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Inductively coupled mass spectrometry analysis of biometals in conditional *Hamp1* and *Hamp1 and Hamp2* transgenic mouse models

S. Lu[1,2], **J. Seravalli**[3], and **D. Harrison-Findik**[1,*]

[1]University of Nebraska Medical Center, Department of Internal Medicine, Omaha, NE., USA

[2]University of Nebraska Medical Center, Department of Biochemistry and Molecular Biology, Omaha, NE., USA

[3]Redox Biology Center, University of Nebraska-Lincoln, NE., USA

Abstract

Hepcidin, a circulatory antimicrobial peptide, is involved in iron homeostasis, inflammation, infection and metabolic signals. Humans express one hepcidin gene, *HAMP* but mice express two hepcidin genes, *Hamp1* and *Hamp2*. Consecutive gene targeting events were performed to produce transgenic mice expressing conditional alleles of either *Hamp1* or both *Hamp1* and *Hamp2 (Hamp1/2)*. The deletion of *Hamp*1 alleles elevated *Hamp2* expression, particularly in males, which was reduced by endotoxin treatment. The tissue levels of iron and other biometals were quantified by inductively coupled mass spectrometry. The ubiquitous or liver-specific deletion of *Hamp1* alleles yielded similar quantitative changes in iron levels in the liver, duodenum, spleen, kidney, heart and brain. The introduction of *Hamp2* null allele did not exacerbate the iron-related phenotype of *Hamp1* null allele. Besides iron, *Hamp1* null allele significantly elevated the levels of selenium in the liver, manganese in the liver and duodenum, and copper in the brain. Mice with conditional *Hamp* alleles will be useful to determine the tissue-specific regulation and functions of *Hamp1* and *Hamp2* in biometal homeostasis and other biological processes.

Keywords

Copper; hepcidin; iron; liver; manganese; selenium

INTRODUCTION

Hepcidin is both an iron-regulatory and acute phase protein, which is primarily expressed in the liver and to a smaller extent in other tissues. (Pigeon, *et al* 2001) Hepcidin serves as the central regulator of iron metabolism by blocking duodenal iron transport and iron release in

[*]**Corresponding Author:** Duygu Dee Harrison-Findik, D.V.M., PhD, University of Nebraska Medical Center, 95820 UNMC, DRC I, Omaha, NE., 68198-5820 USA, dharrisonfindik@unmc.edu, Phone: 1-402-5596355.

DISCLOSURE OF CONFLICT OF INTEREST

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macrophages via the degradation of the iron exporter, ferrroportin. (Lesbordes-Brion, *et al* 2006, Nemeth, *et al* 2004, Nicolas, *et al* 2001) Hepcidin expression itself is responsive to body iron levels. As an acute phase protein, it is induced by endotoxin and inflammatory cytokines.(Lee, *et al* 2005, Nemeth, *et al* 2003) Furthermore, hypoxia, oxidative and endoplasmic reticulum stress, obesity and metabolic signals have also been shown to regulate hepcidin expression. (Bekri, *et al* 2006, Harrison-Findik, *et al* 2006, Nicolas, *et al* 2002, Vecchi, *et al* 2014, Vecchi, *et al* 2009)

Human hepcidin gene, *HAMP* is located on the long arm of chromosome 19 at position 13.1. Unlike humans and rats, mice have 2 hepcidin genes, *Hamp1* and *Hamp2*, which are both located on mouse chromosome 7. Similar to *HAMP*, iron induces the expression of both *Hamp1* and *Hamp2*.(Ilyin, *et al* 2003, Krijt, *et al* 2004) Based on predicted structure analysis, and studies using *Hamp1* knockout or *Hamp2* overexpressing mice, *Hamp1* has been suggested to be the equivalent of *HAMP* regarding the regulation of iron metabolism. (Lesbordes-Brion, *et al* 2006, Lou, *et al* 2004). The role of *Hamp2* is however unknown. There are no transgenic mouse models with targeted deletions of either *Hamp2* or both *Hamp* isoforms. Usf2 knockout mice employed for iron studies harbor coincidental deletions in neighboring chromosomal regions harboring *Hamp1* and *Hamp2* loci. (Nicolas, *et al* 2001) We generated transgenic mice with syntenic conditional alleles of *Hamp1* and *Hamp2 (Hamp1/2*).

Other biometals play an important role in iron homeostasis. Ferrooxidases, haephestin and ceruloplasmin require copper for their activity. (Harris, *et al* 1999, Vulpe, *et al* 1999) Zinc transporters have been shown to participate in iron homeostasis. (Liuzzi, *et al* 2006) By using inductively coupled mass spectrometry (ICP-MS), we quantified iron and other biometals in various organs of transgenic mice with targeted deletions of *Hamp1* or *Hamp1* and *Hamp2*.

This study summarizes the initial investigations related to biometal homeostasis in these mice. Future studies with conditional *Hamp1* single and *Hamp1/2* double knockout mice will involve the analysis of other phenotypes that are not are not directly related to iron metabolism. The double knockout mouse model might also be beneficial for the search of therapeutics for human disorders involving hepcidin regulation.

MATERIAL AND METHODS

Animal experiments

Animal experiments were approved by the animal ethics committee at the University of Nebraska Medical Center. The chow diet (*Harlan Teklad 7012*) used as regular food by the animal facility contained 284.11 mg/kg iron. For LPS experiments, male mice were injected (i.p.) with LPS (1μg/ g body weight) from Escherichia coli serotype O55:B5 (*Sigma*) or PBS (as control for injection) and sacrificed 6 hours later to harvest organs.

Generation of mice with syntenic conditional alleles of Hamp1 and Hamp2

Mouse lines expressing floxed *Hamp1* (*Hamp1*$^{flx/flx}$) or floxed *Hamp1* and *Hamp2* (*Hamp1/2*$^{flx/flx}$) alleles were generated by Ozgene Pty. Ltd. (*Bentley WA, Australia*). Two

separate targeting vectors (*Pelle, Ozgene, Australia*) were designed to flank exons 2 and 3 of *Hamp1* or *Hamp2* with standard (loxP) or variant (lox2272) lox sites, respectively to allow for Cre-mediated deletion. Neomycin selection cassette (PGK-NEO) flanked by FRT recognition sequences was inserted downstream of exon 3 of *Hamp1* and hygromycin selection cassette (PGK-HYGRO) flanked with FLPE recognition sequences was inserted upstream of exon 2 of *Hamp2*, respectively. 5' and 3' homology arms and loxP arms were generated by PCR from C57BL/6 genomic DNA. The *Hamp1* targeting construct was electroporated into C57BL/6 embryonic stem (ES) cell line. (Köntgen, *et al* 1993) Homologous recombinant ES cell clones were identified by Southern hybridization. To create double mutant mice with both genetic modifications on the same chromatid, *Hamp2* was modified by homologous recombination in neomycin-resistant *Hamp1*-targeted ES cell clones. Recombinant ES cell clones were injected into Balb/CJ blastocysts. Male chimeric mice were obtained and crossed to C57BL/6J females to establish heterozygous germline offspring on C57BL/6 background. The germline mice were crossed to a FLP mouse line to remove the flanked selectable marker cassettes. Homozygous floxed mice were bred with PGK-Cre (*Oz-Cre, Ozgene*) or Albumin-Cre (*Jackson Laboratories*) transgenic mice to generate ubiquitous or liver-specific knockout mice, respectively, which were then intercrossed or backcrossed to homozygosity.

Genotyping of Hamp alleles by PCR

Tail genomic DNA, isolated by a commercial kit (*Promega*), was employed as a template in PCR using Taq polymerase (*New England BioLabs*) and allele-specific primers. Floxed *Hamp1* and liver-specific or ubiquitous *Hamp1* knockout mice were genotyped with primers, 5'- actctaatgaggaaggaccagagg-3' and 5'- ctgtctcatctgtgaaagcagaag-3' to amplify floxed (929 bp) and wild-type (860 bp) *Hamp1* alleles, and additionally wild-type *Hamp2* allele (968 bp). Floxed *Hamp1* allele (579 bp) was also verified using primers, 5'-ctatcaggacatagcgttggctac-3'and 5'-agtactgatatcatcgatggcg-3'. Alb-Cre transgene (100 bp) and internal control gene (324 bp) were amplified by primer pairs, 5'-gcggtctggcagtaaaaactatc-3', 5'-gtgaaacagcattgctgtcactt-3' and 5'-ctaggccacagaattgaaagatct-3', 5'-gtaggtggaaattctagcatcatcc -3', respectively. Null *Hamp1* allele (439 bp) was amplified by primers, 5'- actctaatgaggaaggaccagagg-3' and 5'-agtactgatatcatcgatggcg-3'. Floxed *Hamp2* and ubiquitous *Hamp1/2* double knockout mice were genotyped using primer pairs, 5'-attctcatgaggaaggaccagag-3', 5'-ctgtctcatctgtgaaagcagaag-3' and 5'- gtcgccaacatcttcttctggag-3', 5'-ctgtctcatctgtgaaagcagaag-3' to amplify wild-type (968 bp) and floxed (918 bp) *Hamp2* alleles, respectively. Null *Hamp2* allele (792 bp) was amplified by primers, 5'-attctcatgaggaaggaccagag-3' and 5'-gaagcaaacctaggtctagaaagc-3'. PCR parameters were: 1 cycle of 95°C for 5 min., 35 cycles of [95°C for 30s, 58°C for 1.5 min (*Hamp1*), 55°C for 1 min (*Hamp2*), 51.7°C for 1 min (*Cre*), 72°C for 30s], and 1 cycle of 72°C for 5 min.

Inductively coupled mass spectrometry (ICP-MS)

The tissues were weighed and digested with metal grade nitric acid for 2–3 hours at room temperature followed by overnight digestion at 80°C. They were subsequently cooled and diluted 20-fold prior to ICP-MS analysis. Ga (50 ppb) was added as internal standard. Iron was quantitated by measuring at m/z = 56 and 57Fe was used for confirmation. He (5ml/

min) was employed as collision gas for the elimination of polyatomic interferences, as described. (Malinouski, *et al* 2014) Each sample was analyzed in triplicate.

Real-time and Reverse Transcription (RT) Polymerase Chain Reaction (PCR)

Hamp1 and *Hamp2* gene expression was analyzed by real-time PCR with Taqman probes, as described. (Harrison-Findik, *et al* 2006) Mouse glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and beta-actin genes were used as endogenous controls. *Hamp* exons were amplified by RT-PCR using a primer pair (*sense: 5'-actcagcactcggaccca-3'; antisense: 5'-tctttatttcaaggtcattggt-3'*) binding to both *Hamp1* and *Hamp2*, and Taq polymerase [94°C, 5 min (1 cycle), 94°C, 30s, 57°C 30s, 72°C, 30s (35 cycles), 72°C 10 min (1 cycle)].

Statistical Analysis

SPSS software was used for statistical analysis. The significance of difference between groups was determined by non-parametric Wilcoxon Mann-Whitney test and Student's t-test. A value of $p < 0.05$ was accepted as statistically significant.

RESULTS AND DISCUSSION

Human and mouse hepcidin genes are composed of 3 exons encoding 84-amino-acid prepropeptide. (Pigeon, *et al* 2001) Exon 1 encodes the 24-amino-acid signal peptide and 6-amino-acid propeptide whereas exons 2 and 3 encode 54-amino-acid propeptide regions. The *Hamp1* and *Hamp1/2* transgenic mice were created by flanking exons 2 and 3 with lox sites to allow for Cre-mediated deletion of propeptide coding sequences, as described in Methods (figures 1A–E).Both transgenic mouse lines are on the same genetic background of C57BL6/J.

Hamp1 floxed mice were crossed with Albumin-Cre or PGK-Cre transgenic mice to generate liver-specific or ubiquitous *Hamp1* knockout mice, respectively. *Hamp1/2* floxed mice were bred with PGK-Cre to generate ubiquitous *Hamp1/2* double knockout mice. Genotypes for the wild-type, floxed and null *Hamp* alleles in liver-specific (figure 1F) or ubiquitous (figure 1I) *Hamp1* knockout and ubiquitous *Hamp1/2* double knockout (figure 1J) mice were determined by PCR, as described in Methods. Liver-specific targeting of *Hamp1* was confirmed by real-time PCR (figures 1G and 1H). Liver-specific *Hamp1* knockout mice did not display *Hamp1* mRNA expression in the liver compared to *Hamp1* floxed mice (figure 1G). In contrast, *Hamp1* expression was present in kidneys of liver-specific *Hamp1* knockout mice and the level was similar to that in *Hamp1* floxed mice (figure 1H). To amplify *Hamp* exons 1–3, primers binding to sequences shared by *Hamp1* and *Hamp2* were used in RT-PCR (figure 1K). The livers of ubiquitous *Hamp1* single, but not *Hamp1/2* double, knockout mice displayed *Hamp* expression, which was weaker compared to *Hamp1* floxed mice (figure 1K). Real-time PCR analysis also confirmed the absence of both *Hamp1* and *Hamp2 expression* in *Hamp1/2* double knockout mice (*data not shown*). Collectively, these findings show that *Hamp1* knockout mice express *Hamp2*, but not *Hamp1*, whereas double knockout mice lack the expression of both *Hamp*1 and *Hamp2*.

The livers of homozygous *Hamp1* knockout male mice displayed a significant 24-fold increase (24 ± 1.4) in *Hamp2* mRNA expression compared to *Hamp1* floxed male mice (1 ±

0), as determined by real-time PCR (figure 1L). The fold induction of *Hamp2* expression was less prominent (2.5 ± 0.34) in female *Hamp1* knockout mice, which might be due to higher basal *Hamp* expression in female mice (*data not shown*). Wild-type mice have been reported to display sex-related differences in hepcidin expression. (Courselaud, *et al* 2004). *Hamp1* null allele-mediated induction of *Hamp2* might be due to high iron levels in *Hamp1* knockout mice. It might also suggest that the expression of mouse hepcidin genes is regulated by interconnected mechanisms, which requires further investigation. Hepcidin is an acute phase protein and induced by inflammation. Following endotoxin (LPS) injection, *Hamp1*, but not *Hamp2*, expression was elevated in the livers of *Hamp1* floxed mice (supplementary figures 1A and 1B). In contrast, LPS attenuated the induction of *Hamp2* expression in *Hamp1* knockout mice suggesting that endotoxin exerts opposite effects on *Hamp1* and *Hamp2* expression in the liver (supplementary figure 1B). Pancreas *Hamp2* expression in wild-type mice has also been shown to be reduced by LPS treatment. (Krijt, *et al* 2004)

Iron levels in the liver, duodenum, spleen, kidney, heart and brain tissues and sera of *Hamp1* and *Hamp1/2* floxed and knockout mice were quantified by ICP-MS analysis using stable metal isotopes, 56Fe and 57Fe, as described in Methods (figures 2A–G). Male and female mice with *Hamp1* or *Hamp1/2* null alleles displayed altered iron homeostasis in all the tissues tested (figures 2A–F). The spleens of female floxed *Hamp1* and floxed *Hamp1/2* mice contained significantly higher iron than their male counterparts (figure 2C). No significant age-dependent differences in iron accumulation were observed in liver, duodenum and spleen tissues of *Hamp1* or *Hamp1/2* knockout mice (supplementary figures 2 and 3). In contrast, kidney, heart and brain tissues of 7 months old male and female *Hamp1* and *Hamp1/2* knockout mice displayed significantly higher iron content compared to those of 2 months old mice (supplementary figures 2D–F and 3D–F). The deletion of *Hamp1* expression either in the liver or all the organs exhibited similar quantitative changes in tissue and serum iron levels (figures 2A–G). Zumerle et al. reported comparable iron levels in liver-specific and ubiquitous *Hamp1* knockout mice by colorimetric assays. (Zumerle, *et al* 2014) The introduction of *Hamp2* null allele did not exacerbate iron accumulation in either male or female mice. The quantitative changes in tissue and serum iron content of *Hamp1/2* double knockout mice were not statistically different than those in *Hamp1* single knockout mice (figures 2 A–G).

The levels of other biometals were also quantified in the liver, duodenum, spleen, kidney, heart or brain tissues and sera of *Hamp1* floxed and liver-specific or ubiquitous *Hamp1* knockout mice using stable metal isotopes, 63Cu, 65Cu, 64Zn, 24Mg, 55Mn, 44Ca, 78Se, 23Na, 39K. No significant age-dependent changes were observed in the levels of these biometals (*data not shown*). The level of selenium in the livers of ubiquitous, but not liver-specific, *Hamp1* knockout mice was significantly elevated compared to *Hamp1* floxed mice (figure 3A). Similar changes were observed with both male and female knockout mice (figure 3A). The reason why significant changes were observed in ubiquitous, but not liver-specific, knockout mice requires further investigation. On the other hand, the levels of manganese were increased in the liver and duodenal tissues of both ubiquitous and liver-specific *Hamp1* knockout male and female mice (figures 3B, C). Selenium functions as an

antioxidant via selenoproteins.(Lu and Holmgren 2009) Manganese is indirectly involved in antioxidant defense via mitochondrial manganese superoxide dismutase enzyme, SOD2. (Culotta, *et al* 2005) Oxidative stress has not been examined in *Hamp1* transgenic mice but iron is associated with free radical formation and oxidative damage.(Puntarulo 2005) It is therefore feasible that changes in selenium and manganese might be part of a protective mechanism against iron accumulation in *Hamp1* knockout mice. Furthermore, iron importer, DMT1 and exporter, ferroportin can also transport manganese. (Gunshin, *et al* 1997, Madejczyk and Ballatori 2012, Yin, *et al* 2010) Changes in iron transporter expression, due to the lack of hepcidin expression, might also be responsible for altered manganese metabolism in the liver and duodenum of *Hamp1* knockout mice. Interestingly, *Hamp1* null allele induced significant changes in copper levels only in the brain. The brains of both ubiquitous and liver-specific *Hamp1* knockout mice contained more copper than those of *Hamp1* floxed mice (figure 3D). Similar quantitative changes were observed in the brains of male and female knockout mice (figure 3D). Iron and copper metabolism are linked via copper-dependent ferroxidases. The brain also expresses a glycosylphosphatidylinositol-anchored form of ceruloplasmin.(Patel and David 1997) Brain ceruloplasmin has been suggested to play a role in both iron influx and efflux.(Qian and Ke 2001) The interaction of iron and copper homeostasis, and potential role of ceruloplasmins in the brains of *Hamp1* knockout mice requires further investigation. The serum content of selenium and copper were similar in *Hamp1* floxed and knockout mice, as determined by ICP-MS analysis (figure 3E–F). These mice however displayed negligible levels of serum manganese (*data not shown*). Unlike the liver, duodenum or brain, the levels of 78Se, 55Mn, 63Cu and 65Cu were not significantly altered in the spleen, heart or kidney tissues of ubiquitous and liver-specific *Hamp1* knockout mice. In addition, *Hamp1* null allele did not affect zinc, magnesium, calcium, sodium and potassium homeostasis in any of the tested tissues (*data not shown*).

Our conditional transgenic mouse lines will be useful to determine the tissue-specific role of hepcidin genes in biometal homeostasis and related disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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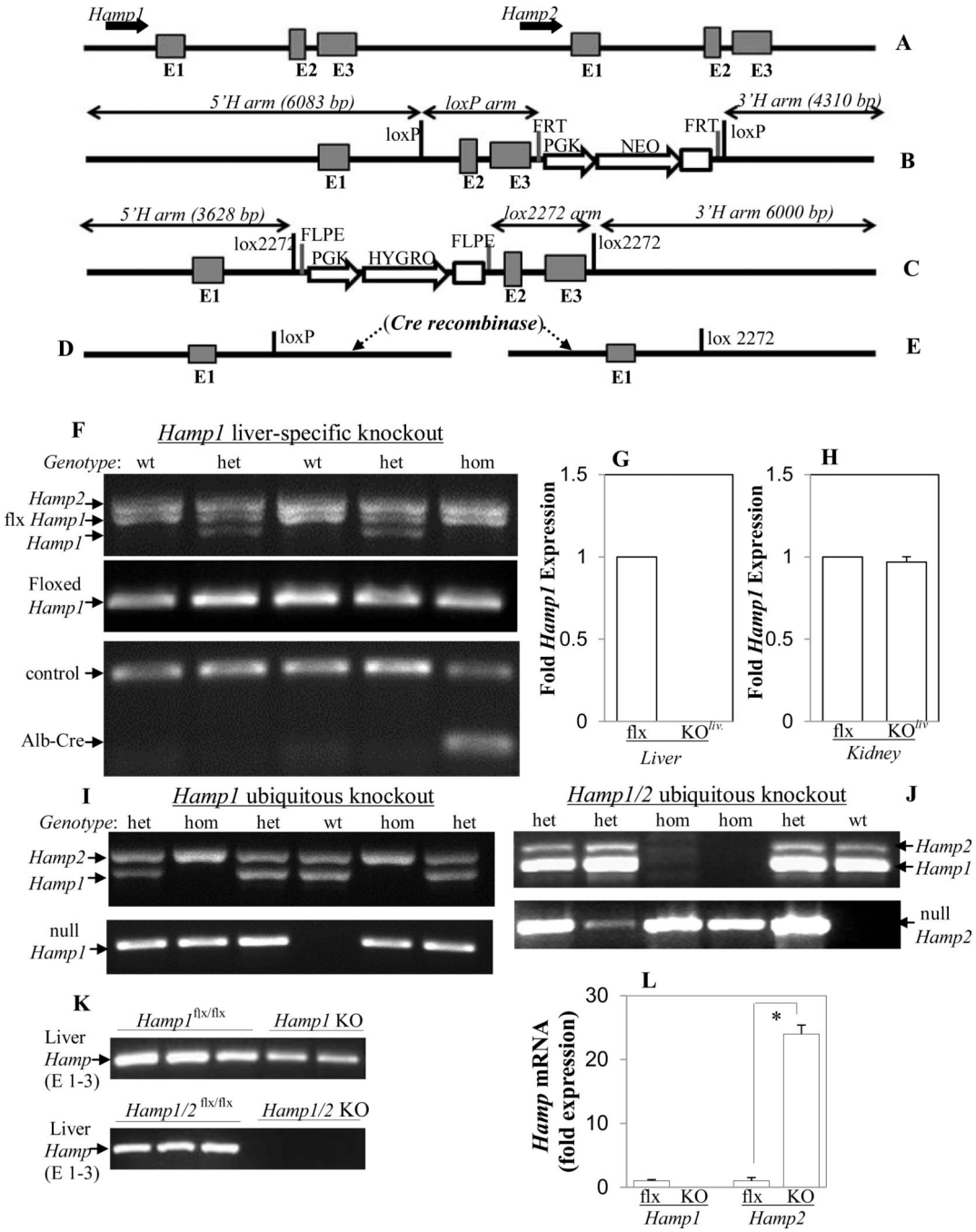

Figure 1. Targeted disruption and genotyping of *Hamp1* and *Hamp2* alleles

(**A):** The genomic structure of wild-type *Hamp1* and *Hamp2* loci. Gray boxes represent exons (E1, E2 and E3). (**B, C):** *Hamp1* (**B**) and *Hamp2* (**C**) targeting vectors flanking exons 2 (E2) and 3 (E3) with standard (loxP) or variant (lox2272) lox sites, and neomycin (PGK-NEO) and hygromycin (PGK-HYGRO) selection cassettes with FRT and FLPE recognition sequences, respectively, have been generated, as described in Methods. (**D, E):** *Hamp1* (**D**) and *Hamp2* (**E**) null alleles after Cre-mediated recombination excising exons 2 and 3 leaving

a single lox P or lox2272 site, respectively and exon 1, which encodes primarily for the signal peptide.

(F): Genotyping of wild-type (wt) mice and heterozygous (het) or homozygous (hom) liver-specific *Hamp1* knockout (KO^{liv}) mice generated by mating *Hamp1* floxed (flx) and Albumin-Cre transgenic mice was performed by PCR. as described in Methods. **(G–H):** Tissue-specific *Hamp1* expression was determined by real-time PCR by employing cDNA synthesized from the liver (G) or kidney (H) RNA of homozygous flx or KO^{liv} mice. mRNA expression in KO^{liv} mice was expressed as fold expression of that in flx mice. **(I–J):** Genotyping of wild-type (wt) mice and heterozygous (het) or homozygous (hom) ubiquitous *Hamp1* (H) and ubiquitous *Hamp1/2* (I) knockout (KO) mice generated by mating *Hamp1* or *Hamp1/2* flx mice with PGK-Cre transgenic mice, was performed by PCR. (**K**)**:** Amplification of *Hamp* exons (E1–3, 300 bp) was performed by RT-PCR using cDNA synthesized from liver RNA of *Hamp1* ($Hamp1^{flx/flx}$) or *Hamp1/2* ($Hamp1/2^{flx/flx}$) floxed and ubiquitous single (*Hamp1 KO)* or double *(Hamp1/2 KO)* knockout mice, and primers binding to both *Hamp1* and *Hamp2*. (**L):** *Liver Hamp1 and Hamp2 expression* in homozygous *Hamp1* flx and ubiquitous *Hamp1* knockout (KO) mice was determined by real-time Taqman PCR. mRNA expression in the livers of KO mice was expressed as fold change of that in the livers of flx mice.

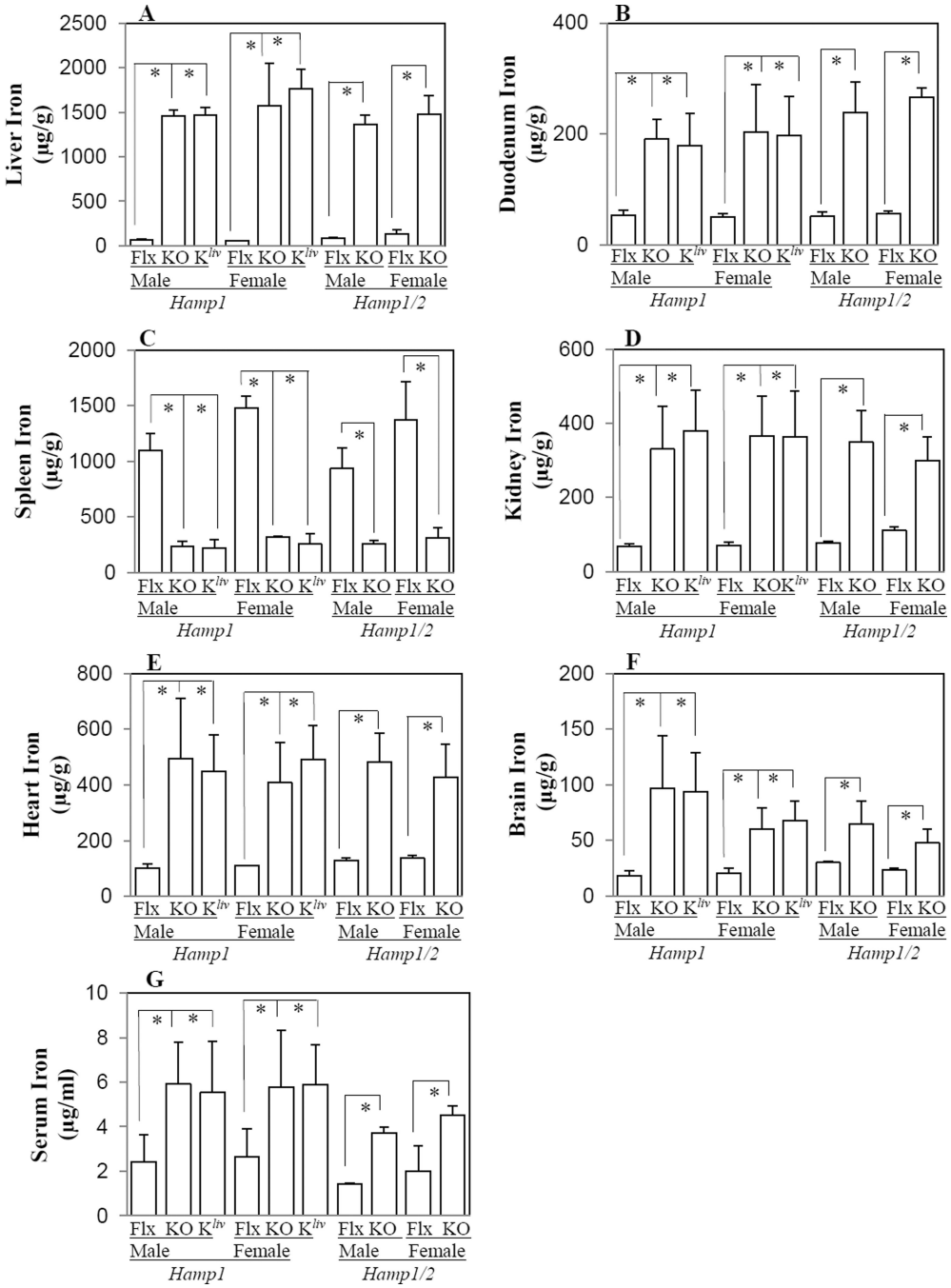

Figure 2. Quantification of iron in Hamp1 and Hamp1/2 knockout mice

Iron in the liver, duodenum, spleen, kidney, heart brain and sera of *Hamp1 or Hamp1/2* floxed (Flx), and ubiquitous *Hamp1* or *Hamp1/2* (KO) and liver-specific *Hamp1* (K^{liv}) homozygous knockout mice (*n= 8/ group, 2–7 months old*) was quantified by ICP-MS (*see Methods*). Similar quantities of iron were detected measuring either 56Fe or 57Fe (*data not shown*). Tissue and serum iron levels are expressed as microgram iron per gram wet tissue weight or per milliliter of serum, respectively. Asterisks indicate statistical significance ($p<0.05$).

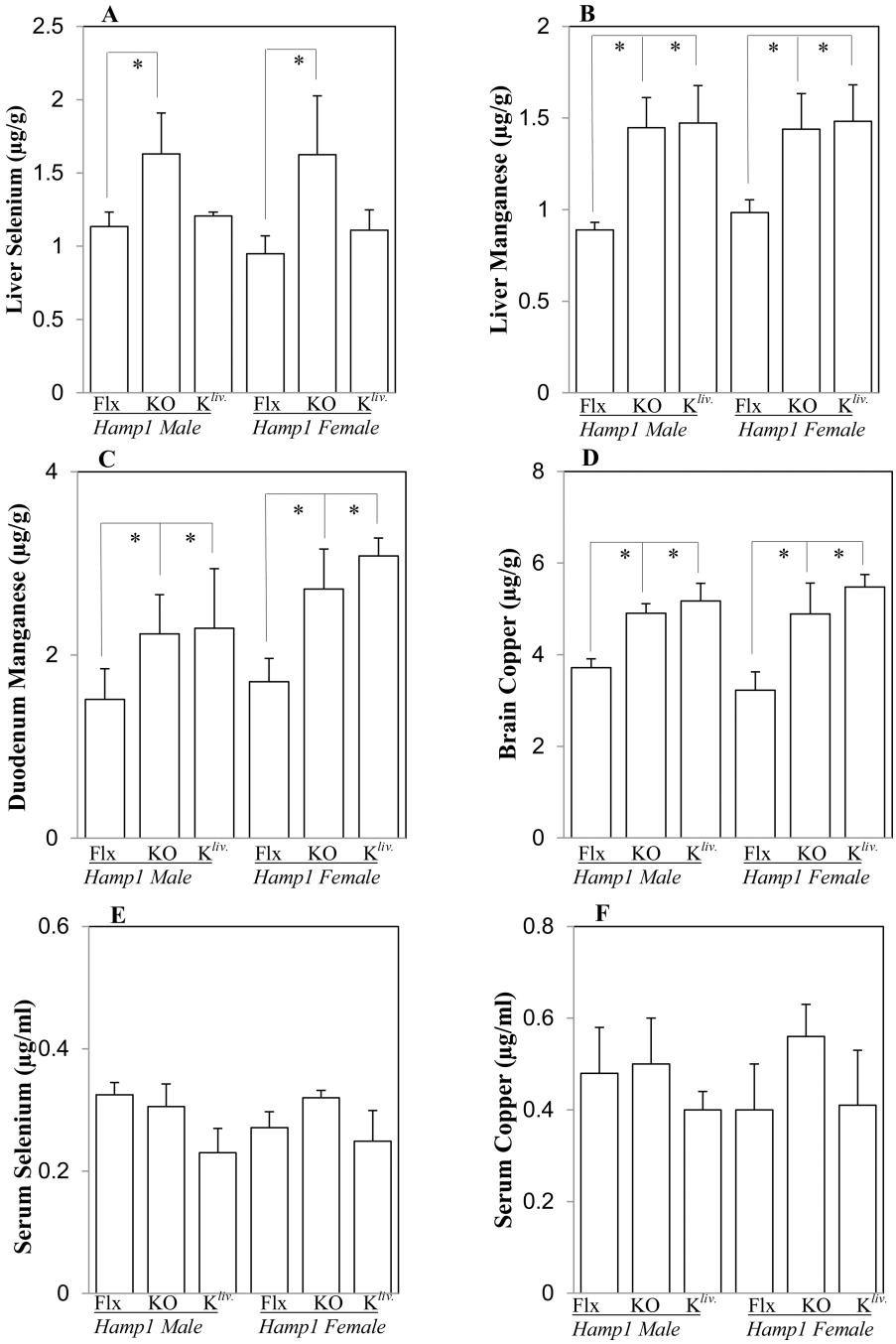

Figure 3. The effect of Hamp1 null allele on selenium, manganese and copper

The levels of selenium (**A**) and manganese (**B**) in the liver, manganese in the duodenum (**C**), copper in the brain (**D**) tissues, and selenium (**E**) and copper (**F**) in sera of floxed (flx) and ubiquitous (KO) or liver-specific (K^{liv}) *Hamp1* homozygous knockout mice (*n= 8/ group, 2–7 months old*) were detected by ICP-MS measuring 78Se, 55Mn, 65Cu or 63Cu. Biometals are expressed as microgram metal per gram wet tissue weight or per milliliter of serum, respectively. Asterisks indicate statistical significance ($p<0.05$).