transferrin protein relative to wild-type mice. Heterozygous mice, though not anemic, express roughly half the normal levels of serum transferrin. Despite the decreased levels of transferrin in serum, the transferrin protein expressed in hpx mice exhibits largely normal structure and stability in vitro (Bernstein 1987; Trenor et al. 2000). The transferrin protein expressed from the hpx allele carries a deletion of nine amino acids near the C-terminus of the protein (Fig. 1).

⁵⁹Fe tracer studies have been employed to investigate iron trafficking in hpx mice. Distribution of administered iron throughout the body is dependent upon route of administration (intravenous, oral or subcutaneous), the form of administered iron (iron salt or transferrin-bound) and the age of mice (neonatal or adult). Administered ⁵⁹Fe salts deposit largely in the liver of adult hpx mice, while they are observed in a wider distribution in wild-type mice including bone marrow and red blood cells (Craven et al. 1987; Bernstein 1987; Kaplan et al. 1988; Bradbury et al. 1994; Dickinson et al. 1996). ⁵⁹Fe bound to transferrin deposits in a pattern in adult hpx mice similar to that observed in wild-type mice (Bernstein 1987). In contrast, ⁵⁹Fe salts accumulate largely in the liver of neonatal hpx mice but also in bone marrow (Takeda et al. 2002). Overall, these studies demonstrate that transferrin is not required for gastrointestinal iron absorption or distribution to non-erythropoietic organs.

Given the near complete transferrin deficiency in hpx mice, the predominant form of circulating iron in hpx mice is, by definition, non-transferrin bound iron (NTBI). Notably, measured levels of NTBI in hpx serum differ depending upon the assay used (Simpson et al. 1991b, 1992; Herrera et al. 2014). The profound tissue iron overload observed in hpx mice

is attributed to tissue uptake of NTBI, implying that NTBI uptake is mediated by pathways distinct from those that mediate uptake of transferrin-bound iron. The profound anemia observed in hpx mice also implies that NTBI uptake is insufficient to meet erythropoietic demands for iron. Possible mechanisms of NTBI uptake in hpx mice are discussed below.

Further studies on iron metabolism in hypotransferrinemic mice

The massive tissue iron overload suggested dietary iron hyperabsorption in untreated hpx mice. Increased duodenal iron absorption in hpx mice has been demonstrated using laser Doppler fluxmetry (Raja et al. 1995). Studies utilizing tied-off duodenal sections also demonstrate both enhanced iron absorption (Raja et al. 1994) and enhanced maximal rate of iron uptake in hpx mice similar to that observed in wild-type mice rendered hypoxic or iron-deficient (Simpson et al. 1991a). These studies indicate that transferrin deficiency alters gastrointestinal iron absorption directly or indirectly. The mechanism underlying this phenomenon would become more clear with the discovery of key iron regulatory factors.

Early studies on the reversibility of the hypotransferrinemic phenotype indicated that the regulation of iron absorption by transferrin is complex. Intraperitoneal transfusion of packed red blood cells normalizes hematocrit, reticulocyte count and the rate of iron absorption in mutant mice (Buys et al. 1991). This suggests that the anemia and/or hypoxia in hpx mice stimulate iron absorption. When maintenance treatments with exogenous transferrin in hpx mice are discontinued and circulating transferrin is permitted to turn over, rates of iron absorption increase even without significant changes in hemoglobin or hepatic iron levels (Raja et al. 1999). This result suggests that transferrin can affect iron absorption independently of its role in erythropoiesis.

The mechanism underlying the severe tissue iron overload in hpx mice became clearer once hepcidin and its role in iron homeostasis were discovered. Hepcidin is a peptide hormone synthesized predominantly by the liver (Ganz 2013). It is abundant under conditions of iron excess and inflammation and suppressed under conditions of anemia and hypoxia. Hepcidin regulates systemic iron homeostasis by inhibiting dietary iron absorption and reticuloendothelial macrophage iron efflux. It acts by binding to the cellular iron exporter ferroportin, expressed in duodenal enterocytes and macrophages, and inducing its internalization and degradation. The finding that hpx mice are deficient in hepcidin and that this deficiency corrects after treatment with exogenous transferrin (Weinstein et al. 2002; Bartnikas et al. 2011) explains, at least in part, the hyperabsorption of dietary iron and the ensuing tissue iron overload observed in untreated mutant mice. While the transport proteins responsible for dietary iron absorption in hpx mice have not been demonstrated experimentally, the expression of genes of known relevance to duodenal iron transport has been analyzed. RNA levels of the cellular iron exporter ferroportin, protein levels of the cellular iron importer DMT1 and activity levels of the ferric reductase duodenal cytochrome b are all increased in hpx duodenum (McKie et al. 2000; Canonne-Hergaux et al. 2001; McKie et al. 2001).

The secondary deficiency of hepcidin in hpx mice can currently be attributed to several mechanisms. First, hpx mice express low levels of transferrin receptor 2 (Tfr2), a component

of the signaling pathway that stimulates hepcidin expression in conditions of iron overload (Robb and Wessling-Resnick 2004). The decreased Tfr2 protein levels in hpx mice are most likely a direct reflection of transferrin deficiency, as diferric transferrin is essential for Tfr2 stability (Johnson and Enns 2004). Second, the anemia and/or hypoxia resulting from transferrin deficiency suppress hepcidin expression by the liver. Intraperitoneal transfusion of hpx mice with wild-type red blood cells increases hemoglobin levels and liver hepcidin levels (Bartnikas et al. 2011). Third, transferrin is essential for stimulation of hepcidin expression by the liver under conditions of iron overload. In hpx mice treated with the myeloablative agent doxorubicin, treatment with exogenous transferrin increases liver hepcidin levels without increasing hemoglobin levels (Bartnikas et al. 2011).

Hepcidin expression in hpx mice has also been shown to require the bone morphogenetic protein co-receptor hemojuvelin (Hjv). Hjv is mutated in juvenile forms of hereditary hemochromatosis, an inherited disorder of iron overload due to hepcidin deficiency (Ganz 2013). Treatment of hpx mice deficient in Hjv with exogenous transferrin fails to increase liver hepcidin levels, indicating that Hjv is essential for transferrin-dependent hepcidin expression (Bartnikas and Fleming 2012). Intraperitoneal transfusion of hpx mice deficient in Hjv with wild-type red blood cells also fails to substantially increase liver hepcidin levels, indicating the anemia and/or hypoxia in untreated hpx mice inhibits hepcidin expression by acting on the Hjv-hepcidin signaling pathway.

Pathophysiology

The effects of hypotransferrinemia have been examined in multiple organ systems.

Liver

Prussian blue staining for iron deposits first demonstrates iron accumulation in hepatocytes (Bernstein 1987). Livers in older mutant mice display lymphocyte-rich inflammatory infiltrates and prominent iron accumulation in both hepatocytes and Kupffer cells (Simpson et al. 1993; Trenor et al. 2000) with periportal and pericanalicular iron distributions (Iancu et al. 1995). Gomori's trichrome stain for reticulin fibers suggests that the liver is fibrotic, while elevated serum aspartate aminotransferase levels in hpx mice suggest liver damage (Simpson et al. 1993). Ultrastructural and laser microprobe mass analysis demonstrates ferritin particles and siderosomes in hepatocytes at all ages and in macrophages in older mice and aggregates similar to non-membrane-bound hemosiderin in bile canaliculi of older mutant mice (Iancu et al. 1995).

Pancreas

The pancreas shows macrophage infiltration and region-specific iron accumulation. Acinar, centroacinar and intercalated duct cells exhibit iron loading while islets of Langerhans are spared (Simpson et al. 1993; Trenor et al. 2000).

Spleen

Hpx mice display prominent splenomegaly (Simpson et al. 1993; Raja et al. 1994), with an increased ratio of white to red pulp, but decreased iron concentration (Iancu et al. 1995).

Splenomegaly results from extramedullary hematopoiesis, with the decreased spleen iron concentration most likely reflecting two processes: First, red cells recycled by splenic macrophages are iron-deficient. Second, hepcidin deficiency leads to unregulated ferroportin-mediated iron efflux from splenic macrophages.

Heart

Hpx mice develop cardiomegaly (Simpson et al. 1993) and iron accumulation in cardiomyocytes (Kaplan et al. 1988; Iancu et al. 1995; Trenor et al. 2000).

Kidney

Iron deposition occurs mainly in the renal tubules in the corticomedullary junction (Kaplan et al. 1988; Trenor et al. 2000).

Immune system

Hpx mice have been used to demonstrate that transferrin is essential for early T cell differentiation in vivo (Macedo et al. 2004) and to suggest a role for cytokines in mediating transferrin synthesis in macrophages (Djeha et al. 1995).

Skeletal system

Hpx mice have also been used to demonstrate that transferrin is essential for bone mineralization (Malecki et al. 2000).

Small intestine

While enterocytes are spared from iron loading, ferritin accumulation has been observed in myocytes and enteric nerve endings of the small intestine (Iancu et al. 1995).

Lungs

In hpx mice, hypotransferrinemia increases resistance to hyperoxia-induced lung injury, possibly due to increased pulmonary ferritin and lactoferrin levels (Yang et al. 1999; Ghio et al. 2000). Upregulation of ferroportin RNA and protein expression is also observed in hpx lungs (Yang et al. 2002).

Adrenal gland

Iron accumulates in medullary cells and in subcapsular cells of the adrenal cortex, but not in other cortical cells (Kaplan et al. 1988; Trenor et al. 2000).

Central nervous system

Both wild-type and hpx adult mice demonstrate similar ^{59}Fe levels after subcutaneous $^{59}\text{FeCl}_3$ injections (Dickinson et al. 1996), while another tracer study demonstrated increased brain ^{59}Fe levels in neonatal hpx mice with ^{59}Fe localized largely to the ventricular system (Takeda et al. 2001). Adult hpx mice exhibit decreased amounts of white matter and neurofilament staining, altered neuronal morphology in brains and spinal cords relative to wild-type mice (Dickinson and Connor 1994) and distinct patterns of iron staining in hippocampus (Dickinson and Connor 1995). Other abnormalities noted in hpx

mice include normal structure and iron levels yet abnormal function and gene expression in the retina (Lederman et al. 2012). Overall, studies suggest that there are transferrin-independent mechanisms for both brain iron uptake (Ueda et al. 1993; Dickinson and Connor 1995; Takeda et al. 2001; Beard et al. 2005) and for cell-specific iron uptake within the brain (Takeda et al. 1998). However, transferrin may play a role in distribution of iron within the brain (Dickinson and Connor 1994, 1995, 1998; Takeda et al. 2001).

Future directions

Our understanding of transferrin's role in iron homeostasis has advanced considerably since the first description of the hpx mouse line (Fig. 2). However, many questions remain regarding the mechanism by which hypotransferrinemia leads to the hpx phenotype. Answering these questions will not only improve our understanding of hypotransferrinemic patho-physiology but also our understanding of mammalian iron biology overall. Here we propose several lines of research.

What factors are responsible for hepcidin suppression in untreated hpx mice?

Likely tissue sources of such factors include liver and bone marrow. Two liver-derived proteins, Bmper and Atoh8, may contribute. Bmper, or bone morphogenetic protein (BMP)-binding endothelial cell precursors-derived regulator, is a BMP-binding protein that has been shown to stimulate and inhibit BMP signaling dependent on the context of activity. Bmper is abundantly expressed in hpx mice; injection of Bmper peptide into wild-type mice leads to decreased liver hepcidin levels (Patel et al. 2012). Atoh8 is an iron-regulated transcription factor, the levels of which are decreased in hpx liver and increased in wild-type mice treated with diferric transferrin (Patel et al. 2014). Bone marrow-derived factors include twisted gastrulation (Twsg1), growth differentiation factor 15 (Gdf15) and erythroferrone (Erfe). Identified by gene expression profiling of erythroblasts, Twsg1 and Gdf15 both inhibit hepcidin expression in vitro (Tanno et al. 2007, 2009). Erfe contributes to suppression of hepcidin in a mouse model of β -thalassemia intermedia, an inherited anemia characterized by inappropriately low hepcidin expression and iron overload (Kautz et al. 2014).

Do factors other than hepcidin contribute to dietary iron hyperabsorption in hpx mice?

Hypoxia-inducible factors (HIFs) are transcription factors essential for the regulation of iron homeostasis in conditions of hypoxia. For example, HIF-2 regulates expression of DMT1 and ferroportin (Mastrogiannaki et al. 2013) and may mediate, at least in part, the hyperabsorption of dietary iron observed in hpx mice.

What factors are responsible for NTBI uptake in untreated hpx mice?

As hpx mice are nearly devoid of transferrin, the progressive tissue iron overload observed in hpx mice reflects NTBI uptake in liver and other tissues. While essential for iron uptake by the gut and erythroid precursors, divalent metal transporter 1 (Dmt1) is not essential for liver iron overload in hpx mice (Wang and Knutson 2013). ZRT/IRT-like protein 14 (Zip14) is a strong candidate for an NTBI uptake protein, as it is abundantly expressed in hpx liver (Nam et al. 2013) and can mediate cellular uptake of iron (Jenkitkasemwong et al. 2012).

How does hypotransferrinemia impact the inflammatory response to infection or other stresses?

Iron homeostasis and inflammation are intimately related (Ganz 2013). The effect of various characteristics of hpx mice—anemia, hypoxia, tissue iron loading and dysregulation of iron homeostasis, for example—on the establishment, progression and resolution of infections from different pathogens has yet to be examined in hpx mice.

Why do the phenotypes of transferrin-deficient and transferrin receptor-deficient mice differ so strikingly?

In contrast to the near complete deficiency of transferrin observed in hpx mice, genetic ablation of transferrin receptor is embryonically lethal in mice (Levy et al. 1999). As previously speculated, in transferrin receptor-deficient mice, iron may be sequestered by transferrin and therefore be unavailable for uptake by erythroid precursors by transferrin-independent mechanisms (Trenor et al. 2000). In hpx mice, NTBI levels are high and may be sufficient to permit iron uptake by these transferrin-independent mechanisms. Alternatively, the low levels of circulating transferrin in hpx mice may be adequate to supply enough iron for erythropoiesis, thereby permitting embryonic development. The difference in phenotype between transferrin and transferrin receptor deficiency may also reflect an unidentified yet essential function of transferrin receptor independent of its role in transferrin-dependent iron uptake.

What can the hpx mouse model tell us about maternal-fetal iron transfer?

To our knowledge, this subject has yet to be approached. ⁵⁹Fe tracer studies, gene expression analysis and tissue histology could be very informative as to the mechanisms of transferrin-dependent and -independent nutrient transfer to developing embryos.

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Fig. 1. Model of transferrin structure. Transferrin consists of two iron-binding lobes, designated N and C, connected by a short loop (*white*). Each lobe consists of two domains (N1 and N2 in *light* and *dark green* respectively and C1 and C2 in *pink* and *red* respectively). One atom of iron (*gray sphere*) is coordinated by four amino acid residues and one carbonate anion in a cleft between the two domains in each lobe. The nine amino acid residues deleted near the carboxy terminus of the murine hpx protein are shown in *yellow*. Shown here is human diferric transferrin, PDB 3V83, visualized using SwissPDB Viewer. Residues absent from hpx transferrin were mapped onto human transferrin by sequence alignment between NP_001054 (human) and NP_598738 (mouse) using Clustal Omega

Fig. 2.

Model of transferrin's role in systemic iron homeostasis. Dietary inorganic iron is absorbed in the duodenum. In wild-type mice, circulating iron exists largely as diferric transferrin ($\text{Fe}_2\text{-TF}$), which plays at least two essential roles: It stimulates hepcidin expression by the liver and it delivers iron to bone marrow for synthesis of red blood cells (RBCs). In hpx mice, circulating iron exists largely as non-transferrin-bound iron (NTBI), a redox active form. Incapable of stimulating hepcidin expression or delivering iron to bone marrow, NTBI accumulates in multiple organs including liver, heart, pancreas and brain. Dietary iron absorption continues unabated in hpx mice because of secondary hepcidin deficiency, attributed to suppression of hepcidin expression by unidentified factors secreted by bone marrow and lack of stimulation of hepcidin expression by transferrin. Not shown here is the spleen, where iron is reclaimed from senescent or damaged RBCs and delivered to transferrin for re-use