IRON-REGULATORY FUNCTION OF HEPCIDIN IN THE CHANNEL

CATFISH AND WESTERN CLAWED FROG

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IRON-REGULATORY FUNCTION OF HEPCIDIN IN THE CHANNEL CATFISH AND WESTERN CLAWED FROG

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DISSERTATION ABSTRACT

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Hepcidin, originally identified as a 25 amino acid (aa) antimicrobial peptide made in the liver, is a small peptide hormone that has been shown to be the long-sought regulator of iron metabolism in humans and mice. Although closely related hepcidin genes and peptides have been identified in other mammals, amphibians and various fish species, it is still largely unknown whether hepcidin can regulate iron metabolism in fish and amphibians. In the current study, it was found that in channel catfish hepcidin transcript levels in the liver were increased by 4, 19, and 22 fold at 4, 24, and 48 hours post Edwardsiella ictaluri challenge, respectively. However, augmented hepcidin expression in the gut and olfactory sac was detected only at 48 h post infection. In naturally occurring anemic fish, the concentration of serum iron, total iron binding

capacity, and liver iron content were half of that in healthy controls. The levels of hepcidin transcript in the livers of catfish affected by anemia were only 14% of that of healthy catfish. Correlation analysis indicated that hepatic hepcidin transcript levels correlated significantly with serum iron concentrations ($r = 0.54$, $P < 0.05$) and with the percent saturation of transferrin ($r = 0.63$, $P < 0.05$). In Xenopus tropicalis study, we demonstrated for the first time that hepcidin may regulate iron metabolism in amphibians. Two hepcidin genes were identified in the X. tropicalis frog but only tHEP2 was responsive to iron loading, indicating a possible role of tHEP2 in the regulation of iron homeostasis. In contrast, tHEP1 was most likely involved in the host defense in response to corticosterone. The study on the promoter areas of the two hepcidin genes supports such notions. These preliminary data suggested that X. tropicalis could be a promising animal model which allows us to separate some features of the regulation of hepcidin expression at the transcriptional level. The phylogenetic study revealed the co-evolution of hepcidin and its receptor ferroportin. Multiple Sequence Analysis (MSA) demonstrated that charged residues (E, D, H, K, and R) within external segments (ESs) are most likely involved in the hepcidin-ferroportin interaction. In this study, highly conserved external segments (ES1-4, and ES6) were predicted to play a critical role during the binding between hepcidin and ferroportin. On the other hand, the most variable external segment ES5 was proposed to be responsible for the endurance of ferroportin to hepcidin variation.

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LIST OF ABBREVIATIONS

AMP	antimicrobial peptide
AP-1	activating protein 1
BMP	bone morphogenetic protein
C/EBP	CCAAT/enhancer-binding protein
CCA	channel catfish anemia
cDNA	complementary deoxyribonucleic acid
DCYTB	duodenal cytochrome B
DMT-1	divalent metal transporter 1
EST	expressed sequence tag
FPN	ferroportin
GPCR	G protein coupled receptor
HBD-1	human beta-defensin 1
HD-5	human defensin 5
HIF	hypoxia inducible factor
HJV	hemojuvelin
HNF	hepatocyte nuclear factor
IRE	iron regulatory element
IRP	iron regulatory protein

LEAP-1	liver expressed antimicrobial peptide 1
LPS	lypopolysaccharide
MCR-1	melanocortin 1 receptor
MMP-7	matrix metalloproteinase 7
mRNA	messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
NF-κB	nuclear factor kappa B
ORF	open reading frame
ROS	reactive oxygen species
SMAD4	mothers against decapentaplegic homolog 4
STAT3	signal transducer and activator of transcription 3
TF	transferrin
TFR2	transferrin receptor 2
TIBC	total iron binding capacity
USF2	upstream stimulating factor 2

CHAPTER I. INTRODUCTION

Antimicrobial peptides (AMPs, also called host defense peptides) are an evolutionarily conserved component of innate immunity and are found in all classes of life, including bacteria, insects, plants, and vertebrates. AMPs are potent, broad spectrum antibiotics which demonstrate potential use as novel therapeutic agents. They have been extensively studied for more than two decades for their various roles in innate immunity and their potential application as new antibiotics. The first plant antimicrobial peptides, thionins, were isolated in the 1970s (Fernandez de Caleya et al., 1972), while the first AMPs in animals are the cecropins isolated from the insect cecropia moth (Steiner et al., 1981). The first AMPs in humans are the α-defenses isolated from neutrophils (Selsted et al., 1985). So far, there are more than 900 AMPs from plants and animals listed in a database in Trieste (http://www.bbcm.univ.trieste.it/~tossi/pag1.htm). Based on their structures, AMPs can be classified into three major groups (Boman, 1998; Bulet et al., 2004): 1) Linear peptides without Cys, often amphipathic helices (cecropins, magainins, etc.); 2) Cyclic and open-ended cyclic peptides with pairs of cysteine residues (defensins, protegrin, etc.); and 3) Linear peptides with a high proportion of one or two amino acids (proline rich, histidine rich, etc.). This dissertation will focus on hepcidin, a recently discovered AMP from humans, which has four disulfide bonds belonging to group 2.

These gene-encoded and naturally occurring AMPs are the major effectors of innate immunity, forming a first line of host defense against pathogens (Boman, 2003).

Most AMPs display hydrophobic and cationic properties, have a molecular weight below 25-30 kDa (15-45 amino acid residues), and adopt an amphipathic structure (α-helix, β-hairpin-like β-sheet, β-sheet, or α-helix/β-sheet mixed structures) which is believed to be essential to their anti-microbial action. The precise details of their mechanism of action remain unknown. However, it is generally accepted that the positively charged peptides act directly on the negatively charged cellular membranes of bacterial cells, causing an increase in membrane permeability, which then leads to rapid cell death (Zasloff, 2002). The outer membrane of higher eukaryotes is made of electrically neutral phospholipids like phosphatidylcholine and sphingomyelin, whereas bacterial membranes have exposed negatively charged phosphatidylglycerol and cardiolipin (Matsuzaki, 1999). Though some AMPs kill bacteria without permeabilization of the membrane but inhibiting the synthesis of specific membrane proteins or DNA (Boman et al., 1993; Carlsson et al., 1998), or breaking single stranded DNA (Bateman et al., 1991), their selectivity of killing is based on the different characteristics between membranes of eukaryotic and bacterial cells.

Most AMP genes are constitutively expressed whereas many others are also inducible. For example, the cecropins were originally discovered after induction of antibacterial activity by vaccination of Cecropia pupae with live non-virulent bacteria (Boman et al., 1972). The breakthrough in the regulation of AMPs was made by Sun & Faye (Sun and Faye, 1992) showing that all immune genes in the Cecropia moth were controlled by a transcription factor (Cif) which is very similar to the mammalian NF-κB discovered 6 years earlier (Sen and Baltimore, 1986). In mammals as well as in insects there is a family of NF-κB-like transcription factors which are homo- or heterodimeric

Rel-containing proteins. These factors, all termed IκB, are maintained in the cytosol as long as they are tightly complexed with one member of a family of proteins with ankyrin repeats. Once liberated from the inhibitor, NF-κB is translocated into the nucleus where it will recognize and bind to an upstream element, inducing gene expression. Such elements have been identified in the genes of most inducible antimicrobial peptides (Zasloff, 2002; Boman, 2003). Glucocorticoid treatment of the frog Rana esculenta inhibits the transcription of all genes encoding antibacterial peptides by inducing the synthesis of IκB alpha (Simmaco et al., 1997), verifing this NF-κB pathway in frog. Another pathway, namely imd (immune deficiency), was also suggested to be involved in the regulation of AMPs (Lemaitre et al., 1995).

In addition to destroying microbes, AMPs have been discovered to have many other functions during last two decades. As a review of the literature, AMPs have demonstrated at least eight different biological functions in vivo.

1. Antimicrobial Activity. This function is what the AMPs are named for. AMPs have potent activities against a broad range of microorganisms including gram-positive and -negative bacteria, fungi, parasites, and enveloped viruses, making them an attractive option for the replacement of some conventional antibiotics. The first human disease directly linked to the malfunction of an antimicrobial peptide is cystic fibrosis, a genetic disease associated with recurrent bacterial infection and inflammation. A defective chloride channel causing the disease increases the salinity of the alveolar fluid and thus impairs the bactericidal activity of human beta-defensin-1 (HBD-1) (Goldman et al., 1997), causing patients to become frequently infected by Pseudomonas aeruginosa. Mouse β-defensin-1 and CRAMP (cnlp) gene in knockout mice are more susceptible to

infections and fail to clear the pathogens (Nizet et al., 2001; Moser et al., 2002). On the other hand, overexpression of human defensin 5 (HD-5) protected mice against enteric salmonellosis (Salzman et al., 2003) and overexpression of the porcine cathelicindin PR-39 enhanced resistance and protection from group A Streptococcus (Lee et al., 2005b). Some AMPs such as defensins also possess antiviral activity (Braida et al., 2004; Howell et al., 2004; Feng et al., 2006). Fungi Candida species (spp) are a significant clinical problem for a variety of immunoincompetent patients worldwide. Alpha defensins and histatins demonstrated good efficacy against C. albicans (Selsted et al., 1985; Lehrer et al., 1988; Xu et al., 1991). In the early studies, AMPs such as magainins and cecropins also showed antiprotozoan activity (Gwadz et al., 1989; Huang et al., 1990).

2. Anti-inflammatory activity. After initial infection, the host innate immune system mounts an acute inflammatory response to destroy invaders. However, the loss of control for this inflammation is frequently the cause of some autoimmune diseases. Besides directly destroying microbes, AMPs also take part in the inflammatory process hence playing an important role in many autoimmune diseases, including psoriasis, arthritis, respiratory diseases, and inflammatory bowel disease (Zaiou, 2007). It was reported that AMPs such as LL-37 and magainin can prevent LPS from binding to the carrier protein lipopolysaccharide-binding protein, or alternatively to its receptor, inhibiting inflammatory cytokine secretion (Scott et al., 2000; Rosenfeld et al., 2006). Using matrix metalloproteinase-7-deficient (MMP-$7^{-/-}$) mice, Shi et al demonstrated that alpha-defensins may play an important role in intestinal homeostasis by controlling the production of IL-1beta by macrophages (Shi et al., 2007).

3. Immuno-modulatory activities. AMPs are not only major effectors but also important regulators of innate immunity. Meanwhile, more and more evidence demonstrated that AMPs are also potent inducers of adaptive immunity. Defensins and PR-39 act as chemoattractants for monocytes and neutrophils, respectively (Territo et al., 1989; Huang et al., 1997), which enhance the innate immunity. Moreover, defensins influence the production of several cytokines. In monocytes, TNFα and IL-1 expression is stimulated by defensins (Chaly et al., 2000); while in lung epithelial cell defensins enhance the expression of IL-8 (Van Wetering et al., 1997). As a linker between innate and adaptive immunity, defensins were shown to induce migration of human naïve T cells and immature dendritic cells (Yang et al., 1999; Yang et al., 2000). The accumulated data has shown that defensins and cathelicidins are the two major families that demonstrated most immuno-modulatory activities (Yang et al., 2002; Bals and Wilson, 2003).

4. Anti-tumor Activity. In comparison to the membrane of normal human keratinocytes, there is 3~7-fold higher phosphatidylserine content in that of melanoma and carcinoma cells (Utsugi et al., 1991). Such differences can result in higher susceptibility of tumor cells to membrane permeabilitzing AMPs. Several reports have demonstrated that AMPs are emerging as a promising class of new natural drugs with antitumor ability. Magainin II was shown to exert a cytotoxic effect against a wide range of cancer cell lines including melanoma, breast and lung cancers, as well as lymphomas and leukemias (Baker et al., 1993; Soballe et al., 1995). More recently, magainin II was reported to exert cytotoxic and antiproliferative effect by pore formation in bladder cancer cells but did not affect normal murine or human fibroblasts (Lehmann et al., 2006).

Cecropins, insect-derived cationic peptides, were also found to be effective in specific lysis of tumor cells (Moore et al., 1994). It was also reported that bovine lactoferricin inhibited liver and lung metastasis of both murine melanomas and lymphomas (Yoo et al., 1998) and induced apoptosis in human leukemia and carcinoma cell lines (Mader et al., 2005). Furthermore, α-defensins were found to be associated with lung tumors, renal cell tumors and bladder cancer (Bateman et al., 1992; Muller et al., 2002; Holterman et al., 2006).

5. Wound repair and angiogenesis. When the skin epithelial barrier function is breached, a wound repair process requires the recruitment of numerous cell types including inflammatory cells (Zaiou, 2007). Neutrophils and macrophages are among cells that invade wound areas and constitute the major source of several angiogenic growth factors and AMPs. It was reported that the expression of LL-37 was increased in wounded skin (Dorschner et al., 2001). Besides protecting the injured tissue from infection, AMPs also play an important role in the tissue angiogenesis involving the wound healing. Using in vitro and in vivo models for wound healing, Koczulla et al have identified another novel biologic activity of human LL-37, which consists of induction of angiogenesis and arteriogenesis (Koczulla et al., 2003). Interestingly, the porcine cathelicidin PR-39 has been shown to induce a robust angiogenic response in both in vivo and in vitro experimental models (Li et al., 2000).

6. Mitogenic activity. AMPs can also stimulate cell proliferation by acting as mitogens. Murphy et al provided the first evidence by showing that in a concentration range required for antimicrobial activity, defensins stimulated growth of fibroblasts and epithelial cells in vitro (Murphy et al., 1993). A similar study (Aarbiou et al., 2002)

showed that neutrophil defensins at 4-10 mg/ml enhanced proliferation of the A549 lung epithelial cell line whereas they decreased cell proliferation at higher concentration. Muller and colleagues further demonstrated that these neutrophil derived defensins stimulated the proliferation of renal carcinoma cells (RCCs) at lower concentration by increasing DNA synthesis (Muller et al., 2002). All these data suggest that neutrophil defensins may possibly be involved in epithelial repair by inducing cell proliferation.

7. Pigmentation. A recent Science paper revealed that a beta-defensin plays a role in pigmentation (Candille et al., 2007). The beta-defensin, CBD103, binds to melanocortin 1 receptor (MCR1), which belongs to the G protein coupled receptor (GPCR) superfamily involved in pigmentation, inflammation, and feeding behavior. Mutant CBD103 has higher production and affinity than wild-type peptide for the dog MCR1, which controls the production of black/brown hair pigment eumelanin. Mutant CBD103 competes with agouti signaling protein (hair with a yellow stripe) for binding with MCR1, resulting in higher production of eumelanin and black hair. Interestingly, the authors also observed that transgenic mice overexpressing the dog beta-defensin have lower body weight, indicating this defensin may also play a role in feeding behavior.

8. Iron Metabolism. So far, only one family of AMPs, hepcidin, was found to be involved in iron metabolism. In this dissertation, I will set up a non-mammalian animal model for the study of hepcidin and will also evaluate the structure and function of hepcidin with an evolutionary perspective.

As we have already appreciated, AMPs are far from being a simple peptide family, as they own versatile biological functions. Though some of them are specialized and complement specific functions beyond antimicrobial activity, we still could find some

common characteristics among them which will help us understand their structure and function. For instance, hepcidin has been already identified as iron-regulatory hormone but we still can group it as an AMP based on its size, structure, and antimicrobial activities. In this dissertation, I will focus on the iron regulatory function of hepcidin in fish and frog, and will also study the structure and function of hepcidin and ferroportin from lower vertebrates (fish and frog) to mammals.

CHAPTER II. LITERATURE REVIEW

THE DISCOVERY OF HEPCIDIN

Hepcidin, originally identified as a 25 amino acid peptide antibiotic predominantly produced in the liver, has turned out to be a key iron regulator in humans and mice. Hepcidin was independently discovered by two groups searching for novel antimicrobial peptides from human fluids (Krause et al., 2000; Park et al., 2001). Krause et al (2000) purified a 25-residue peptide containing four disulfide bonds from human plasma ultrafiltrate, designating it LEAP-1 (Liver-Expressed Antimicrobial Peptide). LEAP-1 mRNA was predominantly detected in the liver and in the heart with a much lower level by real time PCR, and the peptide demonstrated potent activity against bacteria and yeast. Meanwhile, Park et al (2001) isolated the same peptide with three forms (hep20, hep22, and hep25) differing in N-terminal truncation from human urine during an investigation of the antimicrobial properties of various body fluids, designating it hepcidin (hepatic bactericidal protein). Using Northern blot analysis, the authors demonstrated that hepcidin mRNA is expressed in the fetal and adult liver only. Upon its discovery, the researchers showed high enthusiasm for hepcidin. Very quickly, hepcidins have been found from other mammals such as mouse, rat, and pig, and many fish species such as white bass, medaka, rainbow trout, Japanese flounder, winter flounder, long-jawed mudsucker, Atlantic salmon, zebra fish, and channel catfish. The wide distribution of hepcidin in the vertebrates greatly enlarged our view on the structure and functions of

this small peptide.

More interestingly, hepcidin was found to be involved in iron metabolism. This new role of hepcidin was demonstrated by two groups independently. While searching for new genes up-regulated by iron loading, Pigeon et al isolated a 225-base pair mouse hepcidin cDNA by suppressive subtractive hybridization performed between livers from carbonyl iron-overloaded and control mice (Pigeon et al., 2001). Besides experimentally iron-overloaded mice, the authors further found that mouse hepcidin mRNA was overexpressed in livers from spontaneously iron-overloaded mice (β2-microglobulin knockout mice). Another group also from France unexpectedly found massive iron overload in the liver and pancreas from the Usf2 (-/-) mice (Nicolas et al., 2001). To identify genes that may account for the abnormalities of iron homeostasis in Usf2 (-/-) mice, the authors isolated a cDNA encoding mouse hepcidin using suppressive subtractive hybridization between livers from Usf2 (-/-) and wild-type mice. Their following studies confirmed that it is hepcidin but not USF2 that is involved in iron homeostasis (Nicolas et al., 2002a). The authors demonstrated that transgenic mice overexpressing hepcidin in the liver have decreased body iron stores and at birth are affected by severe microcytic hypochromic anemia. On the other hand, targeted disruption of hepcidin gene in mice (Hamp1-/- mice) causes severe iron overload (Viatte et al., 2005). In humans, Weinstain and colleagues (2002) firstly suggested the link between hepcidin and iron metabolism by demonstrating that inappropriate expression of hepcidin in the liver is associated with iron refractory anemia (Weinstein et al., 2002). It has now been found that dysregulation of hepcidin is responsible for anemia of

inflammation and most forms of hereditary hemochromatosis disorders (Andrews and Schmidt, 2007).

HEPCIDIN STRUCTURE AND ACTIVITY

The hepcidin gene contains three exons that encode an 84-amino-acid preprohepcidin with a signal peptide and two introns. This gene structure is conserved in vertebrates from fish to mammals. Mass spectrometry and circular dichroism spectroscopy demonstrated that the hep25 contains four disulfide bonds and two beta-sheets (Park et al., 2001; Hunter et al., 2002). The nuclear magnetic resonance spectroscopy showed that hepcidin forms a simple hairpin, with three disulfide bonds stabilizing the antiparallel strands and a fourth bond linking adjacent Cys in the turn (Hunter et al., 2002). The high degree of disulfide bonding stabilizes hepcidin in the circulation, but the removal of individual bonds did not have a significant impact on hepcidin iron-regulatory function in vitro (Nemeth et al., 2006). When compared with hep25, the truncated peptides (hep20 and hep22) display much reduced iron-regulatory activity (Rivera et al., 2005; Nemeth et al., 2006) and are probably the products of degradation of the 25-amino-acid form.

Like other AMPs, hepcidin also has an amphipathic structure with hydrophobic residues distributed on the convex side and positively charged residues on the concave side (figure 1.1). Although both hep20 and hep25 peptides demonstrated potent antimicrobial activity at a very high (30μM) concentration, it is unlikely that hepcidins exert antimicrobial activity in human urine (Nemeth and Ganz, 2006). This is because the hepcidin concentrations in human urine are usually much lower, normally in the 3~30

nM range. Since the concentrations of hepcidin in other tissues including plasma and liver have not been measured, its direct antimicrobial activity in vivo remains to be established (Shi and Camus, 2006). On the other hand, the iron-regulatory activity of hepcidin is performed at 100-fold lower concentrations than those required for its antimicrobial activity (Nemeth et al., 2004b; Rivera et al., 2005). Particularly, the iron-regulatory activity is exhibited only by hep25 (Nemeth et al., 2005).

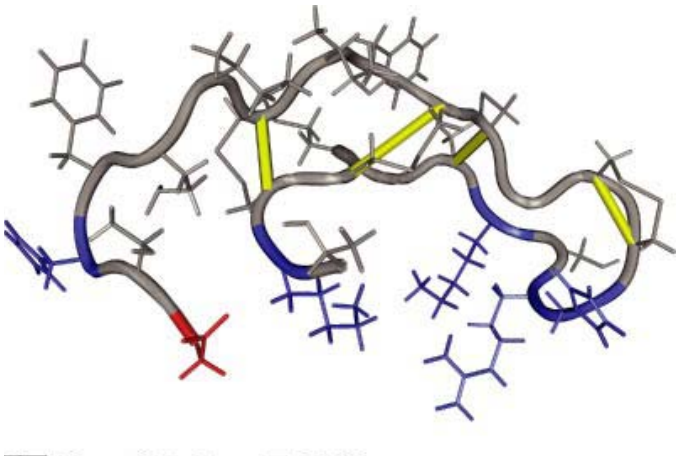


Figure 1.1. Nuclear magnetic resonance structure of hepcidin. The backbone and side chains are shown, with positively charged residues in blue, negatively charged in red, and disulfide bonds in yellow. The model clearly shows the spatial segregation of charged residues in the peptide and the unusual vicinal disulfide bond in the turn. Adapted from Nemeth and Ganz (2006).

In this dissertation, I am focusing on the iron-regulatory activity of hepcidin. The goal is to determine whether this new function is conserved in the lower vertebrates (fish,

frog, and chicken). Before we go further, it is necessary to have a brief talk on iron metabolism and its regulation mechanisms.

IRON METABOLISM

Iron is an essential element for nearly all living organisms. It has many physiological functions such as oxygen transport, storage molecules and enzyme components, various metabolic intermediates and host defense. However, excess free iron promotes the formation of damaging oxygen radicals that attack cellular lipids, proteins and nucleic acids. The average adult human contains 2~4 g of iron (Andrews, 2005; Nemeth and Ganz, 2006). More than half of the body's iron in normal individuals is found in hemoglobin of blood erythrocytes and their precursors. Approximately 25% of the iron is maintained as storage iron (ferritin and hemosiderin) mainly in the liver. The remainder exists in myoglobin, reticuloendothelial macrophages, and heme enzymes. In humans, about 20~25 mg of iron is the daily requirement for adults to produce new erythrocytes, most of which comes from recycling of iron already in the body. Only 1~2 mg a day needs to be absorbed from the dietary food to replace the normal iron losses (around 1~2 mg per day). The tissues and cells that generate major iron flows into the blood plasma include the duodenal enterocytes involved in dietary iron absorption, hepatocytes that are the main site of iron storage, macrophages that recycle iron from senescent red blood cells, and the placental syncytiotrophoblast involved in iron transfer from mother to fetus during pregnancy (Hentze et al., 2004; Andrews, 2005; Nemeth and Ganz, 2006). Since there is no active mechanism for iron excretion through the liver or kidneys, iron losses occur primarily through bleeding and sloughing of mucosal and skin

epithelial cells (Nemeth and Ganz, 2006). As a result, iron homeostasis must be tightly regulated; otherwise it will end up with iron disorders such as iron deficiency anemia and iron overload disorders (hemochromatosis).

Iron Absorption

Normally, mammals obtain iron exclusively from the diet (Ganz and Nemeth, 2006). Inorganic iron presents in a wide variety of foodstuffs but the absorption is inefficient. In contrast, heme iron primarily from animal sources can be more efficiently absorbed (Anderson et al., 2005). Both inorganic and heme iron are taken up by duodenal enterocytes in humans. In the enterocytes, the uptake of iron is performed by a cooperation of a ferric reductase (duodenal cytochrome B, DCYTB) and a divalent metal transporter 1 (DMT1) (Anderson et al., 2005; Donovan et al., 2006). As most dietary non-heme iron is in the ferric (Fe^{3+}) form, it must be firstly reduced to ferrous (Fe^{2+}) iron which is more easily absorbed (Anderson et al., 2005). The ferric iron is reduced into its ferrous form by the ferric reductase DCYTB; and then the ferrous iron transporter DMT1 moves iron across the cell membrane to enter the enterocytes (Anderson et al., 2005). Mice lacking DCYTB (*Cybrd1*-/-) do not develop an iron deficient phenotype, suggesting that another mechanism for iron reduction may exist (Gunshin et al., 2005b). On the other hand, targeted mutation of DMT1 in mice (*Slc11a2*-/- mice) demonstrated that DMT1 plays a critical role in the intestinal iron absorption and erythroid iron uptake (Gunshin et al., 2005a). Heme iron seems to be transported intact from the gut lumen into the enterocytes, but the process is less well understood (Anderson et al., 2005).

Once inside the enterocyte, part of the iron is oxidized back to the ferric state and bound to the ferritin for storage (Andrews and Schmidt, 2007). The remaining iron is transferred across the basolateral surface into the circulation. The major molecule that carries iron across the membrane is ferroportin (FPN; also known as SLC40A1, IREG1, and MTP1) (Ganz and Nemeth, 2006). As the sole known iron exporter, ferroportin is highly expressed in duodenal enterocytes, hepatocytes, macrophages and placental cells (Nemeth and Ganz, 2006). The critical role of ferroportin in intestinal iron absorption and macrophage iron release was confirmed in ferroportin knockout mice (Donovan et al., 2005). Similar to DMT1, ferroportin likely conducts ferrous (Fe2+) irons. However, Fe2+ must be oxidized to Fe3+ before it can enter the circulation and load onto plasma transferrin. Thus another ferroxidase protein, hephaestin in enterocytes or ceruloplasmin in macrophages, is also required for cellular iron export (Ganz, 2005a; Chen et al., 2006). As mentioned above, iron absorption must be tightly regulated to maintain iron balance because humans cannot excrete excess iron other than by bleeding.

Iron Trafficking

Transferrin (TF), an abundant and high-affinity iron-binding protein, is the primary iron transporter in the plasma (Anderson et al., 2007; Atanasiu et al., 2007). TF is a beta globulin and is produced in liver. Each molecule of transferrin can bind and transport two molecules of iron in the ferric (Fe^{3+}) state. In the circulation, TF carries nearly all serum iron while very small amounts of iron may be loosely associated with other proteins including albumin (Andrews and Schmidt, 2007). TF saturation rapidly responds to local circumstances and is usually used as an indicator of body iron status.

Under normal circumstances, approximately one third of the transferrin is bound with iron (Beutler, 2006; Donovan et al., 2006). In mice, the TF saturation is much higher ranging from 60% to 80% (Andrews and Schmidt, 2007). Transferrin mainly carries iron to the marrow but also to other organs if the marrow is damaged or excessive amounts of iron are already stored in the marrow (Andrews, 2005). Once bound to the cell membrane, the transferrin changes shape and releases the iron. It then returns to the portal circulation to bind more iron.

Iron Utilization

Precursor erythroid cells, hepatocytes, macrophages, placental cells, and most other cells in the organism can take up the transferrin-bound iron from circulation using transferrin receptors (TFRs) (Donovan et al., 2006; Ganz and Nemeth, 2006). As we know, the erythroid bone marrow is the largest consumer of iron. Normally, around two-thirds of the body iron is found in developing erythroid precursors and mature red blood cells (Donovan et al., 2006). Erythroid precursors express cell-surface TFRs that take up Fe-TF by receptor-mediated endocytosis (Hentze et al., 2004). Although TFRs are widely expressed, most other cells apparently can use non-TFR mechanisms to assimilate iron. However, the utilization of iron by muscle cells and other cell types such as neuron cells is far less known (Andrews and Schmidt, 2007). Targeted disruption of the murine TFR gene (Trfr-/- mice) causes embryonic lethality because of severe anemia (Levy et al., 1999a). No human mutations in TFR have yet been identified, but it was reported that antibodies against TFR in a patient resulted in severe anemia (Larrick and Hyman, 1984).

Once iron leaves the endosome, it must move to the mitochondrion for incorporation into protoporphyrin IX by ferrochelatase to form heme. Though heme biosynthesis begins and ends in the mitochondrion, the intermediate steps occur in the cytoplasm (Ponka, 1997). Mitoferrin (also called SLC25A37) carries out mitochondrial iron import (Shaw et al., 2006). The authors demonstrated that zebrafish and mice lacking mitoferrin fail to incorporate erythroid iron into heme. Most heme in erythroid precursors is used for hemoglobin production (Donovan et al., 2006).

Iron Storage

Two cell types are important for systemic storage of iron (Ganz, 2005a; Ganz and Nemeth, 2006): macrophages and hepatocytes. Macrophages can recover iron from dying erythrocytes and store it (see iron recycling). Hepatocytes can acquire both TF-bound and non-TF-bound iron from the plasma and also have a large capacity for iron storage. Inside the cells, iron storage occurs primarily in ferritin which can frequently be found within lysosomes (Beutler, 2007). Each ferritin molecule can accommodate up to 4,500 iron atoms in the ferric (Fe^{3+}) state. The iron complex with ferritin is water soluble and the ferritin is believed to be involved in the regulation of iron uptake by the cells (Andrews and Schmidt, 2007). Another iron-storage complex, hemosiderin, also deposits iron in the ferric (Fe3+) state but in a water insoluble form (Chen et al., 2006). Hemosiderin is most commonly found in macrophages and is especially abundant in situations following hemorrhage (Ganz, 2005a). Deposition of iron in hemosiderin can cause organ damage and has been found to be associated with several diseases (Chen et al., 2006).

Iron Recycling

Due to lack of regulated mechanism for iron excretion, iron absorption is limited. Each day less than 0.1% of the total body iron (1~2 mg) enters the circulation through intestinal absorption (Andrews and Schmidt, 2007). And most circulating iron comes from senescent erythrocytes recycled by macrophages. Tissue macrophages recognize old and damaged erythrocytes (particularly in the spleen), phagocytizing and removing them from circulation (Donovan et al., 2006). Within the macrophages the erythrocytes are lysed and the hemoglobin is degraded (Ganz, 2005a). Heme oxygenase catalyzes the release of iron from heme. The amount of iron daily recycled by macrophages approximates the amount needed for erythropoiesis (about 20 mg). Some iron remains in macrophages while another portion is exported to blood plasma (Ganz, 2005a). Ferroportin (FPN) is critical for macrophage iron export and involved in the regulation of the ratio between stored and released iron (Donovan et al., 2005; Ganz, 2005a).

REGULATION OF IRON METABOLISM

Iron balance must be strictly maintained to ensure that adequate amounts of iron are available for vital functions and to avoid the toxicity that results from iron excess. Iron metabolism can be regulated during its absorption, transport, utilization, storage, and recycling processes (Ganz, 2005a). As mentioned above, the uptake of ferric iron in enterocytes is carried out by a ferric reductase which reduces iron to its ferrous (Fe^{2+}) form, and a ferrous iron transporter DMT1 that moves iron across the cell membrane (Anderson et al., 2005). Iron is transported primarily by the iron transport protein Transferrin (TF). Other cells take up diferric transferrin using transferrin receptors

(TFRs) through endocytosis. In the cytoplasm, iron is stored mainly in the form of ferritin. The export of iron out of the cells involves ferroportin and it also requires a ferroxidase to deliver ferric iron to transferrin. Generally, iron concentrations and influxes are regulated at both the cellular and the systemic levels.

At the cellular level, iron homeostasis is maintained by at least two mechanisms (Andrews and Schmidt, 2007). First, all mammalian cells produce ferritin, an iron storage protein. The ferritin can regulate the iron uptake through accepting excess iron and allowing for the mobilization of iron when needed (Andrews, 1999). The more iron required by the body, the less ferritin is manufactured in each enterocyte cell. The second protective mechanism involves iron regulatory proteins (IRPs) (Nemeth and Ganz, 2006). When cytoplasmic iron is low, IRPs bind to iron regulatory elements (IREs) found in the untranslated regions of mRNAs involved in iron transport and storage. If IRPs binds with IREs on 3' untranslated region, they will stabilize mRNA, increasing protein synthesis; whereas binding to the IREs on 5' untranslated region prevents the translation of mRNA, decreasing protein synthesis. The mRNAs of the transferrin receptor, DMT1, ferritin, and ferroportin are found to be regulated by the IRP/IRE mechanism. Therefore, IRPs/IRE system control the cellular iron homeostasis by modifying the production of proteins involved in iron uptake, storage, and exportation, according to the cytoplasmic iron concentrations.

Systemically, iron homeostasis is achieved by regulating the major iron flows into the plasma: absorption of dietary iron by duodenal enterocytes, release of stored iron from hepatocytes, release of iron from macrophages recycling senescent red blood cells (Nemeth and Ganz, 2006). The stimuli known to modulate the iron homeostatic

mechanism are erythroid iron needs, hypoxia, iron deficiency, iron overload, and inflammation (Andrews, 2005). For a long time, people believed that there are two regulators (iron store regulator and erythroid regulator) responsible for the iron homeostasis although nobody knew what they were (Finch, 1994). Until the beginning of this century, the discovery of hepcidin (Krause et al., 2000; Park et al., 2001; Pigeon et al., 2001) and its receptor ferroportin (Donovan et al., 2000; McKie et al., 2000; Njajou et al., 2001) has allowed people to really appreciate iron homeostasis regulation. The expression of hepcidin is increased in response to increased serum iron, iron overload, and inflammation and is diminished in response to increased erythroid drive, hypoxia, and iron deficiency (Pigeon et al., 2001; Nicolas et al., 2002b), all of which are known to affect iron homeostasis. What's more, all forms of genetic hemochromatosis are found to be associated with decreased hepcidin production or activity (Bridle et al., 2003; Roetto et al., 2003; Papanikolaou et al., 2004; Nemeth et al., 2005). Hepcidin has been proven to be the linker between inflammation and anemia (Ganz, 2003). Taken together, hepcidin is the long-sought hormone regulator for iron metabolism.

IRON DISORDERS

Under normal circumstances, the iron concentration in plasma and extracellular fluid remains in a relatively narrow range despite fluctuating iron supply and demand. Disorders of iron deficiency and iron overload occur when iron balance is disrupted. Andrews (2005) listed four situations that lead to measurable changes in intestinal iron absorption and tissue iron distribution: abnormal iron availability (iron overload or deficiency), accelerated erythropoiesis, hypoxia, and inflammation. Under iron overload

or inflammation conditions, iron absorption and plasma availability will be decreased; while they are increased in response to iron deficiency, accelerated erythropoiesis and hypoxia (Anderson et al., 2007).

Iron excess causes widespread organ damage and reduces the ability of host defense (Pietrangelo, 2004). The total body iron of the patients with severe forms of hemochromatosis or iron overload is more than 5 to 10 times normal (Pietrangelo, 2006). Affected patients with genetic hemochromatosis have parenchymal iron deposition in the liver, heart, and endocrine tissues but a paucity of iron in intestinal epithelial cells and tissue macrophages (Pietrangelo, 2006). In severe cases, tissue iron leads to cirrhosis, cardiomyopathy, diabetes, and other endocrinopathies (Pietrangelo, 2004). Bacteria grow faster and form biofilms more readily when iron is abundant, explaining why the patients with iron overload are more susceptible to a number of the infectious pathogens (Ganz, 2003). Due to the host defense mechanisms, infection can result in serum iron deficiency and induce anemia of inflammation. Serum iron levels are diminished by two mechanisms (Andrews, 1999). First, macrophages fail to return recycled iron from old erythrocytes to the circulation. Second, intestinal iron absorption is reduced. These responses can sequester iron and protect the host from the infectious microbes. But the coin has two sides, less iron is available to erythroid precursor cells, at least partially accounting for the development of anemia of inflammation (Ganz, 2003).

In contrast with iron deficiency anemia (IDA) in humans rarely resulting from genetic causes, mutations causing hereditary hemochromatosis (HH) have provided important insights into the genes that regulate iron homeostasis (Pietrangelo and Trautwein, 2004). Homozygous mutations in the *HFE*, transferrin receptor 2 (*TFR2*),

hemojuvelin (*HJV*), ferroportin (*FPN*), and hepcidin (*HAMP*) genes (Feder et al., 1996; Camaschella et al., 2000; Montosi et al., 2001; Njajou et al., 2001; Roetto et al., 2003; Papanikolaou et al., 2004) have similar clinical phenotypes but cause varying severity. Patients with *HFE, TFR2*, and *FPN* mutations typically present in midlife (Ajioka and Kushner, 2003). In contrast, patients with *HJV* and *HAMP* mutations are severely affected early in life, typically dying of cardiomyopathy before the fourth decade if not treated (Pietrangelo, 2006). Based on the fact that the different forms of iron overload disorders are all associated with decreased hepcidin production or activity (Bridle et al., 2003; Roetto et al., 2003; Papanikolaou et al., 2004; Nemeth et al., 2005), hereditary hemochromatosis can be grouped into three functional classes (Andrews and Schmidt, 2007). First, mutations in the hepcidin gene cause dysfunctional hepcidin protein, resulting in unregulated ferroportin activity. Second, mutations in the ferroportin gene prevent hepcidin binding and/or consequent ferroportin internalization and degradation (De Domenico et al., 2005; Drakesmith et al., 2005). Third, mutations in other genes (*HFE, TFR2*, and *HJV*) induce inappropriate hepcidin expression or activity.

HEPCIDIN AND IRON METABOLISM

As I mentioned in the beginning, hepcidin was found to be involved in iron metabolism immediately after its discovery. This was demonstrated first in mice models by two groups (Nicolas et al., 2001; Pigeon et al., 2001; Nicolas et al., 2002a). And later on, transgenic mouse targeted disruption of the hepcidin gene (Hamp1-/- mice) with severe iron overload further verified that hepcidin plays a pivotal role in iron metabolism (Viatte et al., 2005). Meanwhile, it was found that dysregulation of hepcidin is

responsible for anemia of inflammation and most forms of hereditary hemochromatosis disorders in humans (Weinstein et al., 2002; Andrews and Schmidt, 2007).

With its established role in iron metabolism, the molecular activity of hepcidin has been an attractive topic. A breakthrough was made by a Science paper that identified ferroportin, the only known cellular iron exporter in vertebrates, as the receptor of hepcidin (Nemeth et al., 2004b). Hepcidin binds to ferroportin and causes its internalization, thus decreasing iron efflux from iron exporting tissues into plasma (Nemeth et al., 2004). Interestingly, ferroportin was discovered by three groups (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000) at the same time that hepcidin was found. Studies in zebrafish (Donovan et al., 2000) and mouse (Donovan et al., 2005) demonstrated that the complete loss of ferroportin expression is embryonic lethal due to the inability to transfer iron from the mother to the embryo. In addition to placental trophoblasts, ferroportin is found in all other tissues where major iron flows are regulated (duodenal enterocytes, macrophages, and hepatocytes). Ferroportin-deficient animals accumulated iron in enterocytes, macrophages, and hepatocytes, consistent with a key role for ferroportin in those cell types (Donovan et al., 2005). Intestine-specific inactivation of ferroportin confirmed that it is critical for intestinal iron absorption. Additionally, the release of iron from hepatic storage was defective, as was the release of iron from macrophages that recycled red cells. This study indicated that ferroportin is the sole significant iron exporter in tissues involved in iron absorption, recycling, and storage. Mutation in ferroportin is one of the major forms of hereditary hemochromatosis.

With this knowledge, it is becoming clearer how hepcidin regulates the iron homeostasis in our body. Hepcidin acts by directly binding to ferroportin, causing

ferroportin to be internalized and degraded in lysosomes (Nemeth et al., 2004b; Delaby et al., 2005a). As a result, the loss of ferroportin from cell membrane consequently ablates cellular iron export (Nemeth et al., 2004b; Knutson et al., 2005). When iron stores are adequate or high, the liver produces hepcidin and releases it to the circulation. By interacting with ferroportin and causing the latter to be internalized, hepcidin blocks the sole pathway for the transfer of iron from the enterocytes to plasma. When iron stores are low, hepcidin production is suppressed, and ferroportin molecules are displayed on basolateral membranes of enterocytes, transporting iron from the enterocyte cytoplasm to plasma transferrin. A similar mechanism applies to macrophages recycling aged red blood cells and hepatocytes uptaking or immobilizing storage irons. The plasma levels of hepcidin are in turn regulated by iron and anemia/hypoxia, thus completing the homeostatic loop controlling systemic iron concentrations.

However, the direct interaction of hepcidin with ferroportin may not be the only pathway by which ferroportin density on cell membranes is regulated (Nemeth and Ganz, 2006). There is evidence that ferroportin mRNA levels are also regulated by iron and inflammation independent of hepcidin (Frazer et al., 2002; Ludwiczek et al., 2003; Delaby et al., 2005b). Hepcidin may also have an indirect effect on the cellular IRE/IRP system. By blocking cellular iron efflux, hepcidin causes a rise in intracellular iron levels, which would affect expression of the IRE-containing DMT1, the transferrin receptor, and ferroportin. The importance of this interaction between the hepcidin-ferroportin and the IRE/IRP system remains unclear (Ganz, 2005a).

Dysregulation of hepcidin or its receptor ferroportin results in a spectrum of iron disorders. In inflammatory disorders and infections, cytokine-induced hepcidin excess

contributes to development of anemia of inflammation, characterized by hypoferremia and anemia despite adequate iron stores. On the other hand, inappropriately low hepcidin production due to mutations in the hepcidin gene or its putative regulators (such as HFE, TFR2, and HJV) appears to be the cause of most types of hereditary hemochromatosis, the iron overload disease characterized by excessive dietary iron uptake and iron deposition in vital organs (De Domenico et al., 2005; Drakesmith et al., 2005; Schimanski et al., 2005).

REGULATION OF HEPCIDIN

The regulation of hepcidin in humans and mice has been extensively studied. Hepcidin expression is up-regulated by iron; by cytokines (IL-1 and IL-6); and by the bone morphogenetic proteins (BMPs). It is down-regulated by anemia and hypoxia. The regulation of hepcidin appears to occur at the level of transcription, exerted by several distinct inflammatory or non-inflammatory pathways (Andrews and Schmidt, 2007; Truksa et al., 2007b).

Under basal conditions, hepcidin expression depends upon signaling through a bone morphogenetic protein (BMP)/SMAD pathway (Babitt et al., 2006). Liver-specific inactivation of the co-SMAD protein SMAD4 causes a failure of hepcidin production and an iron overload phenotype similar to that observed in hepcidin knockout mice (Wang et al., 2005). Hepcidin production is increased by treatment with BMPs (Wang et al., 2005; Babitt et al., 2006) and inhibited by expression of a dominant-negative BMP receptor or a dominant-negative regulatory SMAD protein (Babitt et al., 2006). The ubiquitous BMP signaling apparatus is co-opted for the regulation of hepcidin expression through the

interaction of hemojuvelin (HJV) with BMP and BMP receptors (Babitt et al., 2006). HJV is a homolog of "repulsive guidance" molecule proteins important in neurodevelopment (Niederkofler et al., 2004). It is mutated in patients with severe, early-onset juvenile hemochromatosis. BMPs regulate the transcription of hepcidin by binding to hemojuvelin as a co-receptor and signaling through SMAD4 (mothers against decapentaplegic homolog 4) (Wang et al., 2005; Babitt et al., 2006).

Under inflammatory conditions, the cytokines (IL-6 and IL-1) were found to be a group of important activators of hepcidin expression (Nemeth et al., 2003; Inamura et al., 2005; Lee et al., 2005a). In human volunteers infused with IL-6, urinary hepcidin excretion was increased an average of 7.5-fold within two hours after infusion, whereas IL-6 knockout mice failed to induce hepcidin in response to turpentine inflammation (Nemeth et al., 2004a). IL-1 also increased hepcidin mRNA expression in vitro, but in human primary hepatocytes, this was blocked by anti-IL-6 antibodies. In mouse hepatocytes, however, the IL-1 effect was independent of IL-6 (Lee et al., 2005a). Recently, it has been shown that signal transducer and activator of transcription 3 (STAT3) plays a role in the inflammatory regulation of hepcidin and that a crucial binding site is located at -148 to -130 from start of translation of human hepcidin gene (Wrighting and Andrews, 2006; Verga Falzacappa et al., 2007). Truksa et al (2007) showed that the regulation of hepcidin expression by IL-6 and BMPs occurs through distinct regulatory elements. The induction of hepcidin by BMPs requires at least two regions of the mouse HEPC1 promoter (one between 140-260 bp and the other between 1.6-2.0 kb upstream of the start of translation). In contrast, the IL-6 response required

only the proximal 260 bp HEPC1 promoter region (Truksa et al., 2007b). Furthermore, there were no regulatory elements located in the non-coding or coding regions of HEPC1.

Hepcidin production is also homeostatically regulated by anemia and hypoxemia (Nicolas et al., 2002b). Anemia due to bleeding or acute phenylhydrazine (PHZ)-induced hemolysis in mice caused a decrease in hepcidin mRNA levels (Frazer et al., 2004; Latunde-Dada et al., 2004; Bondi et al., 2005). Hepcidin mRNA was also suppressed within 2 days in mice housed in hypobaric chambers (Nicolas et al., 2002b) and in rats exposed to 10% oxygen for 30 days (Leung et al., 2005). Most of the iron absorbed from the diet or recycled from hemoglobin is used for creating erythrocytes. When oxygen delivery is inadequate, the homeostatic response is to produce more erythrocytes. Thus, in anemia, hepcidin levels decrease, its inhibitory effects diminish, and more iron is made available from the diet and from the storage pool in macrophages and hepatocytes. Under hypoxia conditions, it was found that it is reactive oxygen species (ROS) but not hypoxia-inducible factors (HIFs) that represses hepcidin gene expression by preventing C/EBPα and STAT-3 binding to hepcidin promoter (Choi et al., 2007).

The mechanism of hepcidin regulation by iron is turning out to be unexpectedly complex, although iron is the first factor found to induce hepcidin expression. In mice, oral or parenteral iron loading increases hepatic hepcidin mRNA expression (Pigeon et al., 2001; Nemeth et al., 2004a). In humans, even a single dose of oral iron ($FeSO_4$) increases urinary hepcidin excretion within several hours (Nemeth et al., 2004a). In vitro iron loading of primary mouse or human hepatocytes, or human hepatic cell lines, does not increase hepcidin mRNA, regardless of whether hepatocytes are loaded with iron-transferrin or other forms of iron (Pigeon et al., 2001; Gehrke et al., 2003; Nemeth et al.,

2003). The hepcidin mRNA lacks any stem-loop structures containing the consensus IRE motif for binding of iron-regulatory proteins (Nemeth and Ganz, 2006). However, some clues about hepcidin regulation by iron came from the studies of genes involved in hereditary hemochromatosis. Despite iron overload, hepcidin was found to be deficient in patients or mice with homozygous mutations in HFE, transferrin receptor 2 (TfR2), and hemojuvelin (HJV) (Nemeth and Ganz, 2006), suggesting that these molecules regulate hepcidin synthesis in response to iron. The study on the hepcidin promoter showed that the promoter region between 1.6 Kb and 1.8 Kb upstream from the start of translation is essential for the response to iron (Truksa et al., 2007a). Furthermore, this region is also critical for hepcidin regulation by IL-6 (Truksa et al., 2007b), indicating that the iron regulatory pathway may crosstalk with the inflammatory response.

Several other transcriptional factors have been identified using reporter gene techniques. First, CCAAT/enhancer-binding proteins (C/EBPα and C/EBPβ) (Courselaud et al., 2002) and the basic helix–loop–helix leucine zipper (bHLH-ZIP) family of transcriptional regulators such as USF and c-Myc/Max (Bayele et al., 2006) were identified as potent hepcidin expression activators. Interestingly, both C/EBPα/β and USF are involved in energy/glucose metabolism, indicating a crosstalk between iron homeostasis and glucose metabolism. More recently, p53 (a tumor suppressor) and leptin were found to regulate the expression of hepcidin (Chung et al., 2007; Weizer-Stern et al., 2007), indicating a crosstalk with these processes. Hence, hepcidin may have additional functions in vivo besides its iron regulatory activity.

Mutations in the HFE, TFR2 and HJV genes are frequently found in hereditary hemochromatosis patients and are also found to cause hepcidin deficiency in spite of

massive iron overload, indicating that all these molecules act as direct or indirect regulators of hepcidin synthesis. *HFE*, the gene most commonly mutated in patients with hemochromatosis, encodes an atypical major histocompatibility class I protein that complexes with β-2 microglobulin but cannot bind small peptides (Feder et al., 1996; Lebron et al., 1998). The discovery that HFE forms a protein-protein complex with TFR suggested that HFE may play a role in iron homeostasis (Roy and Enns, 2000). Although TFR2 can bind and internalize Fe-TF, it probably does not serve a primary role in cellular iron uptake. In contrast, TFR2 more likely acts as an iron sensor because its protein levels increase in response to increased ambient Fe-TF (Johnson and Enns, 2004; Robb and Wessling-Resnick, 2004). As I will discuss in regulation of hepcidin below, HJV acts as a BMP co-receptor and positively regulates hepcidin expression. HFE, TFR2, and hemojuvelin are all expressed on the surface of hepatocytes. One hypothetical model puts HFE, TFR2 and HJV together trying to explain how iron regulates the expression of hepcidin (Nemeth and Ganz, 2006). However, the details of how these factors regulate hepcidin expression has left much to desire.

EVOLUTION OF HEPCIDIN

Unlike antimicrobial peptides whose sequences vary even between closely related species, hepcidin is highly conserved across vertebrates, from fish to humans (Park et al., 2001; Shi and Camus, 2006). Immediately as hepcidin peptide was sequenced, the authors performed a Blast search and found several homologs such as mice, rat, fish, and even an insect AMP (Drosomycin) (Park et al., 2001). All of these hepcidin genes have three exons and two introns and are located down stream of USF2 gene (Haslego-Hilton

and Lambert, 2008). Particularly, the four disulfide bonds and the N-terminal region of hepcidin peptides are most conserved. In support of this notion, zebrafish hepcidin was fully active when tested as a regulator of mouse ferroportin (Nemeth et al., 2006). Interestingly, genomes of some species contain two (mouse) or more (certain fish species) hepcidin genes. And in some fish species such as bass and zebrafish, hepcidin was found likely to function in the same way as in mammals (Shike et al., 2002; Shike et al., 2004). In mice, HEPC2 appears to have arisen by a recent duplication of the region encompassing hepcidin and part of the neighboring gene USF2 (Ilyin et al., 2003). Although the two mouse hepcidins are similarly regulated by iron, HEPC2 does not appear to have a role in iron metabolism. Unlike the transgenic overexpression of mouse HEPC1, which caused severe iron-refractory anemia, transgenic overexpression of HEPC2 had no detectable effect on iron metabolism (Lou et al., 2004). Apparently, the mouse hepcidins can not provide us much evolutionary information.

So far, there are three papers published or in press on the evolution of hepcidin. One paper was published by Shi and Camus in 2006. Based on a comprehensive comparison of hepcidin from fish to mammals, the authors proposed that two groups of hepcidin peptides exist in fishes: one of them may function as iron regulator as in mammalians while the others are still AMPs. Furthermore, the mammalian hepcidins may have evolved from fish hepcidins. This review paper suggested another way to study the structure and function of hepcidin. A recently published paper (Padhi and Verghese, 2007) revealed that, in contrast to mammals, positive Darwinian selection is the likely cause of the accelerated rate of amino acid substitutions in the hepcidin mature peptide region of perciform and pleuronectiform fishes. The authors concluded that the

adaptive evolution of this peptide in these fishes might be directed by pathogens when the host is exposed to new habitats/environments. A third evolutionary paper on hepcidin will be published in the journal Gene. The authors (Haslego-Hilton and Lambert, 2008) collected 67 different hepcidin genes from 50 different species among all vertebrates. Although some species have multiple hepcidin homologues, the authors suggested that each contains only one copy that functions as an iron regulator. Despite the recent report of hepcidin in pigeon (Fu et al., 2007), Haslego-Hilton and Lambert (2008) failed to identify a hepcidin gene in other birds including chickens.

HEPCIDIN: A PROMISING THERAPY FOR IRON DISORDERS

As a negative iron regulator, hepcidin administration should prevent most types of hereditary hemochromatosis. This idea is supported by the fact that transgenic mice with HFE hemochromatosis did not develop iron overload if they also expressed a hepcidin transgene (Nicolas et al., 2003). Furthermore, acute administration of synthetic hepcidin to mice rapidly lowered serum iron, and the effect lasted at least 48 hours (Rivera et al., 2005). However, the current treatment of hemochromatosis, bleeding, is inexpensive and generally well accepted, causing resistance to develop a new expensive drug.

On the other hand, a hepcidin antagonist should be useful in treating anemia of inflammation, probably the most common form of anemia in the United States (Ganz, 2003). The measurement of hepcidin concentrations in biological fluids would be expected to provide a differential diagnosis of anemia of inflammation (elevated hepcidin concentrations) and iron deficiency anemia (low hepcidin concentrations). Recent

research studies have employed urinary hepcidin measurements (Nemeth et al., 2004a), but a serum or plasma assay may also be feasible.

Although there still are no successful AMP drugs on the market so far, the hope always exists. The discovery of hepcidin and its role in iron metabolism could lead to new therapies for anemia of inflammation and other kinds of iron overload disorders. Ganz (2003) suggested that hepcidin or related agonists could be helpful for the treatment of the hereditary hemochromatosis forms that are due to hepcidin deficiency. However, any effort to develop a peptide drug mimicking hepcidin will rely on our knowledge about the structure and function of hepcidin. My study in this dissertation will provide information useful for such a purpose.

CHAPTER III. AIM OF THE DISSERTATION

Human hepcidin, originally identified as a 25 amino acid antimicrobial peptide, is the long-sought iron regulator that is responsible for the iron recycling and iron balance. Dysfunction of hepcidin has been found to be associated with iron disorders such as iron deficiency anemia and most forms of hemochromatosis (Pietrangelo, 2006). With the established role in the iron metabolism, hepcidin provides a promising therapeutic choice for iron disorder diseases. Hepcidin agonists may help to prevent iron overloading while antagonists could be used for the treatment of anemia of inflammation (Ganz, 2003). Clearly, understanding the molecular mechanism of the function and regulation of hepcidin is critical for development of such therapeutics.

The first aim of this dissertation is to develop a non-mammalian model used for the study of hepcidin. Although iron overloading led to the discovery of the hepcidin gene, the mechanism of hepcidin regulation by iron has turned out to be unexpectedly complex. In contrast to in vivo data, iron loading (iron-transferrin or other forms of iron) does not increase hepcidin expression using primary mouse or human hepatocytes, or human hepatic cell lines. Despite the advances in hepcidin study, the regulation of hepcidin by iron is still elusive due to lacking a suitable in vitro model. Closely related hepcidin genes and peptides have been identified in other mammals, amphibians and a number of fish species. Similar to human hepcidin, fish hepcidins can destroy bacteria and be induced by infections. However, fish hepcidin demonstrates a

broader structural diversity at the NH2-terminus, suggesting that not all hepcidins function as an iron regulator in fish. Some fish or amphibian hepcidins may function still as antimicrobial peptides. The hypothesis for this aim is that hepcidin evolved from an antibacterial peptide into an iron regulatory hormone in vertebrate evolution. To verify such a notion, suitable animal models from different evolutionary stages of vertebrates are needed. Because mammalian hepcidin synthesis is regulated by iron overloading, inflammation and anemia, we will use these effectors to test the function of hepcidin in fish (channel catfish) and in frog (western clawed frog).

The second aim of this dissertation is to study the structure and function of hepcidin in an evolutionary view. The advances in the genome information and techniques in bioinformatics allow us to historically appreciate the structure and function of proteins. Rapidly increasing genomic data give us such an opportunity to study the structure and function by phylogenetic analysis. The techniques used in the field of molecular evolution make this type of study feasible. Recently, co-evolutionary studies on physically or functionally related proteins have become popular, providing a new way to look at the structure and function of the proteins. These results also provide the clues for experimental studying. The discovery of ferroportin, the receptor of hepcidin, further prompts the study on the structure and function of hepcidin and ferroportin using such a method.

CHAPTER IV. HEPCIDIN IN FISH: CHANNEL CATFISH HEPCIDIN EXPRESSION IN INFECTION AND ANEMIA

INTRODUCTION

Hepcidin, also termed LEAP-1 (liver-expressed antimicrobial peptide), was originally identified by two independent groups as a 25 amino acid peptide antibiotic made in the liver (Krause et al., 2000; Park et al., 2001). In addition to its direct antimicrobial activity in vitro, human hepcidin production is also increased in patients with bacterial infection (Nemeth et al., 2003), suggesting that hepcidin may play an important role in host defense against infections. The connection between hepcidin and iron metabolism was observed by two other independent groups almost immediately after the hormone was shown to possess antimicrobial properties (Nicolas et al., 2001; Pigeon et al., 2001). Hepcidin mRNA was overexpressed in the livers of mice suffering from iron overload, both experimentally following the administration of carbonyl iron and iron-dextran, and spontaneously in β_2-microglobulin knockout mice (Pigeon et al., 2001). Hepcidin knockout mice progressively develop massive iron overload in the liver and pancreas accompanied by significant increases in serum iron (Nicolas et al., 2001). Transgenic animals overexpressing hepcidin in the liver have decreased body iron levels and at birth are affected by severe microcytic hypochromic anemia typical of an iron deficient state (Nicolas et al., 2002a). In humans, inappropriate expression of hepcidin is associated with both iron refractory anemia in patients with hepatic adenomas (Weinstein

et al., 2002) and iron-overload in patients with hereditary hemochromatosis (HH) (Gehrke et al., 2003; Biasiotto et al., 2004).

Taken all together, it is clear that hepcidin is the long sought-after hormone responsible for the regulation of iron balance and recycling in humans and mice (Ganz, 2003). Because most of the body's iron is destined for developing erythrocytes, it is therefore not surprising that hepcidin production is regulated by anemia and hypoxia (Nicolas et al., 2002a). Anemia and hypoxia induce a dramatic decrease in liver hepcidin gene expression, which may account for the increase in iron release from reticuloendothelial cells and in iron absorption by enterocytes, frequently observed in these conditions (Nicolas et al., 2002a; Ganz, 2005b).

Closely related hepcidin genes and peptides have been identified in a number of mammals, amphibians, and fish species (Douglas et al., 2003; Ganz, 2003; Shike et al., 2004; Bao et al., 2005). Fish hepcidins possess antibacterial activity in vitro and their expression in the liver can be dramatically induced by lipopolysaccharide (LPS), infection, and vaccination (Shike et al., 2002; Douglas et al., 2003; Shike et al., 2004; Straub et al., 2004; Lauth et al., 2005), suggesting that fish hepcidins may be essential elements of the innate immune system. However, compared with other antimicrobial peptides whose sequences have evolved rapidly, and vary significantly even between closely related mammalian species, the evolution of hepcidin is strictly constrained (Shike et al., 2002; Ganz, 2005b).

This high degree of structural conservation between mammalian hepcidins and fish hepcidins prompted us to hypothesize that some fish hepcidins may possess functions corresponding to those found in mammals, including the regulation of iron homeostasis.

Consequently, it would be expected that iron levels in vivo should also regulate hepcidin expression. To test this hypothesis, we took advantage of the existence of channel catfish anemia (CCA), an enigmatic disease commonly encountered in the commercial culture of pond-raised channel catfish (Ictalurus punctatus), and compared hepcidin expression profiles in healthy catfish and fish affected by CCA. Predictably, liver hepcidin levels in anemic fish were markedly suppressed when compared to normal healthy controls. Here we report the transcriptional regulation of hepcidin expression by infection and anemia in the channel catfish.

MATERIALS AND METHODS

Real-time quantitative PCR analysis of hepcidin gene expression

To evaluate the influence of bacterial infection and anemia on the expression of channel catfish hepcidin, a real-time PCR approach was developed. Briefly, two primers (Table 1) were selected for the real-time PCR based on the intron and exon sequence information from channel catfish hepcidin cDNA (GenBank Accession # DQ062122) and genomic DNA (GenBank Accession # DQ200985). The sense primer was designed to avoid potential genomic DNA contamination in the PCR step by selecting the region in the cDNA that spans the second intron. A channel catfish *β*-actin gene transcript (EST BE470101) was used as an internal control for real time PCR. Specificity of real-time PCR products was documented with high-resolution gel electrophoresis and resulted in a single product with the predicted lengths of 102 bp for hepcidin and 206 bp for *β*-actin (data not shown).

Two-step real-time PCR was carried out in an iCycler iQ multicolor real-time PCR detection system (BioRad, Hercules, CA) using the SYBR Green I reagents kit (BioRad). To make cDNA from the total RNA (1 μg), reverse transcription reactions were carried out using thermo-profiles of 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, and 4 °C holds. The conditions of real-time PCR reactions, including annealing temperature, amplification efficiency, and primer concentrations were optimized by gradient and standard curve experiments on both pairs of primers. For real-time PCR, an initial denaturation was carried out at 95 °C for 2 min, followed by 40 cycles of amplification with denaturation at 94 °C for 10 s, primer annealing at 60 °C for 45 s for channel catfish hepcidin and at 62 °C for *β*-actin, followed by elongation at 72 °C for 10 s. A melting curve analysis was performed at 95 °C for 1 min, 55 °C for 1 min and then increased to 95 °C at a rate of 0.5 °C/s. Specificity of real-time PCR products were further confirmed by single product specific melting temperatures of 85.2 °C for hepcidin and 87.4 °C for *β*-actin. No primer-dimers were generated during the 40 real-time PCR amplification cycles.

The amplification efficiency was calculated according to the equation: $E=10^{[-1/\text{slope}]}$ (Pfaffl, 2001). Transcripts showed high real time PCR efficiency rates of 93.7% for hepcidin and 91.2% for *β*-actin in the investigated range from 32 pg to 100 ng cDNA input. The amount of channel catfish hepcidin gene transcription was determined by measuring the relative quantities of the channel catfish hepcidin gene in comparison to the *β*-actin gene using (Pfaffl, 2001).

$$\text{Hepcidin/Actin ratio} = \frac{\left(E_{cHEP}\right)^{\Delta Ct_{cHEP}\ (control\ -\ sample)}}{\left(E_{ref}\right)^{\Delta Ct_{ref}\ (control\ -\ sample)}} \qquad (1)$$

In Eq. (1), *E*cHEP is the real-time PCR efficiency of the catfish hepcidin gene transcript; E_{ref} is the real-time PCR efficiency of the β-actin gene transcript. ΔCt_{cHEP} is the Ct deviation of the control-sample of the hepcidin gene transcript. ΔCt_{ref} is the Ct deviation of the control-sample of the β-actin gene transcript. Ct is the cycle number at which the samples reach the level of detection by exceeding the fluorescent intensity of the background. In the bacterial infection experiment, the control represents the unchallenged catfish and the sample represents catfish infected with Edwardsiella ictaluri. In the CCA experiment, the hepatic hepcidin/actin ratio for individual fish was calculated using a fish with a hematocrit of 15% as the control.

Bacterial challenge experiment

Channel catfish were challenged with E. ictaluri as described previously (Nusbaum and Morrison, 2002). Briefly, fingerling channel catfish hatched from a single egg mass were raised under specific pathogen free (SPF) conditions in an indoor fish culture facility receiving a constant flow of dechlorinated tap water, and were fed a commercially available pelleted diet. At the time of sampling, the fish were 6-17 cm in length and were culture-negative for E. ictaluri and channel catfish virus using routine methods (Nusbaum and Morrison, 2002; Nusbaum et al., 2002).

Prior to challenge, three 20-L plastic buckets were rinsed with 70% ethanol and allowed to dry. Water from the fish culture facility was filtered through 0.45 µm disposable filters and 4 L was placed in each of the three buckets, aerated, and allowed to reach ambient temperature of 19-21 °C. Five control fish were added to one bucket. Fifteen fish were placed in the second bucket to which 50 mL of E. ictaluri inoculum was

added. The final bacterial concentration (6×10^6 CFU/mL) was determined by standard plate count using 10-fold serial dilutions. After 15 min, the experimental fish were removed from the second bucket and placed into the third which is bacteria free.

At time zero, the five control fish were euthanized in 300 mg 3-aminobenzoic acid ethyl ester (AAEE)/100 mL, followed by five experimental fish each at 4, 24, and 48 h post-challenge. All euthanized fish were immediately submitted for necropsy and tissue collection. Because E. ictaluri can invade the body through the gill, gut, and olfactory epithelium (Nusbaum and Morrison, 2002), these organs plus the liver were targeted for gene expression analysis. At each time point, samples from the same organs were pooled and total RNA was isolated using TRIzol reagent. The catfish hepcidin gene transcript levels were then measured by real-time PCR as described above.

Hepcidin expression in fish affected by CCA

During a spontaneous outbreak of CCA, seven 1-2 kg channel catfish were randomly captured by snag line and blood collected by venipuncture of the caudal vein in vacutainers without anticoagulant and with EDTA (Becton Dickinson, Franklin Lakes, NJ). An additional seven fish were collected at random, also by snag line, from an unaffected pond to serve as controls. The fish were euthanized by overdose with 1000 mg/L tricaine methane sulfonate (Western Chemical, Inc., Ferndale, WA) and a portion of the liver was removed for iron (0.5 gm) and hepcidin (1.5 gm) determination. Clotted blood was centrifuged and 200 µl serum was removed for determination of serum iron and total iron binding capacity. Hematocrits (Hct) were performed on unclotted blood using a Crit Spin microhematocrit centrifuge (StatSpin, Inc., Norwood, MA).

Serum iron and total iron binding capacity determinations were performed using a Cobas Mira automated analyzer and reagents as per manufacturers instructions (Roche Diagnostics, Indianapolis, IN). Percent saturation of transferrin was calculated by dividing the serum iron concentration by the total iron binding capacity. Liver iron determinations were performed using modifications of the colorimetric method of Torrance and Bothwell (Torrance and Bothwell, 1968) and Stanbio Laboratory-Iron procedure 0370 reagents (Stanbio Laboratory, Boerne, TX). Analyses were performed using a Spectramax-340PC microplate reader (Molecular Devices, Corporation, Sunnyvale, CA). To determine whether channel catfish hepcidin expression was affected by anemia, the relative quantity of hepcidin mRNA in the liver was determined by real-time PCR as described above. Expression of the hepcidin versus *β*-actin genes (hepcidin/actin ratio) of individual liver samples from healthy catfish and fish affected by CCA was calculated using a fish with a hematocrit of 15% as the control in Eq. (1).

Statistical analysis

Standard ANOVA, correlation, and linear regression analysis were performed on SigmaStat3.11. A *p*-value of 0.05 will be considered significant.

RESULTS

Induction of hepcidin expression in channel catfish challenged with E. ictaluri

To determine whether catfish hepcidin expression was altered in infection, changes in hepcidin mRNA expression in various tissues of catfish challenged with E. ictaluri were determined by real time quantitative PCR. As shown in Figure 4.1, 4 h

following challenge with E. ictaluri, hepatic hepcidin expression had begun to increase and was continuing to increase at the 48 h sampling time. Higher than two-fold increases in hepcidin expression were also seen in the gill, gut, and olfactory sac, but these were not detected until 48 h after challenge.

Inhibition of hepcidin expression in fish affected by CCA

There was no gross evidence of infectious disease processes in any of the sampled fish. Although no established value exists, an average hematocrit value of 10% or less in a population with compatible clinical signs is generally considered diagnostic for CCA. Hematocrits of CCA affected fish averaged 8.71%, while those of unaffected fish averaged 24.71% (Figure 4.2A). Serum iron, total iron binding capacity, liver iron, and percent saturation of transferrin were approximately half that of healthy controls (Figure 4.2B-E). The hepatic hepcidin mRNA levels present in CCA affected fish were significantly lower than that in controls (Figure 4.2F). The average hepcidin/actin ratio in CCA fish was 13.9% of healthy controls or 18.7 versus 135, respectively.

This study also revealed significant correlations between hepatic hepcidin and serum iron levels (r=0.54, p<0.05), as well as percent saturation of transferrin (r=0.63, p<0.05) in the 14 fish sampled (Figure 4.3A and 4.3B). Linear regression analysis also confirmed significant correlations between hepcidin mRNA expression and serum iron concentrations (p<0.05) and between hepcidin mRNA expression and percent saturation of transferrin (p<0.02). These correlations were not statistically significant when examined in either the control or CCA group alone (data not shown). In addition, no

significant correlation was found between hepcidin mRNA level and hepatic iron concentration in CCA fish (r=- 0.28, p>0.5).

DISCUSSION

Our studies have demonstrated that hepatic hepcidin gene expression was dramatically induced in channel catfish challenged with E. ictaluri. These findings are in agreement with studies in pigs (Sang et al., 2006), hybrid striped bass (Shike et al., 2002), Atlantic salmon (Douglas et al., 2003), and Japanese flounder (Hirono et al., 2005) where early and marked induction of hepatic hepcidin expression, as well as tissue-specific and time-dependent induction, occurred in other tissues following bacterial infection and/or LPS challenge. However, these findings contradict those in a recent report by Bao et al. (Bao et al., 2005) where significant induction of hepcidin gene expression was detected only in the spleen and head kidney, but not in the liver in channel catfish challenged with E. ictaluri.

The sequence data and intron analysis in our studies (GenBank Accession #s DQ062122 and DQ200985, data not shown) support the notion by Bao et al. (Bao et al., 2005) that there is only one hepcidin gene in the channel catfish. Therefore, differences in the hepcidin expression profiles seen here and in the study by Bao et al. (Bao et al., 2005) could be the result of differences in the testing methods used (real time quantitative PCR versus reverse transcription PCR), duration of bacterial challenge (15 min versus 2 h), and the physiological state of the fish prior to bacterial exposure. Husbandry conditions under which the fish were maintained could be a major contributing factor, as fish grown indoors under SPF conditions are much less likely than pond raised fish to

have been exposed to the organism prior to experimentation. In this study, fish were grown indoors from a single egg mass, while in the study by Bao et al., fish were raised in ponds. E. ictaluri is a common waterborne pathogen distributed throughout all areas in the southeast that produce pond-raised catfish (Plumb, 1999). The widespread nature of the pathogen was illustrated in a recent survey where E. ictaluri infection caused losses for 52.9% of the 739 catfish operations evaluated (USDA, 2003).

Up-regulation of hepcidin expression is associated with infection in humans and fishes (Shike et al., 2002; Douglas et al., 2003; Nemeth et al., 2003; Bao et al., 2005). This has led to the suggestion that the hepcidin-mediated hypoferremic state may increase resistance to infection by restricting iron availability to invading microbes. However, a hepcidin-mediated hypoferremic response to infection does not explain how hepcidin responds to the body's iron needs. Iron deficiency depresses certain aspects of cell-mediated immunity, including lymphocyte, neutrophil and macrophage function (Oppenheimer, 2001). In addition, although the growth of a variety of bacteria and fungi are inhibited in vitro by iron-binding proteins such as transferrin and lactoferrin (Kochan, 1973; Weinberg, 1978), the significance of hypoferremia on the growth of extracellular microbial pathogens in vivo remains a matter of debate (Oppenheimer, 2001). The role of increased hepcidin expression in host defense to infection has yet to be clearly defined.

Besides their in vitro antimicrobial properties, it has been shown that the predominant role of human and murine hepcidins is to regulate iron balance and recycling (Andrews, 2004; Ganz, 2005b). The high degree of structural conservation among hepcidins prompted us to speculate that some fish hepcidins might function in the regulation of iron homeostasis as has been observed in mammals. If so, expression of

fish hepcidin would also be expected to be influenced by anemia through a mechanism similar to that in humans and mice (Nicolas et al., 2002a; Detivaud et al., 2005). To investigate this hypothesis, hepatic expression of hepcidin was examined in catfish suffering from CCA, a common disease of catfish aquaculture, and in non-anemic controls. Our studies clearly demonstrated that CCA affected fish are depleted of body iron stores and that hepcidin gene expression is significantly inhibited in the anemic fish. In addition, hepatic hepcidin expression was also significantly associated with serum iron levels and percent transferrin Fe saturation.

Recently, decreased hepcidin mRNA levels were associated with anemia in *web*$^{Tp85c-/-}$ zebrafish, an animal model of ferroportin-1 deficiency anemia (Fraenkel et al., 2005); however, this is the first report that impaired fish hepcidin gene expression is closely associated with reduced body iron stores. Diminished hepcidin expression is also seen in mice with experimental anemia induced by phlebotomy and hemolytic agents (Nicolas et al., 2002a). Thus, emerging evidence suggests that, similar to mammalian hepcidins, some fish hepcidins, including those of catfish and zebrafish, are also regulated by anemia.

It is generally accepted that mammalian hepcidins regulate iron metabolism in various pathophyiological states, including iron overload, infection, inflammation, and anemia, but the nature of the relationship between hepcidin mRNA level and liver iron concentration appears to be disease specific. There is a significant correlation between hepcidin mRNA expression and hepatic iron concentration in anemia of inflammation, but this relationship is less predictable in patients with hereditary iron disorders. For example, a significant correlation between hepcidin mRNA level and liver iron

concentration exists in patients with chronic hepatitis C (Aoki et al., 2005) and in patients with liver disease, but without HFE genetic hemochromatosis (Detivaud et al., 2005). Mutations in the hereditary hemochromatosis gene HFE lead to progressive tissue iron overload and HFE-deficient mice develop iron overload mimicking human hereditary hemochromatosis (Zhou et al., 1998; Levy et al., 1999b). No correlation between hepcidin mRNA level and hepatic iron concentration is found in adult HFE-deficient mice (Ahmad et al., 2002; Herrmann et al., 2004). Interestingly, a significant correlation between hepcidin mRNA level and hepatic iron concentration was not found in the CCA fish. This suggests that CCA is not an anemia of inflammation.

Although the use of old feed, abnormal folate metabolism, infectious agents, and both naturally occurring and anthropogenic toxins have all been investigated as possible causes of CCA, no specific nutritional deficiency, infectious agent, or toxin exposure has been shown to experimentally reproduce anemia of this severity, and the etiology of the condition remains unclear (Butterworth et al., 1986; Klar et al., 1986; Plumb et al., 1986; Tucker et al., 1989; Burtle GJ et al., 1998). Decreased hepcidin mRNA expression in CCA fish appears to be a normal physiological response to low serum iron concentrations and anemia-induced hypoxia. In light of the lack of a correlation between hepatic hepcidin mRNA levels and liver iron concentrations in CCA fish and HFE-deficient mice, CCA could be a disease resulting from somatic mutations in iron transporters such as ferroportin and divalent metal transporter (DMT-1). However, at present there is no direct evidence to support this hypothesis.

To further understand the molecular evolution of hepcidin genes, we examined the peptide sequence similarity among mammalian, amphibian and fish hepcidins (Figure

4.4). The NH_2-termini of the mature peptides were assigned based on the amino acid sequence of human hepcidin (Krause et al., 2000; Park et al., 2001) and the proximity to the RXK/RR motif characteristic of processing sites for propeptide convertases. It is worth noting that the predicted NH_2-terminus (HSHLSIC-) of hepcidin in the African clawed frog (Xenopus laevis) is highly homologous to that (QSHLSLC-) of hepcidin in some fish, while the C-terminus (-CCLT) of the frog hepcidin is similar to that (-CCIT) of rat hepcidin (Figure 4.4). Although their NH_2-termini may vary significantly, the C-terminus (-CCR/KF) is conserved among most fish hepcidins.

A search of the EST databases at the NCBI identified more than 20 fish hepcidin cDNA sequences (data not shown). However, current investigations on the molecular and biological properties of fish hepcidins have been limited to a few species (Shike et al., 2002; Douglas et al., 2003; Shike et al., 2004; Bao et al., 2005; Hirono et al., 2005). Among the fish hepcidins listed in Figure 4.4, the predicted mature channel catfish peptide is 100% homologous to that found in zebrafish (Danio rerio) and is least homologous (79.8%) to that found in the hybrid striped bass (Morone chrysop*s* x *M.* saxatilis). To date, the only known fish hepcidin peptide sequence is a 21-residue bass hepcidin, which was isolated from gill extracts of adult hybrid striped bass (Shike et al., 2002). The cleavage site for the bass hepcidin is similar to that for hep20, the smallest human hepcidin (Figure 4.4).

We and others have demonstrated that hepcidins may function as iron-regulators in some fish species, including catfish and zebrafish (Fraenkel et al., 2005). However, hepcidins may act only as antimicrobial peptides in other fish species. Because the NH_2-terminus of human hepcidin is essential for its iron regulatory function (Nemeth et al.,

2006), it seems unlikely that the 21-aa hybrid bass hepcidin and Japanese flounder hepcidins (Hep-JF1 and Hep-JF2, Figure 4.4) can function as iron-regulators. Consistent with this speculation, it has been reported that the expression of Hep-JF2, which has the consensus (eight cysteine residues) of hepcidin and is more similar to human hepcidin than Hep-JF-1, is not regulated by iron overload (Hirono et al., 2005). While hepcidin expression is enhanced by iron overloading in humans and mice (Pigeon et al., 2001; Nemeth et al., 2003), the expression of Hep-JF1, a hepcidin-like molecule with only six cysteine residues in the predicted mature peptide region, is dramatically decreased during experimental iron overloading in the Japanese flounder (Hirono et al., 2005). Hybrid bass and flounder hepcidins have demonstrated potent antimicrobial activity (Shike et al., 2002; Douglas et al., 2003; Hirono et al., 2005; Lauth et al., 2005); therefore, it is reasonable to suggest that hepcidins may be antimicrobial peptides but not iron-regulators in hybrid bass and Japanese flounder.

This hypothesis is consistent with observations that some fish hepcidins, which may function only as antimicrobial peptides, are dramatically induced after LPS administration, vaccination, and bacterial infections (Shike et al., 2002; Douglas et al., 2003; Straub et al., 2004; Hirono et al., 2005). However, in animals in which hepcidin functions as an iron-regulatory hormone, its increased expression in response to infection probably represents part of the host acute phase inflammatory response, rather than an antimicrobial defense mechanism. The induction of iron-regulatory hepcidin in infection in mammalians and some other fish species may be best explained by the hypothesis that hepcidins evolved from an antimicrobial peptide and its structure and transcriptional regulatory mechanisms have been conserved throughout vertebrate evolution.

In summary, we have demonstrated that hepcidin expression is enhanced by bacterial infection and, consistent with expected physiologic responses, is lower in catfish suffering from severe anemia. Additional studies are needed to prove that catfish hepcidin plays an essential role in host defense against infection. In light of the recent finding that the NH_2-terminus of human hepcidin is essential for its iron-regulatory function (Nemeth et al., 2006), and the fact that significant structural differences are present at the NH_2-termini of human and catfish hepcidins, further exploration of the role of hepcidin in catfish iron homeostasis will provide new insights into the molecular mechanisms by which hepcidin acts as an iron-regulatory hormone.

Table 4.1. Primers for real time RT-PCR analysis of catfish hepcidin

Primer name	Sequence (5′→3′)	Location in cDNA(nt.)	GenBank accession #
Sense (Hepc)	GAGACGAGTCCTGAGGTGCT	281–300	DQ062122
Antisense (Hepc)	GCAGAACCCACAGCCTTT	382–365	
Sense (β-actin)	GTCTTCCCTTCCATCGTCG	141–159	BE470101
Antisense (β-actin)	TTCTCCCTGTTGGCTTTGG	406–388	

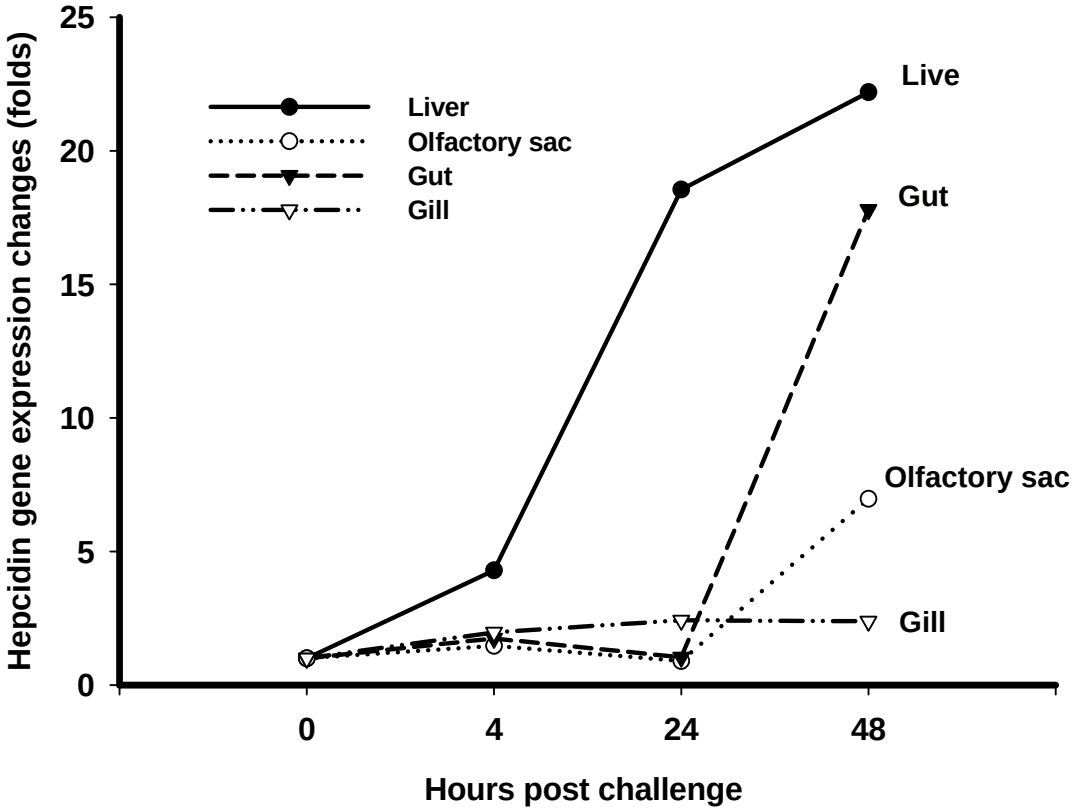

Hepcidin gene expression changes (folds)
25
20
15
10
5
0
Liver
Olfactory sac
Gut
Gill
Live
Gut
Olfactory sac
Gill
0
4
24
48
Hours post challenge

Figure 4.1

Figure 4.1. Time and tissue specific induction of hepcidin expression in channel catfish challenged with E. ictaluri. Shown are results from a real-time PCR experiment. At each time point, total RNA from tissue samples was isolated using TRIzol reagent and the catfish hepcidin gene transcript levels were measured by real time RT-PCR separately. Relative hepcidin expression was calculated based on the hepcidin/actin ratio. Baseline hepcidin expression levels in various tissues were obtained from the control fish (time 0, not challenged with E. ictaluri). Data are representative of two independent real time PCR experiments.

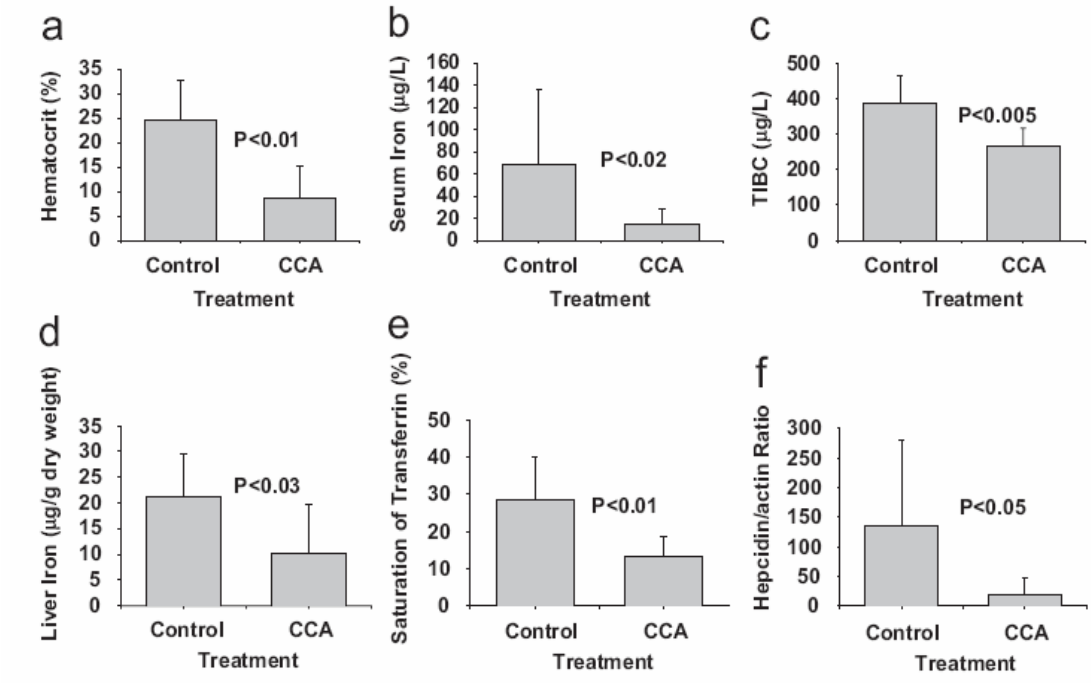

a
Hematocrit (%)
35
30
25
20
15
10
5
0
P<0.01
Control
CCA
Treatment
b
Serum Iron (µg/L)
160
140
120
100
80
60
40
20
0
P<0.02
Control
CCA
Treatment
c
TIBC (µg/L)
500
400
300
200
100
0
P<0.005
Control
CCA
Treatment
d
Liver Iron (µg/g dry weight)
35
30
25
20
15
10
5
0
P<0.03
Control
CCA
Treatment
e
Saturation of Transferrin (%)
50
40
30
20
10
0
P<0.01
Control
CCA
Treatment
f
Hepcidin/actin Ratio
300
250
200
150
100
50
0
P<0.05
Control
CCA
Treatment

Figure 4.2

Figure 4.2. Hepatic hepcidin expression was inhibited in fish affected by CCA. Shown are comparisons between healthy channel catfish and fish affected by CCA in hematocrit (a), serum iron concentration (b), total iron binding capacity (TIBC; c), liver iron concentration (d), percent saturation of transferrin (e), and hepcidin/actin ratio by real time PCR analysis (f). There was no significant difference between the CCA group **(*n* = 7)** and the control group **(*n* = 7)** on gender (3 male and 4 female in each group) and body weight (1.09 ± 0.41 versus 0.81 ± 0.10 pound, respectively). Values are means ± SE. *p*-values are indicated between the paired groups.

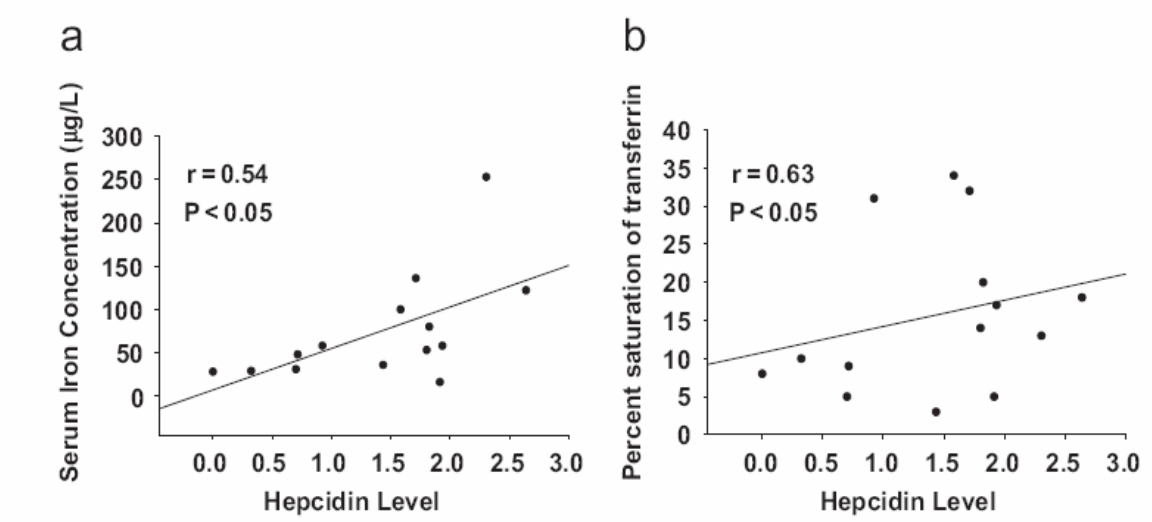

a
Serum Iron Concentration (µg/L)
300
250
200
150
100
50
0
r = 0.54
P < 0.05
0.0 0.5 1.0 1.5 2.0 2.5 3.0
Hepcidin Level
b
Percent saturation of transferrin
40
35
30
25
20
15
10
5
0
r = 0.63
P < 0.05
0.0 0.5 1.0 1.5 2.0 2.5 3.0
Hepcidin Level

Figure 4.3

Figure 4.3. Hepatic hepcidin mRNA level significantly correlated with serum iron concentration and percent saturation of transferrin in channel catfish. Shown are the results of linear regression analysis of catfish hepatic hepcidin mRNA expression level (log [hepcidin/actin ratio]) on serum iron concentration (μg/L; panel a) and percent saturation of transferrin (panel b) in all fish populations **(*n*=14)**. The *p*-values for the correlation coefficient (*r*) in control group **(*n*=7)** or CCA group **(*n*=7)** alone were all larger than 0.05 (data not shown).


Pre-prosegment
...+....+++++.+++....++.. .. .... +..+.+.+ ....**
HUMAN (1) M-ALSSQIWAACLLLLLLLASLTSGS----VFPQQTGQLAELQ-PQ-----DRAGARASWM-PMFQRRRRR
MOUSE (1) M-ALSTRTQAACLL-LLLLASLSSTT----YLHQQMRQTTELQ-PL----HGEESRADIAI--PMQKRRKR
RAT (1) M-ALSTRIQAACLL-LLLLASLSSGA----YLRQQTRQTTALQ-PW----HGAESKTDDSA-LLMLKRRKR
FROG (1) MKSTPI-CCLLLLLSLICYRGHSASL----------SGNEIRVSG--NQISETAMEESNVLEPLI-RSKR
c-catfish (1) MRPMSIACAVAVIIACVCALQSAALPSEVRLDPEVRLEEPEDSEAArSIDQGVAAALAKETSPEVLFRTKR
ZEBRAFISH (1) MKLSNVFLAAVVILTCVCVFQITAVPFIQQVQDEHHVESEELQENQ-----HLTEAEHRLTDPLVLFRTKR
SALMON (1) MKAFSV--AVVLVIACMFILESTAVP----FSEVRTEEVGSFDSPV--GEHQQPGGESMHLPEPF—RFKR
BASS (1) MKTFSVAVAVAVVLAFICLQESSAVP---------VTEVQELEEPM-SNEYQEMPVESWKM—PYNNRHKR
Mature peptide
Similarity to c-hepcidin
(%)
++*..+*.+**+**.+ .+**.**.+
Full / mature
HUMAN (59) DTHFPICIFCCGCCHR-SKCGMCCKT (84) 38.4/70.5
MOUSE (58) DTNFPICIFCCKCCNN-SQCGICCKT (83) 40.8/70.5
RAT (59) DTNFPICLFCCKCCKN-SSCGLCCIT (84) 38.1/72.4
FROG (56) HSHLSICIHCCNCCKF-KGCGKCCLT (81) 45.3/87.3
c-catfish (71) QSHLSLCRYCCNCCKN-KGCGFCCRF (96) 100/100
ZEBRAFISH (66) QSHLSLCRFCCKCCRN-KGCGYCCKF (91) 57.6/100
SALMON (58) QIHLSLCGLCCNCCHN-IGCGFCCKF (86) 54.2/97.1
BASS (59) HSSPGGCRFCCNCCPNmSGCGVCCRF (85) 50.7/79.8

Figure 4.4

Figure 4.4. Amino acid sequences of representative mammalian, amphibian and fish hepcidins predicted from ESTs. The NH_2-termini of mature peptides were assigned based on the amino acid sequence of human hepcidin and the proximity to the RX(K/R)R motif characteristic of processing sites for the subtilisin family of mammalian propeptide convertases (Wise et al., 1990). The alignment was performed using GeneBee programs (http://www.genebee.msu.su/index.html). The meaning of signs at the top of the alignment is following: ( ), no consensus; (.), low consensus; (+), high consensus; and (*), identical. (–) is used for gaps only. Percent identity of each sequence to channel catfish hepcidin (full length prepropeptide and predicted mature peptide) was also determined using the GeneBee program. GenBank accession numbers of the aligned sequences are human (Homo sapiens), BC020612; mouse (Mus musculus), BC021587; rat (Rattus norvegicus), NM053469; African clawed frog (Xenopus laevis), CB199190; channel catfish (Ictalurus punctatus), DQ062122; zebrafish (Danio rerio), NM205583; Atlantic salmon (Salmo salar), BQ036900; Japanese flounder (Paralichthys olivaceus), AB198060 (Hep-JF1), AB198061 (Hep-JF2); and white bass (Morone chrysops), AF394246. Except for the Japanese flounder, when two or more hepcidin ESTs in one species are present in the database, only one EST is listed here. The known cleavage sites for human hepcidin and bass hepcidin mutations are indicated by ↓ and ↑, respectively.

CHAPTER V. HEPCIDIN IN FROG: COMPARATIVE ANALYSIS OF XENOPUS TROPICALIS HEPCIDIN I AND HEPCIDIN II

INTRODUCTION

Hepcidin was originally identified as a 25 amino acid antimicrobial peptide produced in the liver (Krause et al., 2000; Park et al., 2001). However, hepcidin is now known as the key regulator of iron homeostasis in both humans and mice and presumably all mammals (Nicolas et al., 2001; Pigeon et al., 2001; Nicolas et al., 2002a; Weinstein et al., 2002; Roetto et al., 2003). Hepcidin binds to the cellular iron export channel, ferroportin and causes its internalization, thus decreasing iron efflux from iron exporting tissues into plasma (Nemeth et al., 2004b). Hepcidin transcription is increased by either iron loading or inflammation, and is decreased by anemia or hypoxia (Pigeon et al., 2001; Nicolas et al., 2002b). Hepcidin deficiency plays a central role in most known forms of hereditary hemochromatosis (Pietrangelo, 2004; Fleming et al., 2005). On the other hand, overexpression of hepcidin under inflammatory conditions is the major cause of anemia of inflammation (Ganz, 2003; Andrews, 2004). The hepcidin involvement in iron homeostasis and its pathologies suggests that therapeutic targeting hepcidin would be useful in treating iron disorders (Ganz, 2006). However, a structure/function study demonstrated that the hepcidin-ferroportin interaction is highly tolerant of hepcidin mutations (Nemeth et al., 2006), making it difficult to understand the molecular mechanism for the interaction between hepcidin and its receptor, ferroportin.

In contrast to other antimicrobial peptides, hepcidin is highly conserved throughout vertebrate evolution (Shi and Camus, 2006; Padhi and Verghese, 2007). Closely related hepcidin genes and peptides have been identified in mammals and a number of fish species, providing us a rich resource for hepcidin structure/function analysis. Shi and Camus (2006) reported at least two groups of hepcidins in fishes and hypothesized that the iron-regulatory hormone, hepcidin in humans may have evolved from fish antimicrobial peptide during vertebrate evolution. In support of this hypothesis, some studies showed that hepcidin may complement iron hormone function in some fish species (Fraenkel et al., 2005; Rodrigues et al., 2006; Hu et al., 2007). The multiple copies of hepcidin found in some fish species also strengthens the possibility of a dual function for hepcidin, either iron regulation or as an antimicrobial peptide (Hirono et al., 2005; Kim et al., 2005; Huang et al., 2007; Yang et al., 2007). To further understand the two hepcidin functions, suitable animal models from different evolutionary stages of the vertebrates are needed.

Xenopus has long been used as a major vertebrate model for cellular and developmental biology research (Klein et al., 2002). In the past, most work on Xenopus focused on the African clawed frog (Xenopus laevis). However, recently more attention has been given to the western clawed frog (Xenopus tropicalis), a close relative of X. laevis. Two obvious reasons exist (Beck and Slack, 2001; Hirsch et al., 2002): first, X. tropicalis offers one of the smallest genomes among the amphibians and is the only diploid Xenopus, greatly simplifying genetic studies. Second, X. tropicalis has a relatively short (3~41/2 months) life cycle, in contrast to the slower-growing tetraploid X. laevis with a life cycle of 8~12 months. These advantages in X. tropicalis greatly reduce

the time and space needed to make inbred lines and to perform genetic screens. Presently, there are useful mammalian (mouse) and fish (zebrafish) models for hepcidin study; however, an evolutionary link between these two vertebrate groups is lacking. To fill this gap, X. tropicalis may be a good candidate since it has two hepcidin genes.

Here, we describe results of cloning copies of hepcidin cDNA (tHEP1 and tHEP2) in X. tropicalis and analyzing their genomic organization. We identified the tissue expression patterns of these two hepcidins and their different responses to iron loading and to the stress responsive hormone, corticosterone, using real time RT-PCR. Our results show that tHEP2 has iron regulatory hormone function in this frog species while tHEP1 is likely an ancestral antimicrobial peptide.

MATERIALS AND METHODS

Frogs

Frogs for all experiments were purchased from Harland Lab (Berkeley, CA). In all experiments adult male western clawed frogs (X. tropicalis) were housed communally (5/aquaria) in 37.8 L glass aquaria. The water in all aquaria was approximately 20 cm deep. Each aquarium was lined with approximately 4 cm of clean aquaria rock and contained 2 refugia made of broken 7 cm clay pots. The aquaria were equipped with a 300 L/hr filter and a 50 watt heater and allowed to equilibrate for 1 week prior to introduction of frogs. For all experiments, except noted, water temperature was maintained at 27 ºC. The frogs were kept on a 12:12 L:D cycle throughout the experiment and fed every other day with Xenopus food (Carolina Biological, Burlington,

NC), approximately 10 pellets/frog. Excess food was removed after 2 hrs. Frogs were allowed to acclimate to the aquaria for 1 week prior to experimentation.

All frogs were euthanized prior to sampling using 300 ppm MS222 (also called tricaine or 3-aminobenzoic acid ethyl ester, Sigma), with absorption through the skin.

Tissue preparation and RNA extraction

Frogs were euthanized by MS222 after one week of acclimation in the aquaria. For the tissue expression experiment, portions of fourteen tissues (bladder, blood, bone marrow, brain, fat, heart, kidney, large intestine, liver, lung, muscle, skin, small intestine, and stomach) from one frog were removed and stored in 0.5mL RNAlater until RNA was extracted. All other experiments were performed as indicated. Total RNA was extracted from the tissues using TRIzol (Invitrogen Company), following the protocol recommended by the manufacturer. The concentration and quality of total RNA was determined by both spectrophotometer and electrophoresis. RNA samples were stored at -20ºC. Blood and liver were also used to determine Fe concentrations (see below).

RT-PCR and cDNA cloning

A search was performed in the expressed sequence tag (EST) database for sequences similar to human hepcidin using the BLAST program from National Center for Biotechnology Information (NCBI). Two ESTs from X. tropicalis (accession numbers are DN020182 and DN039682) were identified that predicted two peptides consistent with the consensus amino acid residues of human hepcidin. Based on these ESTs, two pairs of primers were designed for RT-PCR (see table 5.1). Total RNA samples prepared

as described above were used for gene expression analysis. The RT-PCR was performed using the one step AccessQuick™ RT-PCR System (Promega). Thirty nanograms total RNA was used per reaction. The reactions were completed in a thermocycler with the following thermo-profiles: 46ºC for 45 min, 95ºC for 2 min, followed by 30 cycles of 94ºC 20s, 56ºC for 45s, and 72º C for 30s. Upon completion of the PCR, the reaction was incubated at 72ºC for 5 min and held at 4ºC.

The RT-PCR products were analyzed by electrophoresis on a 1.2% agarose gel showing a specific band of about 430bp for tHEP1 and 570bp for tHEP2. The PCR products were purified from an agarose gel using a QiaQuick gel purification Kit (Qiagen), cloned into TOPO TA vector (Invitrogen), then were submitted for sequence verification.

Iron and Corticosterone Treatment

Twenty frogs were randomly divided into one of 4 experimental groups (n = 5); high dose Fe acute treatment (acute), low dose Fe chronic (chronic), control, and corticosterone. Acute frogs received 0.2 mL daily injections, 10 mg/mL Fe dextran interperitoneally (IP) for 2 days. Corticosterone treated frogs received 0.1 mL daily injections of 1 mg/mL corticosterone in corn oil IP for 4 days. Chronic and control frogs received 0.1 mL injections, 5 mg/ml Fe dextran and 0.9% saline IP respectively. Chronic and control frogs were injected for 5 days and not injected on the 6^{th} and 7^{th} days for 3 weeks.

Acute Fe and corticosterone treated frogs were euthanized the day after the last injection, while chronic and control frogs were euthanized the third day after the last

injection. Blood, heart, kidney, liver, stomach and testis were removed from all frogs. Blood and liver were used for Fe analysis and liver, heart, kidney, stomach and testis were used for hepcidin analysis as described below.

Real time RT-PCR

A primer pair (see table 1), tHEP1 F209 and tHEP1 R346, was designed for the tHEP1 gene amplifying a 138 bp fragment of cDNA. For the tHEP2 gene, the same primers (the size is 208 bp) used in RT-PCR reactions were applied for real time RT-PCR analysis. A pair of primers for 18S RNA which amplifies the conserved region of 18S RNA cDNA (size is 82 bp), was used as an internal control for real time RT-PCR. A two-step real time PCR was carried out by a LightCycler (BioRad company) using the SYBR Green I reagents kit (BioRad) following the protocol from the provider with some modifications. First step is to make cDNA from total RNA (1 μg). The reactions were completed in a thermo-cycler with 25ºC 5 min, 42ºC 30 min, 85ºC 5 min and a 4ºC hold. The real time RT-PCR reactions were optimized using an annealing temperature gradient and a DNA concentration standard curve on the primers. The optimized real time PCR conditions are: 2 min at 95ºC; 40 amplification cycles of denaturation for 10s at 94ºC; annealing for 45s at 60ºC (fHEP-1 and fHEP-2), and for 45s at 64ºC (18S RNA). Melting curve analysis: 1 min at 95ºC, 1 min at 55ºC and then increase to 95ºC at a rate of 0.5ºC per second. The relative expression of fHEP-1 or fHEP-2 was calculated relative to the 18S RNA internal control expression (Pfaffl, 2001; Hu et al., 2007).

Blood and liver Fe determination

Serum iron determinations were performed in a Cobas Mira automated analyzer using reagents as per manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). Liver iron determinations were performed using a modification of the colorimetric method of Torrance and Bothwell (Torrance and Bothwell, 1968) using Stanbio Laboratory-Iron procedure 0370 reagents (Stanbio Laboratory, Boerne, TX). Analyses were measured using a Spectramax-340PC microplate reader (Molecular Devices, Corporation, Sunnyvale, CA). To determine whether *X. tropicalis* hepcidin expression was affected by iron loading, the relative quantity of hepcidin mRNA in the liver was determined by real-time PCR as described above. Real time expression of the hepcidin versus 18S RNA genes (hepcidin/18S RNA ratio) of individual liver samples from control frogs and frogs injected with Fe-dextran or corticosterone was calculated as described previously (Hu et al., 2007).

Computer analysis and data presentation

A homology search was performed using blastp 2.1.2 and tblastn 2.1.3 against NCBI and ENSEMBL genome databases. Sequence alignment was performed with the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) with default parameters. Putative transcription factor binding sites were predicted by TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html). Potential cleavage sites of the signal peptide were identified by SignalP, and propeptide cleavage sites were predicted using ProP1.0 (http://www.cbs.dtu.dk/services/).

SigmaPlot 9.0 and SigmaStat3.01 (Systat Software, Inc., 2004) were used to analyze the data. ANOVA was used to compare the hepcidin expression levels and iron status between the different groups.

RESULTS

Xenopus tropicalis hepcidin cDNAs and gene organization

In the EST Genebank, we identified three sets of frog ESTs highly homologous to channel catfish hepcidin (DQ062122): DN020182, DN039682 (X. tropicalis) and CB199190 (X. laevis). Based on these ESTs, we cloned and sequenced the two hepcidin-like cDNA sequences from X. tropicalis by RT-PCR using a high fidelity Taq polymerase. The two frog hepcidin cDNAs were named tHEP1 and tHEP2 (accession numbers are EF140888 and EF140889 respectively). The tHEP1 cDNA contains an open reading frame (ORF) of 246 bases and encodes an 81-amino acid prepro-hepcidin-peptide, while the tHEP2 cDNA contains an ORF of 243 bases encoding an 80-amino acid prepro-hepcidin-peptide. The homology percent between the complete tHEP1 and tHEP2 sequences is 73.9% and between the mature peptides is 88.4%. The predicted signal peptides of both have 22 amino acid residues, and the mature peptides have 25 and 24 amino acid residues in tHEP1 and tHEP2 respectively. The predicted mature peptide of tHEP1 starts with -QSHLSIC- motif, which is similar to some fish hepcidins (Shi and Camus, 2006). Though tHEP2 is short two amino acid residues at the NH_2 terminus (-HLNIC-), it is still very possible to be functional *in vivo* (Nemeth et al., 2006). The C-terminus of both tHEP1 (-CCLT) and tHEP2 (-CCFT) are similar to mammalian hepcidins (-CCK/IT).

From a tblast search with tHEP1 and tHEP2 cDNAs against the ENSEMBL genome database, we identified a scalfffold_351 as the frog hepcidin gene locus. By aligning the cDNAs and scalfffold_351, we showed that the two hepcidin genes have a 3 exon and 2 intron structure, conserved with the mammalian orthologs (Figures 5.1 and 5.2). Comparing tHEP1 and tHEP2, the first two exons are the same sizes (81 bp and 63 bp) but the third exon is slightly different (102 bp for tHEP1 and 99 bp for tHEP2) (Figures 5.1 and 5.2). Further analysis revealed that an upstream stimulatory factor 2 (USF2) is located upstream of tHEP1 and tHEP2 in a tail-to-head manner with a distance of 10,087 bp, while the distance between tHEP1 and tHEP2 is 7,230 bp. Using TFSEARCH, we analyzed 2.0 kb upstream of the translation start and identified several putative transcriptional factor binding sites (Table 5.2). We found that tHEP2 promoter region has ten short repeats of 52 bp length containing NF-κB binding elements at the region from -809 to -1327 (Figure 5.6). Also tHEP2 does not have any USF elements, but has 5 C/EBPβ elements while tHEP1 has 3 C/EBPα elements in the analyzed area. There is no general similarity in the 2.0 kb regions that we examined. However, we did find a 115 bp sequence at the proximal region of the two promoters (tHEP1: -168~-54; tHEP2: -155~-44) that shares a high similarity (85%). Further blast searching the X. tropicalis genome database revealed that this small piece of conserved sequence is specific to the two hepcidin genes.

Different tissue expression pattern of tHEP-1 and tHEP-2 (Figure 5.3)

Analysis of gene expression using RT-PCR indicated that X. tropicalis hepcidins have different specific tissue expression patterns. The tHEP1 gene is predominantly

expressed in the liver and kidney and has reduced expression in the heart. The tHEP2 gene is predominantly expressed in the liver, kidney and stomach and has much lower expression level in the testis and heart (Figure 5.3).

tHEP1 and tHEP2 are differently regulated by iron loading (Figure 5.4)

To determine the response of tHEP1 and tHEP2 to iron loading, we developed an iron loading assay and applied quantitative real time RT-PCR to measure the expression levels of tHEP1 and tHEP2 in the frog liver upon iron loading. The result (Figure 5.4) showed that only tHEP2 was significantly up-regulated (3.6 x) by acute iron loading in the liver. The expression of tHEP1 was not significantly changed in either acute or chronic iron injections. Serum iron concentration significantly increased (2x) upon acute iron injection while liver iron concentration significantly increased (5.1x) upon chronic iron injection (Figure 5.4), which was to be as expected.

tHEP1 and tHEP2 are differently regulated by corticosterone (Figure 5.5).

Corticosterone is the most abundant and potent glucocorticoid in frogs. It is important in the regulation of glucose/energy metabolism, immune reactions and stress responses. Previous study showed that glucocorticoid treatment of the frog Rana esculenta inhibits the transcription of all genes encoding antibacterial peptides (Simmaco et al., 1997). Since hepcidins have been demonstrated to be involved in the immune response such as infection (inflammation) and stress responses such as hypoxia (Nicolas et al., 2002b), it was of interest to evaluate the response of the hepcidin genes to the stress responsive hormone, corticosterone. The result showed that the expression of tHEP1 is

significantly repressed (43%) by corticosterone injection while tHEP2 was not affected (Figure 5.5).

DISCUSSION

We report here the molecular characterization of two hepcidins in X. tropicalis that share striking similarity in gene organization and peptide primary sequence with hepcidins from other vertebrates spanning fishes to humans (Shi and Camus, 2006). Both hepcidin genes have three exons and two introns and have high similarity in primary amino acid sequences (88.4% for mature peptides). The tissue expression of the two hepcidin genes suggests that they may have different functions and be regulated differently. The expression of tHEP2 was increased upon acute iron loading, indicating that it is involved in iron metabolism. In contrast, tHEP1 was repressed by stress responsive hormone corticosterone and not induced by iron loading, demonstrating a potential role in the stress response. These results suggest that X. tropicalis is a promising animal model for the hepcidin study, and provides an evolutionary link between fish (zebrafish) and mammalian (mouse) animal models.

Hepcidin expression and regulation in humans and mice have been extensively studied. Hepcidin expression is up-regulated by iron; by the cytokines (IL-1 and IL-6); and by bone morphogenetic proteins (BMPs). The hepcidin gene is down-regulated by anemia and hypoxia. The transcriptional regulation of hepcidin is complex. CCAAT/enhancer-binding proteins (C/EBPα and C/EBPβ) (Courselaud et al., 2002) and the basic helix-loop-helix leucine zipper (bHLH-ZIP) family of transcriptional regulators such as USF and c-Myc/Max (Bayele et al., 2006) were identified as potent activators for

hepcidin expression. Interestingly, both C/EBPα/β and USF are also involved in the energy/glucose metabolism, indicating crosstalk between iron homeostasis and glucose metabolism. The cytokines (IL-1 and IL-6) were found to be another group of important activators of hepcidin expression under inflammatory conditions (Nemeth et al., 2003; Inamura et al., 2005; Lee et al., 2005a). Recently, it has been shown that signal transducer and activator of transcription 3 (STAT3) plays a role in the inflammatory regulation of hepcidin and that a crucial binding site is located at -148 to -130 from the translation start of the human hepcidin gene (Wrighting and Andrews, 2006; Verga Falzacappa et al., 2007). Under hypoxia conditions, it was found that reactive oxygen species (ROS) but not hypoxia-inducible factors (HIFs) repress the hepcidin gene expression by preventing C/EBPα and STAT-3 from binding to the hepcidin promoter (Choi et al., 2007). BMPs regulate the transcription of hepcidin by binding to hemojuvelin as a coreceptor and signaling through Smad4 (mothers against decapentaplegic homolog 4) (Wang et al., 2005; Babitt et al., 2006). Truksa et al (2007) showed that the regulation of hepcidin expression by IL-6 and BMPs occurs through distinct regulatory elements. The induction of hepcidin by BMPs requires at least two regions of the mouse HEPC1 promoter (one between 140-260 bp and the other between 1.6-2.0 kb upstream of the start of translation). In contrast, the IL-6 response required only the proximal 260 bp HEPC1 promoter region (Truksa et al., 2007b). Furthermore, there were no regulatory elements located in the non-coding or coding regions of HEPC1. The same group showed that the region of the promoter between 1.6 kb and 1.8 kb upstream from the start of translation is essential for the response to iron (Truksa et al., 2007a).

In order to better understand the regulation of frog hepcidin genes, we compared their promoter areas. We found that there was no general similarity between the promoter regions as well as the intron areas of the two hepcidin genes, suggesting that it is likely the duplication of these two frog hepcidin genes happened ancestrally. A short piece of sequence (155 bp) at the proximal region of the promoters was found to share high similarity of 85%, which further supports the duplication hypothesis. Using the online tool TFSEARCH, several transcriptional factors in the 2.0 kb upstream of the start of translation were identified. Consistent with the human and mouse data, many of these elements (such as C/EBPα, C/EBPβ, HNF3β and STAT3) are located within the proximal 260 bp and 1.6-2.0 kb upstream of the start of translation. There are three striking characteristics found in frog hepcidin genes: 1) the tHEP1 promoter does not have a NF-κB element but has four USF elements; 2) the tHEP2 promoter does not have USF element but has ten NF-κB elements (Figure 5.5); 3) the tHEP1 promoter has 3 C/EBPα but no C/EBPβ elements, while the tHEP2 promoter has five C/EBPβ but no C/EBPα elements. These differences between the two promoter areas may help to explain the different tissue expression patterns and responses to iron loading or hormone stimulation.

Both mouse HEPC1 and HEPC2 are up-regulated by iron loading although only HEPC1 is critical to iron metabolism (Ilyin et al., 2003; Lou et al., 2004). In contrast, in X. tropicalis only tHEP2 is responsive to iron loading, indicating the two frog hepcidin genes are regulated differently. The finding that only tHEP1 was repressed by treatment with the hormone corticosterone supports this speculation. However, it is difficult to reconcile these facts to our findings. Based on the alignment analysis of the primary sequences tHPE1 is more likely to act as an iron-regulatory hormone (Shi and Camus,

2006). The fact that the upstream stimulatory factor 2 (USF2) is located in the upstream of tHEP1 and tHEP2 in a tail-head manner (similar to human and mouse) further suggests that tHEP1 could be the ortholog of mammalian hepcidins. However, this discrepancy could partially be explained by evaluating the promoter regions of the genes. Both C/EBPα and USF are involved in the energy/glucose metabolism and are potent activators of hepcidin in humans and mouse (Nicolas et al., 2001; Courselaud et al., 2002; Bayele et al., 2006). On the other hand, one of the functions of corticosterone is to inversely regulate the concentration of serum glucose. Upon stress or infection, the body increases the serum corticosterone concentration (cortisol in humans) that thereby decreases the serum glucose (Fernandez-Real et al., 2002). Considering that C/EBPα and USF are only found in the promoter area of tHEP1, it suggests that corticosterone represses tHEP1 through pathway(s) involving C/EBPα and/or USF. For tHEP2, its response to iron loading most likely relies on an inflammatory pathway involving the NF-κB elements found in the promoter area of tHEP2. However, the NF-κB element in the promoter or enhancer region is a hallmark of the genes encoding antimicrobial peptide (Boman, 1998; Boman, 2003). Therefore, more studies are needed to determine the role of tHEP2 in iron metabolism.

Finally, it is worthwhile to point out that the origins of mouse hepcidins and X. tropicalis hepcidins appear different. As many others have noted, mouse hepcidins evolved from a very recent duplication event. This notion is supported by the very high similarity (>90%) between their DNA sequences. In particular, the similarity between the promoter regions (>2.0 kb upstream of the start of translation) of the mouse hepcidin genes is also very high (>90%), which may explain why they are both up-regulated by

iron (Ilyin et al., 2003). However, the functions of these two mouse hepcidin peptides are definitely different. It is HEPC1 but not HEPC2 that is involved in the iron homeostasis (Lou et al., 2004), which could be due to some critical mutation in HEPC2 peptide. In contrast, we did not find a significant similarity between the two frog hepcidin genes in terms of the promoter regions as well as the intron areas in spite of the high similarity shared by their ORFs. This suggested that the two frog hepcidin genes evolved from an ancestral duplication event. The significant difference between the promoter areas of the two frog hepcidin genes correlates with their different responses to various stimuli. Most importantly, our study also indicates that both frog hepcidin genes, at least tHEP2, could represent the intermediate(s) of mammalian hepcidins during evolution. At this point, X. tropicalis could serve as a complementary animal model for the analysis of hepcidin, and provides us an evolutionary component to evaluate the regulation and function of hepcidin.

Table 5.1. Primers and their sequences used in this study

Primers	Sequences (5′–3′)	Utilization
fHEP-1 forward	GCAAGAGACATTCCCACCTCTC	RT-PCR
tHEP1 reverse	ACTCCTGTACATGCCTA	RT-PCR
tHEP2 forward	CTCCCTCAGTGGGAATGAAA	RT-PCR and Real time RT-PCR
tHEP2 reverse	TGTTCCTCCTTTCTGGATCG	RT-PCR and Real time RT-PCR
tHEP1 F209	AGATCCCTGAAACCCAGATGGAG	Real time RT-PCR
tHEP1 R346	AGGTCAGACAGCATTTGCCACAG	Real time RT-PCR
18S RNA forward	AGCTCGCGTTGATTAAGTCC	RT-PCR internal control
18S RNA reverse	CCGAGGACCTCACTAAACCA	RT-PCR internal control

Table 5.2. Putative transcription factor binding sites

Transcription factors	tHEP1	tHEP2
TATA box	-92	-82
C/EBPa	-235, -1799, -1962	
C/EBPβ		-252, -379, -530, -1428, -1787
HNF3β	-187, -1542	-181, -190, -1704
USF	-671, -830, -998, -1856	
AP-1	-120, -1570, -1779, -1940	-707
STATx	-1798	-230
NF-κB		-860, -912, -964, -1016, -1068, -1120, -1172, -1224, -1276, -1327

Putative transcription factor binding sites were predicted in the upstream -2.0 kb of the start of translation of tHEP1 and tHEP2 using TFSEARCH. C/EBP: CAAT enhancer-binding protein; USF: upstream stimulating factor; AP-1: activating protein 1; HNF: hepatocyte nuclear factor; NF-κB: nuclear factor-kappaB.

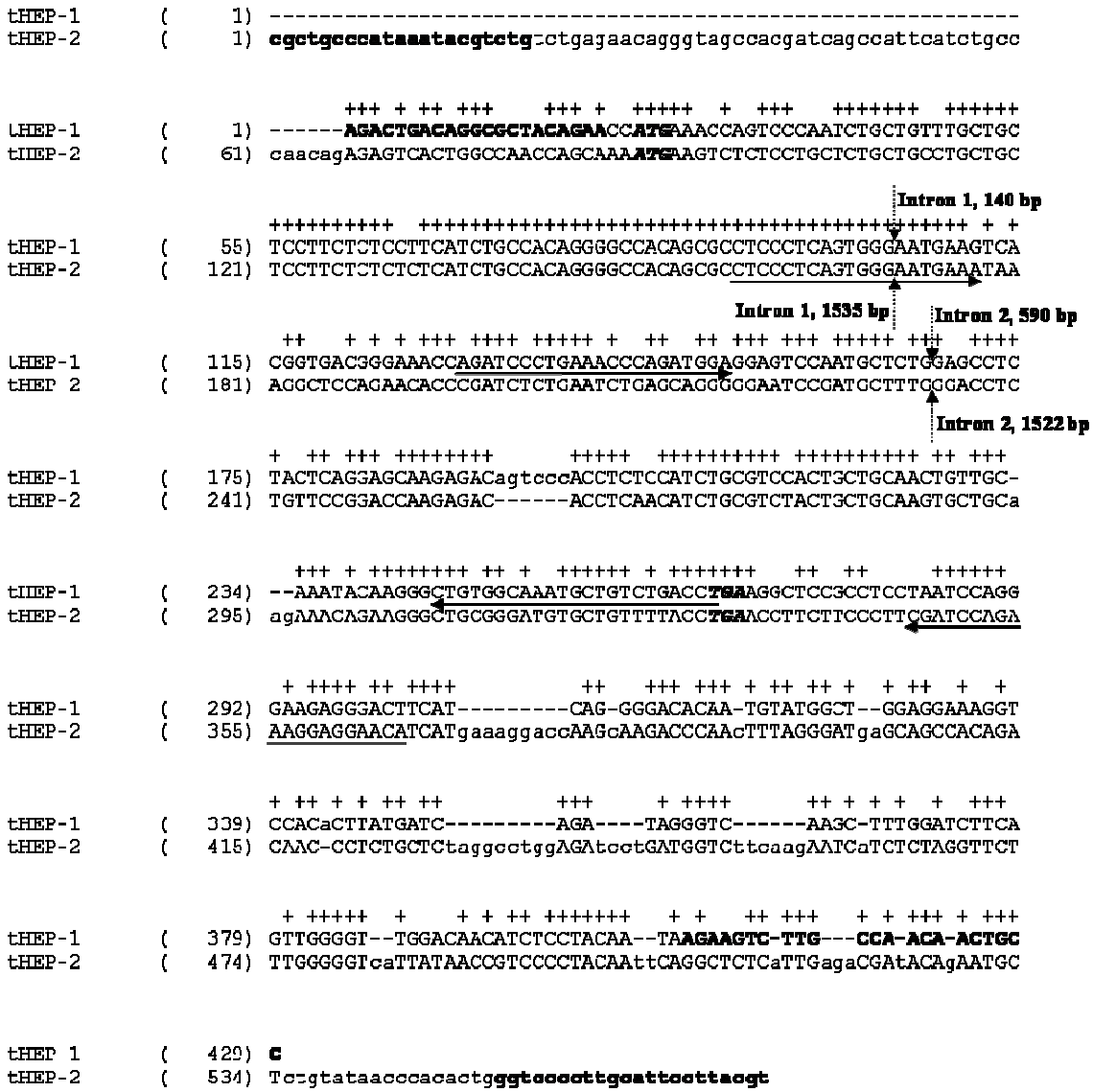

Intron 1, 140 bp
Intron 1, 1535 bp
Intron 2, 590 bp
Intron 2, 1522 bp

Figure 5.1A

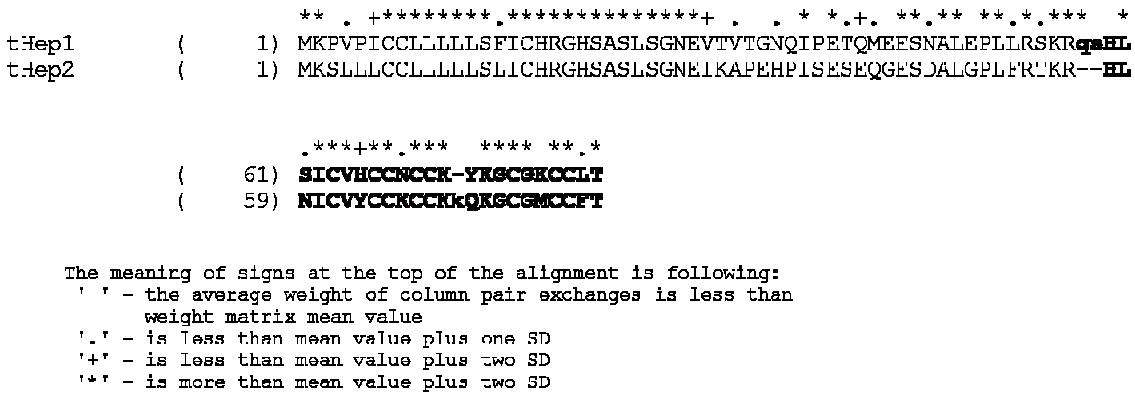

tHep1 (1) MKPVPICCLLLLSFICHRGHSASLSGNEVTVTGNQIPETQMEESNALEPLLRSKRqaHL
tHep2 (1) MKSLLLCCLLLLSLICHRGHSASLSGNEIKAPEHPISESEQGESDALGPLFRLKR--HL
(61) SICVHCCNCCK-YKSCGKCCLT
(59) NICVYCCKCCKkQKSCGMCCFT
The meaning of signs at the top of the alignment is following:
' ' - the average weight of column pair exchanges is less than
weight matrix mean value
'.' - is less than mean value plus one SD
'+' - is less than mean value plus two SD
'*' - is more than mean value plus two SD

Figure 5.1B

Figure 5.1. Alignment of cDNA and protein sequences of tHEP1 and tHEp2. A) The alignment of cDNA sequences of tHEP1 and tHEp2: arrows show the positions of introns and the primers for real-time PCR. B) The alignment of protein sequence of tHEP1 and tHEP2: the predicted mature peptides shown in bold; the cleavage sites of the mature peptides are predicted by SignalP 3.0.

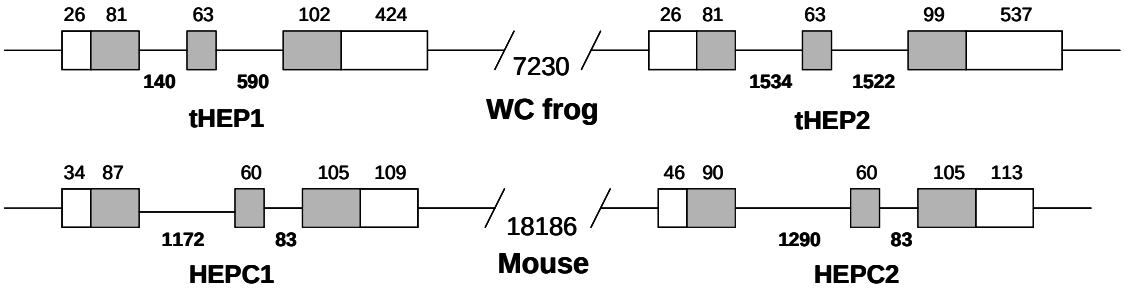

26 81 63 102 424
140 590
tHEP1
7230
WC frog
26 81 63 99 537
1534 1522
tHEP2
34 87 60 105 109
1172 83
HEPC1
18186
Mouse
46 90 60 105 113
1290 83
HEPC2

Figure 5.2

Figure 5.2. Comparison of genomic structures of Western clawed frog and mouse hepcidin genes. Information on Western clawed frog (WC frog) was extracted from mRNAs EF140888 (tHEP1) and EF140889 (tHEP2), ESTs DN020182 (tHEP1) and DN039682 (tHEP2) and scaffold_351. Mouse information comes from the alignment of mRNAs AF503444 (HEPC1) NM_183257 (HEPC2), DNAs NC_000073 (HEPC1) NC_000073 (HEPC2), and mouse chromosome 7. Boxes represent the exons; grey parts indicate the coding regions while open parts show the 5' upstream regions or 3' downstream regions.

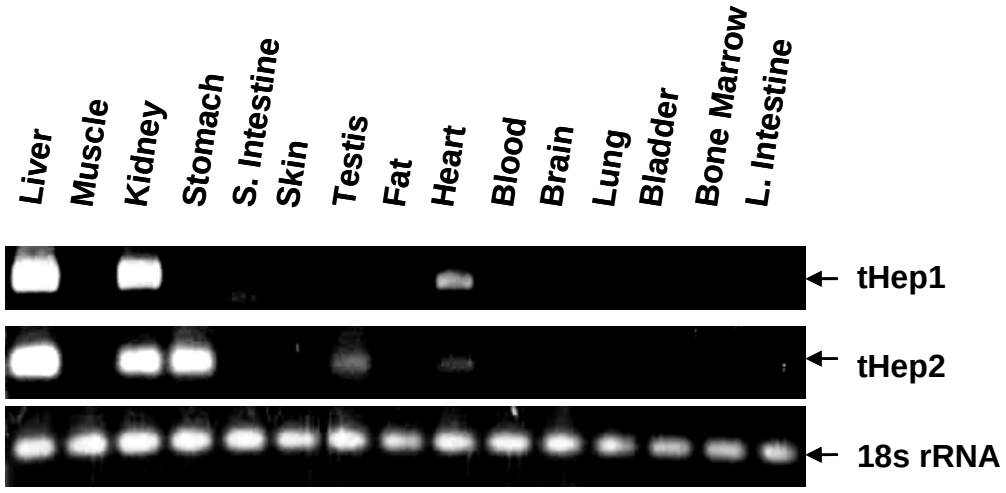
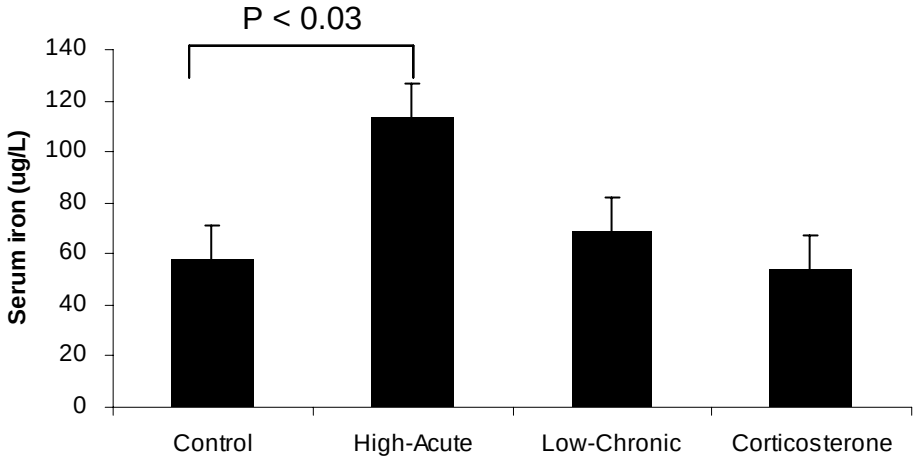

Liver
Muscle
Kidney
Stomach
S. Intestine
Skin
Testis
Fat
Heart
Blood
Brain
Lung
Bladder
Bone Marrow
L. Intestine
tHep1
tHep2
18s rRNA

Figure 5.3

Figure 5.3. Analysis of tissue expression pattern of tHEP1 and tHEP2 using RT-PCR. Fifteen tissues were sampled and studied: Liver, muscle, kidney, stomach, small intestine, large intestine, skin, testis, fat, heart, blood, brain, lung, bladder, and bone marrow.

A


P < 0.03
140
120
100
80
60
40
20
0
Serum iron (ug/L)
Control
High-Acute
Low-Chronic
Corticosterone

B

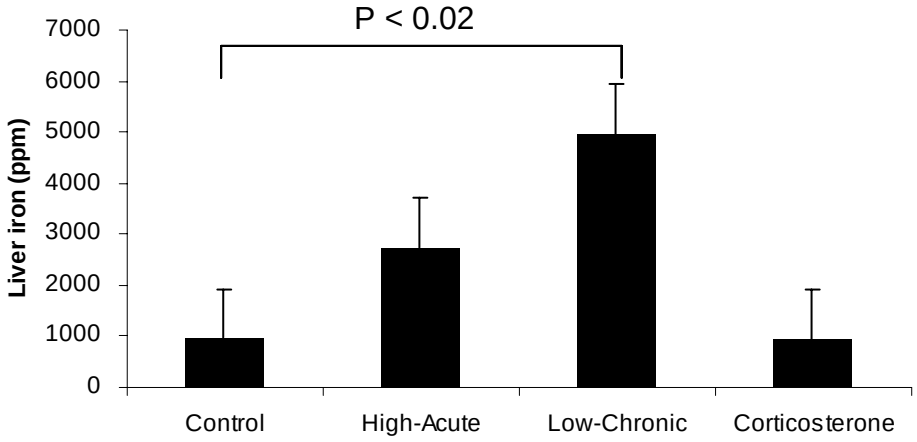
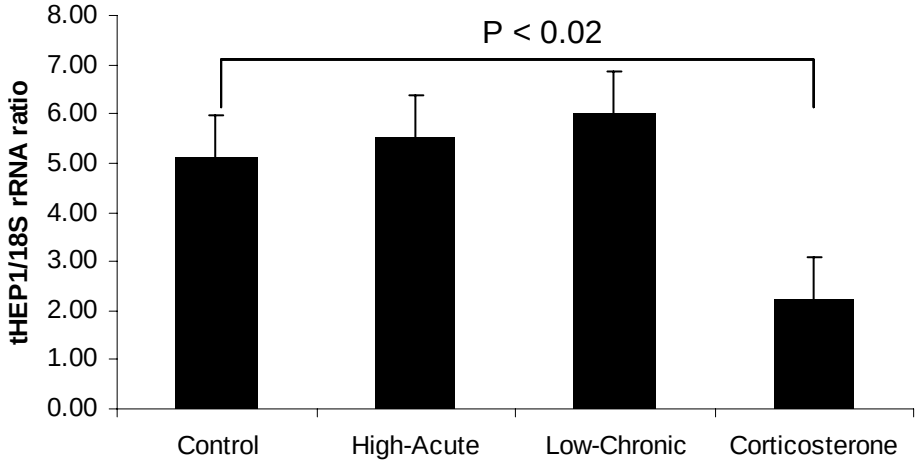

P < 0.02
7000
6000
5000
4000
3000
2000
1000
0
Liver iron (ppm)
Control
High-Acute
Low-Chronic
Corticosterone

Figure 5.4

Figure 5.4. Iron conditions are different among different treatments. Groups of frogs were treated with glucose (control), high-acute iron, low-chronic iron, and corticosterone. A) Serum iron; B) Liver iron.

A


P < 0.02
tHEP1/18S rRNA ratio
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Control
High-Acute
Low-Chronic
Corticosterone

B

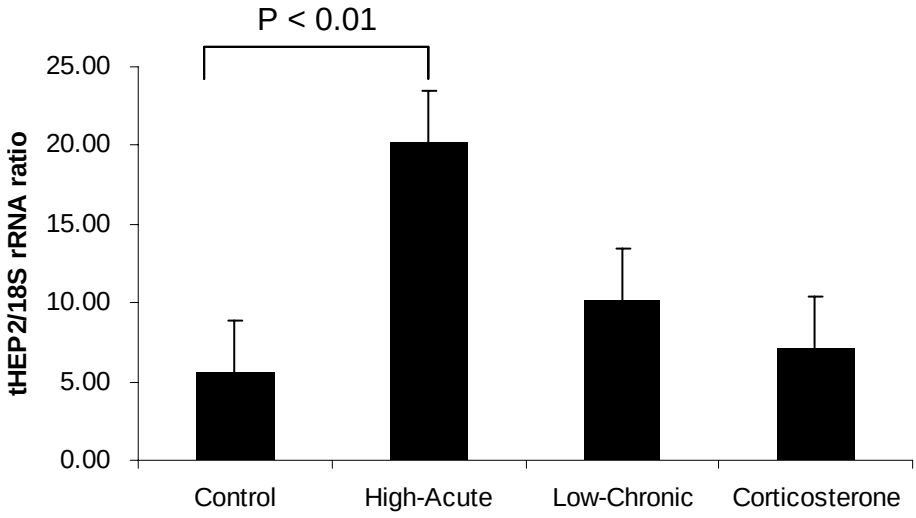

P < 0.01
tHEP2/18S rRNA ratio
25.00
20.00
15.00
10.00
5.00
0.00
Control
High-Acute
Low-Chronic
Corticosterone

Figure 5.5

Figure 5.5. tHEP1 and tHEP2 respond differently to iron loading or corticosterone. Groups of frogs were treated with glucose (control), high-acute iron, low-chronic iron, and corticosterone. The expression levels of hepcidins were measured by real-time PCR using 18S rRNA as control gene. A) tHEP1 responds to the treatments differently; B) tHEP2 responds to the treatments differently.

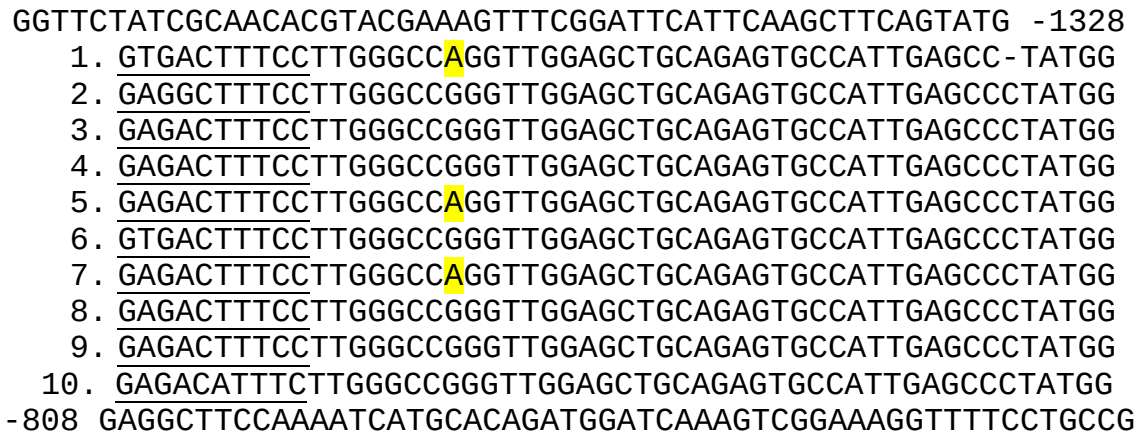

GGTTCTATCGCAACACGTACGAAAGTTTCGGATTCATTCAAGCTTCAGTATG -1328
1. GTGACTTTCCTTGGGCCAGGTTGGAGCTGCAGAGTGCCATTGAGCC-TATGG
2. GAGGCTTTCCTTGGGCCGGGTTGGAGCTGCAGAGTGCCATTGAGCCCTATGG
3. GAGACTTTCCTTGGGCCGGGTTGGAGCTGCAGAGTGCCATTGAGCCCTATGG
4. GAGACTTTCCTTGGGCCGGGTTGGAGCTGCAGAGTGCCATTGAGCCCTATGG
5. GAGACTTTCCTTGGGCCAGGTTGGAGCTGCAGAGTGCCATTGAGCCCTATGG
6. GTGACTTTCCTTGGGCCGGGTTGGAGCTGCAGAGTGCCATTGAGCCCTATGG
7. GAGACTTTCCTTGGGCCAGGTTGGAGCTGCAGAGTGCCATTGAGCCCTATGG
8. GAGACTTTCCTTGGGCCGGGTTGGAGCTGCAGAGTGCCATTGAGCCCTATGG
9. GAGACTTTCCTTGGGCCGGGTTGGAGCTGCAGAGTGCCATTGAGCCCTATGG
10. GAGACATTTCTTGGGCCGGGTTGGAGCTGCAGAGTGCCATTGAGCCCTATGG
-808 GAGGCTTCCAAAATCATGCACAGATGGATCAAAGTCGGAAAGGTTTTCCTGCCG

Figure 5.6

Figure 5.6. Ten short repeats containing NF-κB element found within the promoter area of tHEP2 gene. These short repeats with a length of 152 bp share very high similarity (> 99%). These repeats are located from -808 bp to -1328 bp (the start of translation is 0 bp)

CHAPTER VI. STRUCTURE AND FUNCTION: CO-EVOLUTION OF IRON-REGULATORY HORMONE HEPCIDIN AND ITS RECEPTOR FERROPORTIN

INTRODUCTION

Molecular evolution is the process of evolution at the scale of DNA, RNA and proteins. Research has sought to understand the relationship between the structure and function of nucleic acids or proteins, such as the function of enzymes and "molecular clock". Recent advances in genomics including whole-genome sequencing and bioinformatics have lead to a dramatic increase of studies in this field. Using the genomic information combined with developmental data, comparative genomics studies are starting to discover evolutionary mechanisms such as the role of gene duplication in the emergence of novel gene function and the identification of molecular changes responsible for various human diseases.

Unlike antimicrobial peptides whose sequences vary even between closely related species, hepcidin is highly conserved across vertebrate species, from fish to humans (Park et al., 2001; Shi and Camus, 2006). Particularly, the four disulfide bonds and the N-terminal region of hepcidin peptides are most conserved. And in some fish species such as channel catfish and zebrafish, hepcidin was found likely to function in the same way as in mammals (Fraenkel et al., 2005; Hu et al., 2007). Based on a comprehensive

comparison of hepcidin from fish to mammals, Shi and Camus (2006) proposed that two hepcidin peptide groups exist in fishes: one of them may function as an iron regulator like mammalians while the others are AMPs. Furthermore, the mammalian hepcidins may have evolved from a fish hepcidin. Another evolutionary paper on hepcidin will soon be published in the journal Gene. The authors (Haslego-Hilton and Lambert, 2008) collected sequences of 67 different hepcidin genes from 50 different species, all among the vertebrates. Although some species have multiple hepcidin homologues, the authors suggested that each contains only one copy that functions as an iron regulator. A recently published paper (Padhi and Verghese, 2007) revealed that, in contrast to mammals, positive Darwinian selection is the likely cause of accelerated rate of amino acid substitutions in the hepcidin mature peptide region of perciform and pleuronectiform fishes. These findings suggested another way to study the structure and function of hepcidin.

During the studies on molecular evolution, comparative biologists found that some proteins interacting with each other physically and/or functionally co-evolve. On one hand, it is useful to find out which proteins co-evolve; On the other hand, we can find out which amino acids or region are critical for the function of the proteins. The discovery of the hepcidin receptor (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000), ferroportin, provides such a chance to look at the structure and function of hepcidin or ferroportin in an evolutionary view. In the present paper, we analyzed the co-evolution of hepcidin and ferroportin using multiple alignments of the protein sequences. The information from bioinformatics analysis plus the clinical

information of mutations allow us to predict the critical amino acid (s) of hepcidin and ferroportin that are involved in their interaction and necessary to their functions.

MATERIALS AND METHODS

Sequence Sources

Initial sequences were obtained using the BLASTP program and the NCBI non-redundant protein database. Additional sequences were extracted from ENSEMBL genome database using BLAST program. In this research, only the species for which both hepcidin and ferroportin protein sequences are accessible are included (except chicken: no hepcidin gene found yet). Table 6.1 lists species and accession codes of sequences used in this study. More complete collections of hepcidin peptide sequences can be found in other evolutionary papers (Padhi and Verghese, 2007; Haslego-Hilton and Lambert, 2008).

Sequence Analysis

The propeptide cleavage sites were predicted by ProP1.0 (Duckert et al., 2004). Distance matrixes were generated from the multiple sequences alignments using AliBee (http://www.genebee.msu.su/services/malign_reduced.html). The correlation coefficients were obtained using SigmaStat3.01 software.

RESULTS

Sequences alignment and phylogenetic analysis

Eighteen species were found to have both hepcidin and ferroportin protein sequences in the databases (Table 6.1). As revealed by Shi and Camus (2006), the fish hepcidins are very different from hepcidins from mammals both in the NH2 terminus and C-terminus. In fishes, the common NH2 terminus starts with motif "QSHLS" and end with C-terminus motif "CCR/KF", whereas the NH2 terminus starts with the motif "DTN/HFPI" in mammals and ends with a C-terminus motif "CCR/KT" (Figure 6.1). The frog hepcidins are the evolutionary intermediates with the similar C-terminus (CCL/FT) to mammals and with the NH2 terminus (Q/HSHLS) similar to fishes (Figure 6.1). The percent homology among the 19 hepcidin mature peptides is 79.7%, and 45% among full sequences of hepcidins.

The multiple alignments of ferroportins (Figure 6.2) identify a homology percent 78.2%. The conserved region (291-350aa) containing transmembrane domain 7 (TM7) and external segment 4 (ES4) share a much higher homology of 94.4%; while the variable region (384-460aa) containing ES5 has low homology of 55.1%. According to the proposed FPN topological model (Liu et al., 2005), six external segments (ESs) were defined in the iron exporter (Figure 6.3). Considering that ESs are the most likely positions that hepcidin may interact with, a comparative study was performed on these domains. First, the ESs are highly conserved (except ES5, others share a homology greater than 90%). Second, except ES3 (very short with 3 residues), at least one charged residue is conserved in all species. Except the positive residue (K117) found in ES2, other ESs have one or more conserved negatively charged residues among all the species. Especially for ES5, there are five negatively charged residues (D339, D407, E416, E425, and E444) and one positively charged residue (R/H411) found across all the species.

Since human hepcidin has five positively charged residues (H/R) and one negatively charged residue (D), the multiple charged residues found in the extracellular domains of FPN could be the interaction sites with hepcidin.

Co-evolution Analysis

The phylogenetic distances for ferroportin and hepcidins were obtained using GeneBee software. The phylogenetic distances among the species are list in the table 6.2. The full sequences of ferroportin and hepcidin had the highest correlation coefficient (r) of 0.904 with p = 0.0001, indicating that a highly conserved interaction exists between hepcidin and ferroportin not only physically but also through other processes. The r value is also very high (0.872, p = 0.0001) between ferroportin (full length) and mature hepcidin. Two fragments of ferroportin, ferroportin (291-350aa) which is highly conserved and ferroportin (384-460aa) which is more variable, were also analyzed in our co-evolution model. The r values (table 6.3) between the different fragments of ferroportin or hepcidins strongly suggest that these two molecules co-evolved during vertebrate evolution.

DISCUSSION

As mentioned in the analysis of multiple ferroportin alignments, all the ESs containing charged residues (except ES3) could be involved in hepcidin interaction. Clearly, E52 (ES1), K117 (ES2), D325 (ES4), and E518 (ES6) plus the charged sites in ES5 (D399, D407, R/H411, E416, E425, and E444) play a role in hepcidin binding. In contrast to ES5, other ESs are highly conserved among all the investigated species

indicating a conserved function. With the most charged sites and highest variation, ES5 may be more valuable for studying the interaction between hepcidin and ferroportin. A structure and function study (Nemeth et al., 2006) demonstrated that the hepcidin-ferroportin interaction is highly tolerant of hepcidin mutations, indicating that ferroportin has a variable area that allows reduced ligand specificity. The external segments 5 (ES5) has high variation, supporting this idea.

Natural mutants often provide clues about gene function. These naturally occurring mutations also tell us about critical domains of the encoded proteins. In humans, disease-associated mutations of the ferroportin gene result in two phenotypes: hemochromatosis (Y64N, A77D, N144H, N144T, ΔV162, Q248H, C326Y, and G490D) and hyperferrritinaemia (N174I, Q182H, and G323V) (Schimanski et al., 2005). In vitro functional analysis of human ferroportin revealed that the FPN mutants A77D, ΔV162, L170F, and G490D are retained inside the cell, resulting in loss of function; while the mutants Y64N, N144H, N144D, Q248H, and C326Y locate to the cell membrane and export iron comparably to wide-type FPN (Schimanski et al., 2005). The authors postulated two mechanisms for disease causing ferroportin mutations: one causing disease by haploinsufficiency, and the other retaining function but resisting hepcidin signaling. The latter group of mutations are critical either in hepcidin binding or in subsequent events. The proposed model of FPN topology (Figure 6.3) (Liu et al., 2005) allows us to determine whether the mutations are located extra-cellularly and most likely responsible for the binding with hepcidin or located inside the membrane where they are possibly involved in subsequent events.

Among the second group of mutations, Drakesmith (2005) found that the Y64N and C326Y mutants of ferroportin are completely resistant to hepcidin inhibition whereas N144D and N144H are partially resistant (Drakesmith et al., 2005). This finding indicates that Y64N and C326Y mutants can not bind hepcidin, while N144D and N144H mutants disturb subsequent events. This work also demonstrated the critical role of ES4 (containing mutation C326Y) for binding hepcidin. Such an analysis has provided useful information for designing experiments such as site-directed mutagenesis.

Although more than 60 hepcidins have been discovered or predicted, only very recently were the first bird (pigeon) hepcidins reported (Fu et al., 2007). Two hepcidin cDNAs consisting of 295 and 380 nucleotides, respectively, were named HP1 and HP2. Despite the very low similarity to X. tropicalis hepcidin (25.6%), these two cDNA were recognized as hepcidin-like sequences with the eight conserved cysteines, responsiveness to LPS, and iron loading induction (only HP1 responds to iron). However, nobody has been able to identify the hepcidin-like molecule from chickens, the only completely sequenced animal model for birds. Since we have identified a candidate ferroportin gene from the chicken genome (Figure 6.2), there should be a hepcidin-like molecule in chicken if the birds share a similar mechanism of iron regulation as in mammals. However, the nucleotide sequence of a hepcidin counterpart in chickens must be very different from other species including pigeons. Two facts support this notion: 1) searches of the chicken genomic, EST, and HTGs databases did not reveal any hepcidin-like sequences in birds; 2) mine and others' efforts failed to acquire the cDNA sequences of hepcidin-like molecule from chicken liver samples using molecular cloning techniques.

Future work might use techniques such as suppressive subtractive hybridization, which was used to discover hepcidin cDNA from mice.

In summary, the analysis of the multiple alignments of ferroportins revealed that all extra-cellular domains except ES3 in the putative model (Liu et al., 2005) have at least one conserved charged residue which may be involved in the binding with hepcidin. Meanwhile, this finding provides some supportive evidence for the twelve-transmembrane-domain model of ferroportin. The co-evolutionary data provides a new way to evaluate the interaction between hepcidin and ferroportin. Additional work should focus on the interaction between the ES5 domain and mature hepcidin. With new computational or experimental techniques, we may finally understand the interactions in more detail.

Table 6.1. List of hepcidin and ferroportin protein sequences used

Species	Hepcidin	Ferroportin
Homo sapiens (human)	NP_066998	NP_055400
Pan troglodytes (chimp)	XP_001158692	ENSPTRP00000021763
Pongo pygmaeus (orangutan)	Q5NVR8	CAH91088
Macaca mulatta (monkey)	XP_001094273	ENSMMUP00000004671
Otolemur garnettii (bushbaby)	ENSOGAP00000001319	ENSOGAP00000012330
Mus musculus (mouse)	NP_115930 (HEPC1) NP_899080 (HEPC2)	NP_058613
Rattus norvegicus (rat)	NP_445921	ENSRNOP00000005228
Myotis lucifugus (microbat)	ENSMLUP00000005668	ENSMLUP00000008208
Bos taurus (cow)	XP_589792	ENSBTAP00000013868
Canis familiaris (dog)	NP_001007141	ENSCAFP00000013769
Monodelphis domestica (opossum)	ENSMODP00000038372	ENSMODP00000014260
Gallus gallus	Not available	NP_001012931
Xenopus tropicalis (western frog)	NP_001090729 (tHEP1) NP_001090730 (tHEP2)	AAH77514
Xenopus laevis (African frog)	CB199190 (EST)	NP_001090746
Danio rerio (zebrafish)	NP_991146	AF226612
Oryzias latipes (medaka)	ENSORLP00000018865	ENSORLP00000019126
Gasterosteus aculeatus (stickleback)	ENSGACP00000016484	ENSGACP00000003561
Tachifugu rubridpes (fugu)	SINFRUP00000181117	SINFRUP00000129888

Table 6.2. Distance matrixes of hepcidin and ferroportin in vertebrates.

DISTANCE MATRIX (hepcidin full length)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 HOMO	0.000	0.000	0.005	0.000	0.198	0.376	0.436	0.365	0.271	0.326	0.482	0.785	0.724	0.794	0.797	0.799	0.807
2 PAN	0.000	0.000	0.000	0.000	0.190	0.376	0.428	0.357	0.263	0.326	0.475	0.785	0.724	0.787	0.797	0.801	0.814
3 PONGO	0.005	0.000	0.000	0.000	0.208	0.403	0.447	0.367	0.261	0.337	0.473	0.806	0.745	0.810	0.821	0.824	0.832
4 MACACA	0.000	0.000	0.000	0.000	0.181	0.394	0.432	0.370	0.236	0.341	0.465	0.785	0.724	0.792	0.779	0.793	0.823
5 OTOLEMUR	0.198	0.190	0.208	0.181	0.000	0.407	0.423	0.406	0.260	0.377	0.503	0.795	0.748	0.800	0.806	0.797	0.858
6 MUS1	0.376	0.376	0.403	0.394	0.407	0.000	0.301	0.503	0.439	0.439	0.568	0.786	0.744	0.778	0.814	0.795	0.836
7 RATTUS	0.436	0.428	0.447	0.432	0.423	0.301	0.000	0.473	0.411	0.433	0.598	0.838	0.786	0.753	0.781	0.771	0.803
8 MYOTIS	0.365	0.357	0.367	0.370	0.406	0.503	0.473	0.000	0.374	0.319	0.553	0.875	0.833	0.829	0.835	0.829	0.855
9 BOS	0.271	0.263	0.261	0.236	0.260	0.439	0.411	0.374	0.000	0.326	0.464	0.782	0.737	0.838	0.819	0.827	0.878
10 CANIS	0.326	0.326	0.337	0.341	0.377	0.439	0.433	0.319	0.326	0.000	0.513	0.754	0.702	0.801	0.803	0.810	0.834
11 MONODELPH	0.482	0.475	0.473	0.465	0.503	0.568	0.598	0.553	0.464	0.513	0.000	0.833	0.791	0.847	0.792	0.823	0.871
12 TROPIC1	0.785	0.785	0.806	0.785	0.795	0.786	0.838	0.875	0.782	0.754	0.833	0.000	0.172	0.846	0.797	0.810	0.877
13 LAEVIS	0.724	0.724	0.745	0.724	0.748	0.744	0.786	0.833	0.737	0.702	0.791	0.172	0.000	0.846	0.776	0.796	0.868
14 DANIO	0.794	0.787	0.810	0.792	0.800	0.778	0.753	0.829	0.838	0.801	0.847	0.846	0.846	0.000	0.583	0.539	0.709
15 ORYZIAS	0.797	0.797	0.821	0.779	0.806	0.814	0.781	0.835	0.819	0.803	0.792	0.797	0.776	0.583	0.000	0.163	0.481
16 GASTEROST	0.799	0.801	0.824	0.793	0.797	0.795	0.771	0.829	0.827	0.810	0.823	0.810	0.796	0.539	0.163	0.000	0.484
17 TAKIFUGU	0.807	0.814	0.832	0.823	0.858	0.836	0.803	0.855	0.878	0.834	0.871	0.877	0.868	0.709	0.481	0.484	0.000

DISTANCE MATRIX (ferroportin full length)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 HOMO	0.000	0.015	0.031	0.028	0.106	0.114	0.108	0.200	0.116	0.098	0.191	0.239	0.248	0.331	0.312	0.315	0.393
2 PAN	0.015	0.000	0.031	0.028	0.106	0.114	0.108	0.200	0.116	0.098	0.191	0.239	0.248	0.331	0.312	0.315	0.393
3 PONGO	0.031	0.031	0.000	0.027	0.105	0.114	0.119	0.213	0.118	0.099	0.191	0.236	0.245	0.339	0.311	0.314	0.388
4 MACACA	0.028	0.028	0.027	0.000	0.100	0.107	0.115	0.206	0.110	0.092	0.185	0.234	0.243	0.336	0.310	0.313	0.389
5 OTOLEMUR	0.106	0.106	0.105	0.100	0.000	0.124	0.135	0.222	0.130	0.116	0.172	0.247	0.256	0.340	0.330	0.324	0.404
6 MUS	0.114	0.114	0.114	0.107	0.124	0.000	0.063	0.201	0.125	0.113	0.192	0.246	0.252	0.347	0.327	0.331	0.411
7 RATTUS	0.108	0.108	0.119	0.115	0.135	0.063	0.000	0.205	0.134	0.127	0.198	0.247	0.256	0.331	0.324	0.330	0.410
8 MYOTIS	0.200	0.200	0.213	0.206	0.222	0.201	0.205	0.000	0.214	0.205	0.281	0.304	0.310	0.374	0.347	0.349	0.458
9 BOS	0.116	0.116	0.118	0.110	0.130	0.125	0.134	0.214	0.000	0.084	0.188	0.242	0.255	0.340	0.332	0.333	0.412
10 CANIS	0.098	0.098	0.099	0.092	0.116	0.113	0.127	0.205	0.084	0.000	0.175	0.228	0.236	0.330	0.331	0.328	0.408
11 MONODELPH	0.191	0.191	0.191	0.185	0.172	0.192	0.198	0.281	0.188	0.175	0.000	0.271	0.261	0.342	0.346	0.347	0.419
12 TROPICALI	0.239	0.239	0.236	0.234	0.247	0.246	0.247	0.304	0.242	0.228	0.271	0.000	0.070	0.317	0.323	0.321	0.392
13 LAEVIS	0.248	0.248	0.245	0.243	0.256	0.252	0.256	0.310	0.255	0.236	0.261	0.070	0.000	0.316	0.329	0.328	0.402
14 DANIO	0.331	0.331	0.339	0.336	0.340	0.347	0.331	0.374	0.340	0.330	0.342	0.317	0.316	0.000	0.281	0.292	0.377
15 ORYZIAS	0.312	0.312	0.311	0.310	0.330	0.327	0.324	0.347	0.332	0.331	0.346	0.323	0.329	0.281	0.000	0.153	0.270
16 GASTEROST	0.315	0.315	0.314	0.313	0.324	0.331	0.330	0.349	0.333	0.328	0.347	0.321	0.328	0.292	0.153	0.000	0.290
17 TAKIFUGU	0.393	0.393	0.388	0.389	0.404	0.411	0.410	0.458	0.412	0.408	0.419	0.392	0.402	0.377	0.270	0.290	0.000

Distance (to human) values were calculated using Genebee program (www.genebee.msu.su/cgi-bin/nph-malign.pl).

Table 6.3. Correlation coefficient (r) of phylogenetic distances of hepcidin and ferroportin

	Ferroportin (full length)	**Ferroportin (ES4) (291-350aa)**	**Ferroportin (ES5) (384-460aa)**
Hepcidin (full length)	r = 0.904 p = 0.0001	r = 0.804 p = 0.0001	r = 0.712 p = 0.0001
Hepcidin (mature)	r = 0.872 p = 0.0001	r = 0.833 p = 0.0001	r = 0.512 p = 0.0001

Correlations of phylogenetic distances between ferroportin and hepcidin genes were determined by Pearson's correlation coefficient method using XLSTAT (Mantel test).

```
The meaning of signs at the top of the alignment is following:
      ' ' - the average weight of column pair exchanges is less than
            weight matrix mean value
      '.' - is less than mean value plus one SD
      '+' - is less than mean value plus two SD
      '*' - is more than mean value plus two SD

                  ++*+++*.***.**.....**.**++
         HOMO     DTHFPICIFCCGCCHR-SKCGMCCKT
         PAN      DTHFPICIFCCGCCHR-SKCGMCCKT
         PONGO    DTHFPIYIFCCGCCHR-SKCGMCCKT
         MACACA   DTHFPICIFCCGCCHR-SKCGMCCRT
         OTOLEMUR DTHIPICLFCCKCCRA-SSCGICCRT
         MUS1     DTNFPICIFCCKCCNN-SQCGICCKT
         MUS2     DINFPICRFCCQCCNK-PSCGICCEE
         MYOTIS   DTHFPICIFCCGCCYP-SKCGICCKT
         RATTUS   DTNFPICLFCCKCCKN-SSCGLCCIT
         BOS      DTHFPICIFCCGCCRK-GTCGMCCRT
         CANIS    DTHFPICIFCCGCCKT-PKCGLCCKT

         LAEVIS   HSHLSICIHCCNCCKF-KGCGKCCLT
         TROPIC1  QSHLSICVHCCNCCKY-KGCGKCCLT
         TROPIC2  --HLNICVYCCKCCKKQKGCGMCCFT

         DANIO    QSHLSLCRFCCKCCRN-KGCGYCCKF
         GASTEROST QSHLSMCRWCCKCCRSYKGCGYCCKF
         ORYZIAS  QSHISMCTMCCNCCKNYKGCGFCCRF
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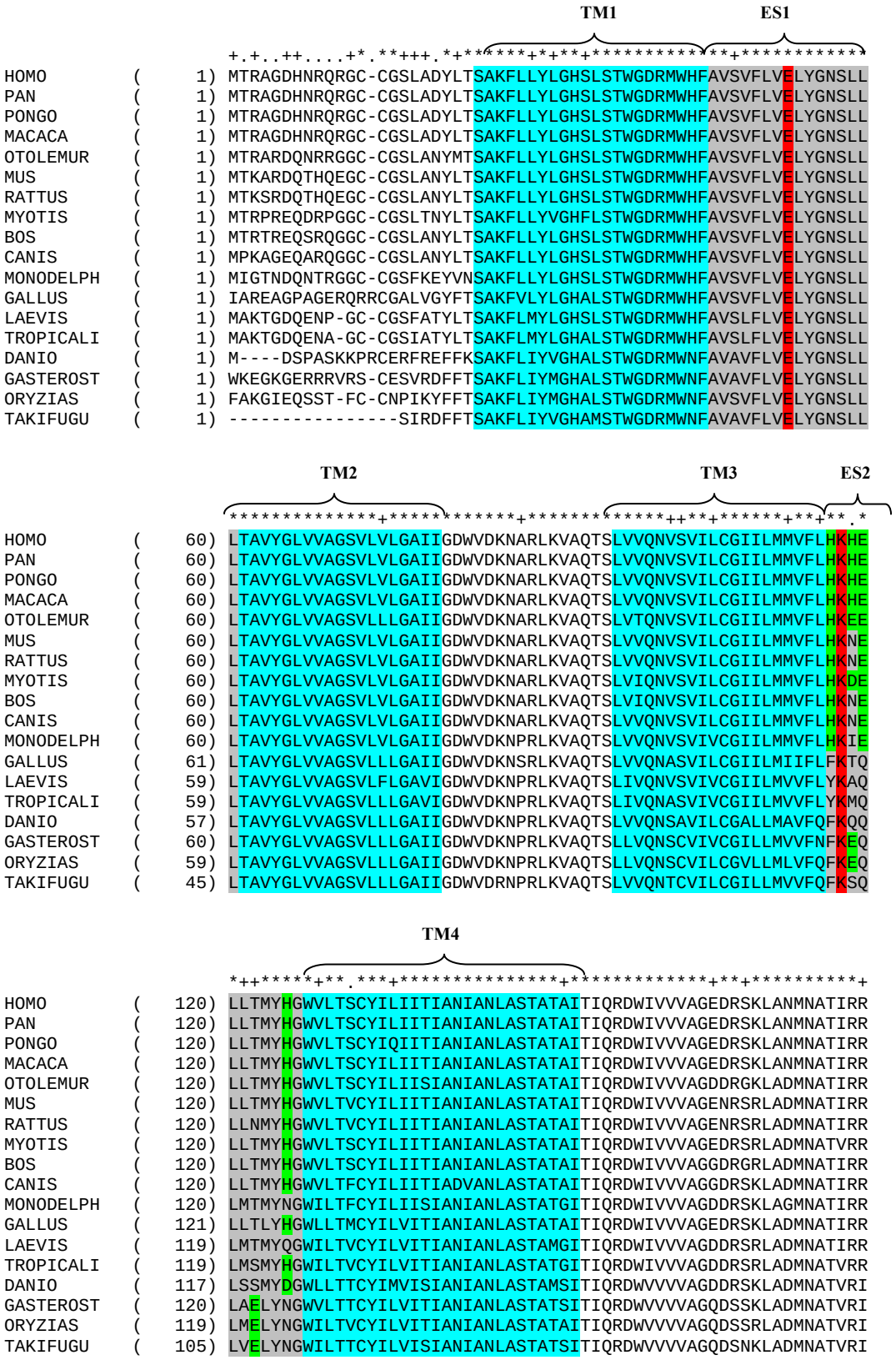
Figure 6.1. Multiple alignments of hepcidin mature peptides.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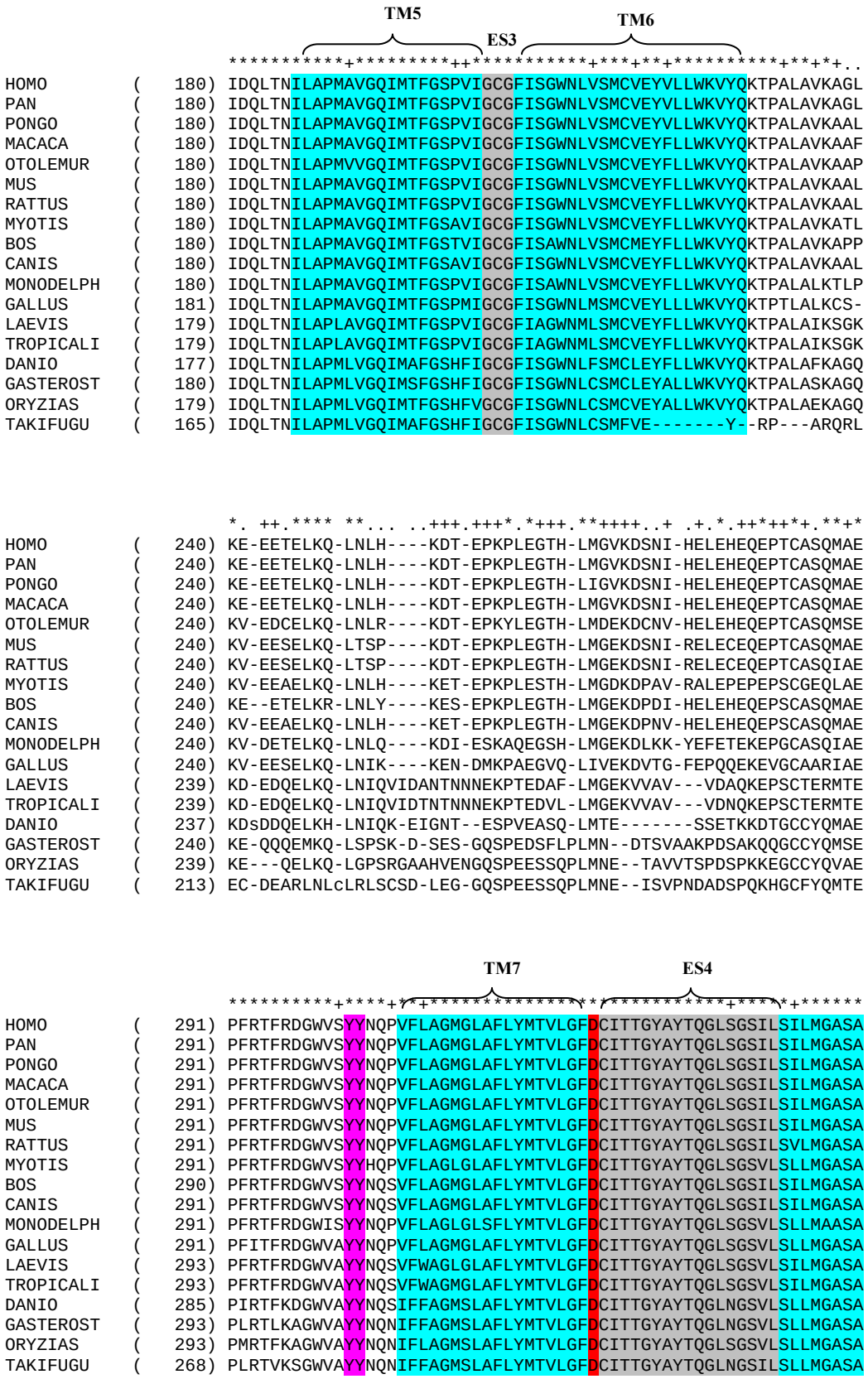
ES1
TM2
TM3
ES2
TM4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ES3
TM6
TM7
ES4
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PAN (240) KE-EETELKQ-LNLH----KDT-EPKPLEGTH-LMGVKDSNI-HELEHEQEPTCASQMAE
PONGO (240) KE-EETELKQ-LNLH----KDT-EPKPLEGTH-LIGVKDSNI-HELEHEQEPTCASQMAE
MACACA (240) KE-EETELKQ-LNLH----KDT-EPKPLEGTH-LMGVKDSNI-HELEHEQEPTCASQMAE
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MONODELPH (240) KV-DETELKQ-LNLQ----KDI-ESKAQEGSH-LMGEKDLKK-YEFETEKEPGCASQIAE
GALLUS (240) KV-EESELKQ-LNIK----KEN-DMKPAEGVQ-LIVEKDVTG-FEPQQEKEVGCAARIAE
LAEVIS (239) KD-EDQELKQ-LNIQVIDANTNNNEKPTEDAF-LMGEKVVAV---VDAQKEPSCTERMTE
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DANIO (237) KDsDDQELKH-LNIQK-EIGNT--ESPVEASQ-LMTE-------SSETKKDTGCCYQMAE
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DANIO (285) PIRTFKDGWVAYYNQSIFFAGMSLAFLYMTVLGFDCITTGYAYTQGLNGSVLSLLMGASA
GASTEROST (293) PLRTLKAGWVAYYNQNIFFAGMSLAFLYMTVLGFDCITTGYAYTQGLNGSVLSLLMGASA
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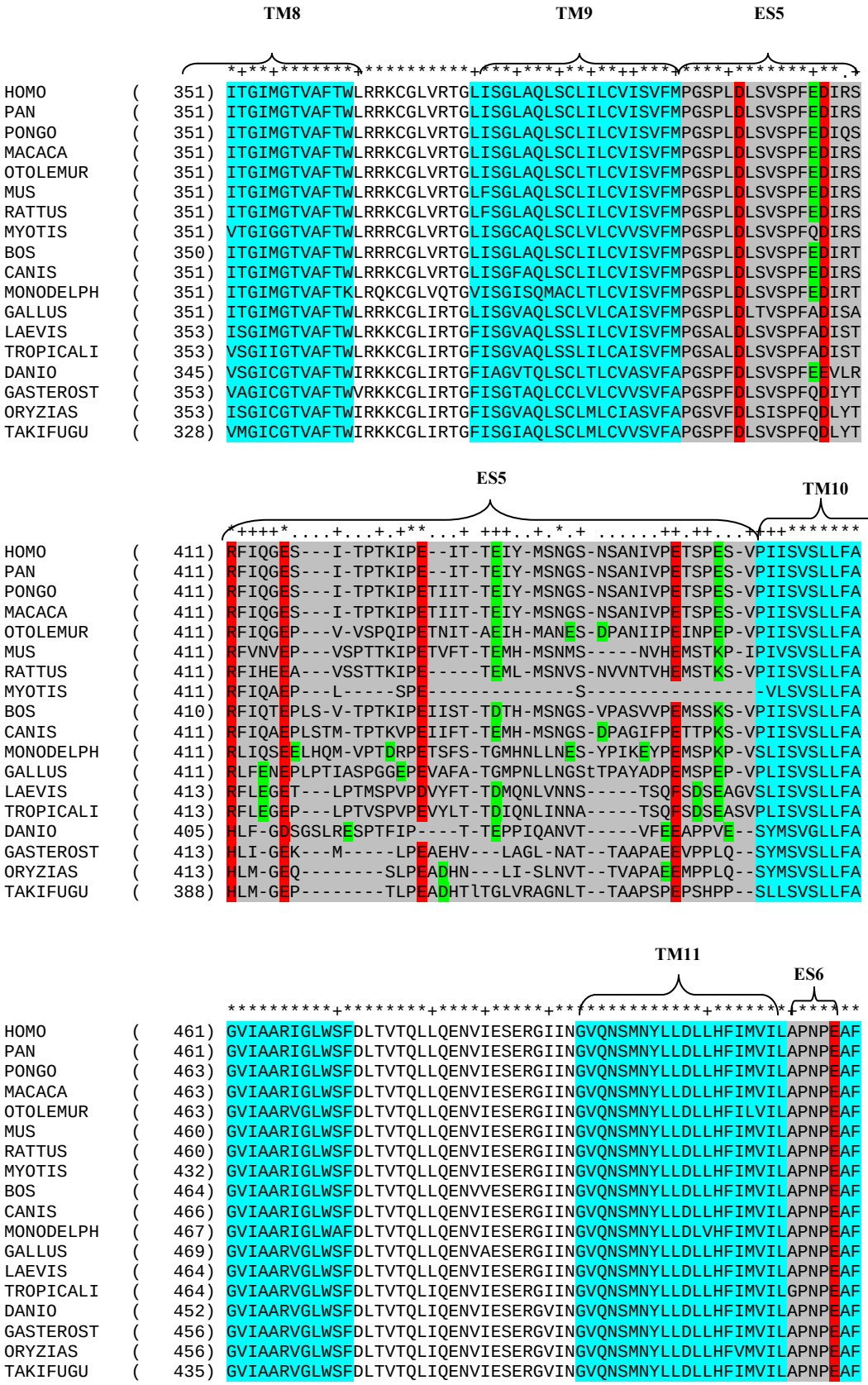
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TM9
ES5
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PAN (351) ITGIMGTVAFTWLRRKCGLVRTGLISGLAQLSCLILCVISVFMPGSPLDLSVSPFEDIRS
PONGO (351) ITGIMGTVAFTWLRRKCGLVRTGLISGLAQLSCLILCVISVFMPGSPLDLSVSPFEDIQS
MACACA (351) ITGIMGTVAFTWLRRKCGLVRTGLISGLAQLSCLILCVISVFMPGSPLDLSVSPFEDIRS
OTOLEMUR (351) ITGIMGTVAFTWLRRKCGLVRTGLISGLAQLSCLTLCVISVFMPGSPLDLSVSPFEDIRS
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RATTUS (351) ITGIMGTVAFTWLRRKCGLVRTGLFSGLAQLSCLILCVISVFMPGSPLDLSVSPFEDIRS
MYOTIS (351) VTGIGGTVAFTWLRRRCGLVRTGLISGCAQLSCLVLCVVSVFMPGSPLDLSVSPFQDIRS
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DANIO (345) VSGICGTVAFTWIRKKCGLIRTGFIAGVTQLSCLTLCVASVFAPGSPFDLSVSPFEEVLR
GASTEROST (353) VAGICGTVAFTWVRKKCGLIRTGFISGTAQLCCLVLCVVSVFAPGSPFDLSVSPFQDIYT
ORYZIAS (353) ISGICGTVAFTWIRKKCGLIRTGFISGVAQLSCLMLCIASVFAPGSVFDLSISPFQDLYT
TAKIFUGU (328) VMGICGTVAFTWIRKKCGLIRTGFISGIAQLSCLMLCVVSVFAPGSPFDLSVSPFQDLYT
ES5
TM10
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PAN (411) RFIQGES---I-TPTKIPE--IT-TEIY-MSNGS-NSANIVPETSPES-VPIISVSLLFA
PONGO (411) RFIQGES---I-TPTKIPETIIT-TEIY-MSNGS-NSANIVPETSPES-VPIISVSLLFA
MACACA (411) RFIQGES---I-TPTKIPETIIT-TEIY-MSNGS-NSANIVPETSPES-VPIISVSLLFA
OTOLEMUR (411) RFIQGEP---V-VSPQIPETNIT-AEIH-MANES-DPANIIPEINPEP-VPIISVSLLFA
MUS (411) RFVNVEP---VSPTTKIPETVFT-TEMH-MSNMS-----NVHEMSTKP-IPIVSVSLLFA
RATTUS (411) RFIHEEA---VSSTTKIPE-----TEML-MSNVS-NVVNTVHEMSTKS-VPIISVSLLFA
MYOTIS (411) RFIQAEP---L-----SPE----------S----------VLSVSLLFA
BOS (410) RFIQTEPLS-V-TPTKIPEIIST-TDTH-MSNGS-VPASVVPEMSSKS-VPIISVSLLFA
CANIS (411) RFIQAEPLSTM-TPTKVPEIIFT-TEMH-MSNGS-DPAGIFPETTPKS-VPIISVSLLFA
MONODELPH (411) RLIQSEELHQM-VPTDRPETSFS-TGMHNLLNES-YPIKEYPEMSPKP-VSLISVSLLFA
GALLUS (411) RLFENEPLPTIASPGGEPEVAFA-TGMPNLLNGStTPAYADPEMSPEP-VPLISVSLLFA
LAEVIS (413) RFLEGET---LPTMSPVPDVYFT-TDMQNLVNNS-----TSQFSDSEAGVSLISVSLLFA
TROPICALI (413) RFLEGEP---LPTVSPVPEVYLT-TDIQNLINNA-----TSQFSDSEASVPLISVSLLFA
DANIO (405) HLF-GDSGSLRESPTFIP----T-TEPPIQANVT-----VFEEAPPVE--SYMSVGLLFA
GASTEROST (413) HLI-GEK---M-----LPEAEHV---LAGL-NAT--TAAPAEEVPPLQ--SYMSVSLLFA
ORYZIAS (413) HLM-GEQ--------SLPEADHN---LI-SLNVT--TVAPAEEMPPLQ--SYMSVSLLFA
TAKIFUGU (388) HLM-GEP--------TLPEADHTlTGLVRAGNLT--TAAPSPEPSHPP--SLLSVSLLFA
TM11
ES6
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PAN (461) GVIAARIGLWSFDLTVTQLLQENVIESERGIINGVQNSMNYLLDLLHFIMVILAPNPEAF
PONGO (463) GVIAARIGLWSFDLTVTQLLQENVIESERGIINGVQNSMNYLLDLLHFIMVILAPNPEAF
MACACA (463) GVIAARIGLWSFDLTVTQLLQENVIESERGIINGVQNSMNYLLDLLHFIMVILAPNPEAF
OTOLEMUR (463) GVIAARVGLWSFDLTVTQLLQENVIESERGIINGVQNSMNYLLDLLHFILVILAPNPEAF
MUS (460) GVIAARIGLWSFDLTVTQLLQENVIESERGIINGVQNSMNYLLDLLHFIMVILAPNPEAF
RATTUS (460) GVIAARIGLWSFDLTVTQLLQENVIESERGIINGVQNSMNYLLDLLHFIMVILAPNPEAF
MYOTIS (432) GVIAARIGLWSFDLTVTQLLQENVIESERGIINGVQNSMNYLLDLLHFIMVILAPNPEAF
BOS (464) GVIAARIGLWSFDLTVTQLLQENVVESERGIINGVQNSMNYLLDLLHFIMVILAPNPEAF
CANIS (466) GVIAARIGLWSFDLTVTQLLQENVIESERGIINGVQNSMNYLLDLLHFIMVILAPNPEAF
MONODELPH (467) GVIAARIGLWAFDLTVTQLLQENVIESERGIINGVQNSMNYLLDLVHFIMVILAPNPEAF
GALLUS (469) GVIAARVGLWSFDLTVTQLLQENVAESERGIINGVQNSMNYLLDLLHFIMVILAPNPEAF
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TROPICALI (464) GVIAARVGLWSFDLTVTQLIQENVIESERGIINGVQNSMNYLLDLLHFIMVILGPNPEAF
DANIO (452) GVIAARVGLWSFDLTVTQLIQENVIESERGVINGVQNSMNYLLDLLHFIMVILAPNPEAF
GASTEROST (456) GVIAARVGLWSFDLTVTQLIQENVIESERGVINGVQNSMNYLLDLLHFIMVILAPNPEAF
ORYZIAS (456) GVIAARVGLWSFDLTVTQLIQENVIESERGVINGVQNSMNYLLDLLHFVMVILAPNPEAF
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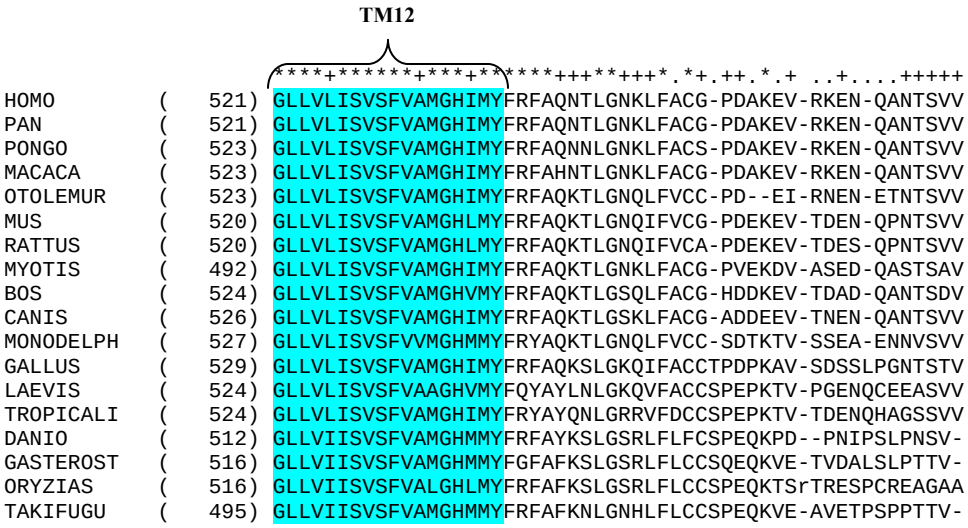
TM12
****+******+***+*******+++**+++*.*+.++.*.+ ..+....+++++
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PAN (521) GLLVLISVSFVAMGHIMYFRFAQNTLGNKLFACG-PDAKEV-RKEN-QANTSVV
PONGO (523) GLLVLISVSFVAMGHIMYFRFAQNNLGNKLFACS-PDAKEV-RKEN-QANTSVV
MACACA (523) GLLVLISVSFVAMGHIMYFRFAHNTLGNKLFACG-PDAKEV-RKEN-QANTSVV
OTOLEMUR (523) GLLVLISVSFVAMGHIMYFRFAQKTLGNQLFVCC-PD--EI-RNEN-ETNTSVV
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RATTUS (520) GLLVLISVSFVAMGHLMYFRFAQKTLGNQIFVCA-PDEKEV-TDES-QPNTSVV
MYOTIS (492) GLLVLISVSFVAMGHIMYFRFAQKTLGNKLFACG-PVEKDV-ASED-QASTSAV
BOS (524) GLLVLISVSFVAMGHVMYFRFAQKTLGSQLFACG-HDDKEV-TDAD-QANTSDV
CANIS (526) GLLVLISVSFVAMGHIMYFRFAQKTLGSKLFACG-ADDEEV-TNEN-QANTSVV
MONODELPH (527) GLLVLISVSFVVMGHMMYFRYAQKTLGNQLFVCC-SDTKTV-SSEA-ENNVSVV
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ORYZIAS (516) GLLVIISVSFVALGHLMYFRFAFKSLGSRLFLCCSPEQKTSrTRESPCREAGAA
TAKIFUGU (495) GLLVIISVSFVAMGHMMYFRFAFKNLGNHLFLCCSPEQKVE-AVETPSPPTTV-

Figure 6.2

Figure 6.2. Multiple alignments of ferroportins with a percent homology of 76.7%. The extra-cellular domains are marked in grey; the transmembrane domains are marked in blue; the conserved charged sites are marked in red; the less conserved charged sites are marked in green. **TM**: transmembrane domains; **ES**: external segment.

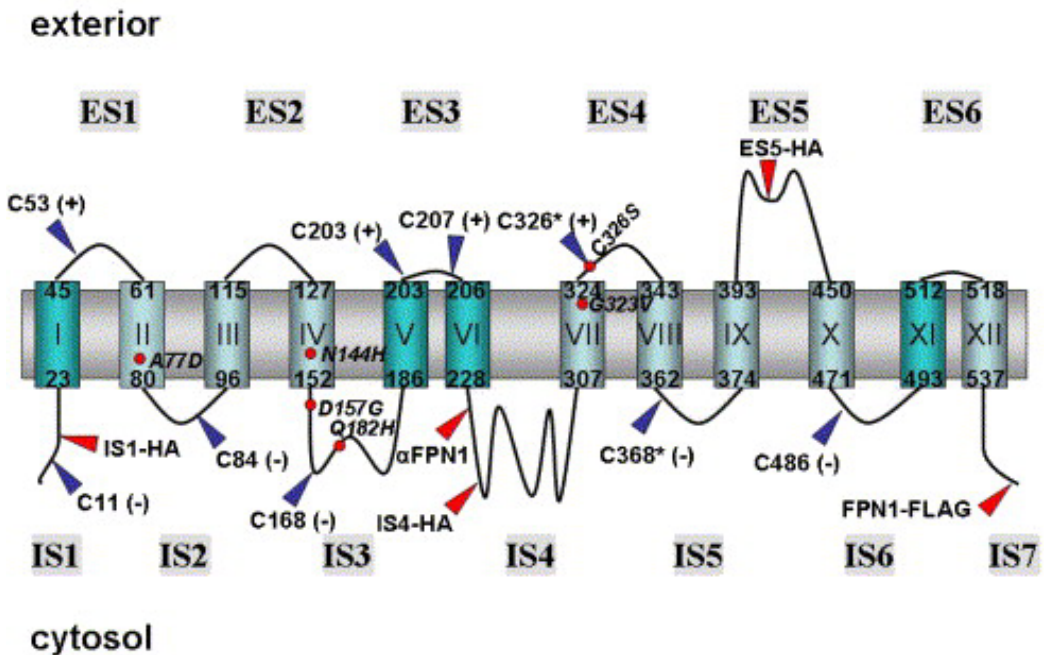
exterior
ES1
ES2
ES3
ES4
ES5
ES6
ES5-HA
C53 (+)
C203 (+)
C207 (+)
C326* (+)
C326S
G323D
45
61
115
127
203
206
324
343
393
450
512
518
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
23
80
96
152
186
228
307
362
374
471
493
537
A77D
N144H
D157G
Q182H
IS1-HA
C84 (-)
αFPN1
C368* (-)
C486 (-)
C11 (-)
C168 (-)
IS4-HA
FPN1-FLAG
IS1
IS2
IS3
IS4
IS5
IS6
IS7
cytosol

Figure 6.3

Figure 6.3. Proposed model of FPN1 topological structure. I–XII indicate proposed transmembrane regions. Blue arrowheads indicate areas of cysteine insertions or substitutions or native cysteines (indicated with asterisk). The plus or minus sign indicates whether or not the cysteine was labeled with cell impermeable biotinylation reagent. Red arrowheads indicate areas of informative epitope insertions or location of epitope of anti-FPN1 antibody. Modified from Liu et al., 2005.

CHAPTER VII. DISCUSSION AND CONCLUSION

Hepcidin, originally identified as a 25 amino acid (aa) antimicrobial peptide made in the liver, is a small peptide hormone that functions both as a homeostatic regulator of iron metabolism and as a mediator of host defense and inflammation in humans and mice. The discovery that hepcidin functions as an iron-regulatory hormone, has provided important insights into the pathogenesis of various forms of hemochromatosis and has changed our understanding of the anemia of inflammation pathogenesis. Closely related hepcidin genes and peptides also have been identified in other mammals, amphibians and various fish species. Fish hepcidins possess antimicrobial activities in vitro and are thought to function as endogenous antibiotics in host defense against infection. However, it remains to be investigated whether or not hepcidin can regulate iron metabolism in fish and amphibians.

The objectives of this catfish study were to clone the channel catfish (Ictalurus punctatus) hepcidin gene and to evaluate its tissue expression pattern and transcriptional regulation by infection and anemia. Using RT-PCR and PCR, the channel catfish hepcidin cDNA and genomic DNA sequences were cloned. Similar to human hepcidin, the channel catfish hepcidin gene comprises three exons and two introns. The predicted mature channel catfish hepcidin is also a 25 aa peptide with eight cysteine residues. RT-PCR analysis showed that channel catfish hepcidin transcript was predominantly expressed in the liver and weakly expressed in the testis, ovary, stomach, and olfactory

sac. Hepcidin expression in catfish challenged with E. ictaluri and in fish affected by channel catfish anemia (CCA) was measured by real time quantitative PCR. It was found that hepcidin transcript level in the liver was increased by 4, 19, and 22 fold at 4, 24, and 48 hours post bacterial challenge, respectively. However, augmented hepcidin expression in the gut and olfactory sac was detected only at 48 h post infection. In CCA fish, the concentration of serum iron, total iron binding capacity, and liver iron content were half of that in healthy controls. The hepcidin transcript levels in catfish livers affected by CCA were only 14% of healthy catfish levels. Correlation analysis indicated that hepatic hepcidin transcript levels correlated significantly with serum iron concentrations ($r = 0.54$, $P < 0.05$) and with the percent saturation of transferrin ($r = 0.63$, $P < 0.05$).

This study identified the channel catfish hepcidin gene and demonstrated that its expression is enhanced by bacterial infection and repressed by anemia. This is one of the only two pieces of evidence (the other is in zebrafish) that decreased fish hepcidin gene expression occurs in association with reduced body iron stores. Similar to mammalian hepcidins, channel catfish hepcidin is an iron-responsive gene and may also play important roles in innate host defense to infection and in iron homeostasis. Mammalian hepcidins may have evolved from an antimicrobial peptide and its structure and transcriptional regulatory mechanisms have been conserved throughout vertebrate evolution. Additional studies are needed to prove that catfish hepcidin plays an essential role in host defense against infection.

Consistent with its essential function, the structure of hepcidin is conserved across vertebrate evolution. More and more data demonstrates that hepcidin is involved in the

regulation of iron metabolism in mammals and fish, but little is known about this iron regulatory hormone in amphibians and birds. The frog paper described cloning two hepcidin cDNAs (tHEP1 and tHEP2) from western clawed frog (X. tropicalis) and showed that tHEP2 is up-regulated by iron loading while tHEP1 is repressed by the stress responsive hormone, corticosterone. Based on an analysis of their promoter and 5' un-translated regions (-2.0 kb upstream of translation start), tHEP2 has sequences homologous to the cytokine responsive elements, STAT3 and NF-κB while energy /glucose metabolism related elements, USF and C/EBPα were found in the promoter region of only tHEP1. These results indicated that tHEP2 may be involved in iron metabolism through an inflammatory pathway, while tHEP1 may crosstalk with energy/glucose metabolism and demonstrate a role in innate immunity. The frog paper demonstrated that both tHEP1 and tHEP2 share some characteristics with mammalian hepcidins, suggesting that X. tropicalis could be a promising animal model for the study of hepcidin. Because the transcriptional regulation of hepcidin is not well understood, we may throw new light on hepcidin regulation by comparing the two frog hepcidins to other vertebrates.

Co-evolutionary analysis of hepcidin and ferroportin provides another way to study the structure and function of these two proteins. Multiple alignment of ferroportins from GeneBank and ENSEMBL databases revealed that all extra-cellular domains except ES3 have at least one conserved charged site. These are most likely involved in the binding with hepcidin. Combined with human disease phenotypes and experimental data, the critical sites involved in hepcidin and ferroportin interactions were found. Multiple alignment analysis of ferroportin amino acid sequences also revealed a highly variable

external segment (ES5), which provides a possible explanation for the high acceptance of ferroportin to hepcidin sequence variation. With 3D structures and suitable computational techniques, we may be able to study the amino acids interactions and thereby provide more detailed information for further experimental explorations.

OVERALL CONCLUSIONS

1. For the first time, the channel catfish study using fish with naturally occurring anemia demonstrated the iron regulatory function of hepcidin in fish. This finding is different from a zebrafish study which had similar results but used transgenic fish. The infection assay using E. ictaluri showed the common feature of hepcidin responsiveness to infection, which also demonstrated that catfish hepcidin may play an important role in the infectious disease in channel catfish caused by E. ictaluri. Considering that the genome of channel catfish is almost complete, it may become an acceptable replacement model for zebrafish.
2. For the first time, the Xenopus frog study demonstrated iron regulatory function of hepcidin in amphibians. Two hepcidin genes were found in the X. tropicalis frog but only tHEP2 is responsive to iron loading, indicating a possible role of tHEP2 in the regulation of iron homeostasis. In contrast, tHEP1 is most likely involved in the host innate defense by its responsiveness to corticosterone. The evaluation of the promoter areas supports such notions. This preliminary data suggests that X. tropicalis could be a promising animal model which allows us to separate some features of the regulation of hepcidin expression at the transcriptional level. X. tropicalis will provide an evolutionary link between fish (zebrafish or catfish) and

mammal (mouse), which will help us understand the structure and function of hepcidin in an evolutionary view.

3. For the first time, the phylogenetic study evaluated the co-evolution of hepcidin and its receptor ferroportin. Genomic information combined with clinical and experimental data allow us to predict the critical sites of interaction between two physically or functionally related proteins. In this study, highly conserved external segments (ES1-4, and ES6) were predicted to play a critical role during the binding of hepcidin to ferroportin. On the other hand, the variable external segment, ES5, was proposed to be responsible for the acceptance of ferroportin to hepcidin variation. The co-evolutionary study also provides clues for searching for hepcidin-like molecules in birds.

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