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Short Communication

# Hepcidin deficiency undermines bone load-bearing capacity through inducing iron overload

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ABSTRACT

Osteoporosis is one of the leading disorders among aged people. Bone loss results from a number of physiological alterations, such as estrogen decline and aging. Meanwhile, iron overload has been recognized as a risk factor for bone loss. Systemic iron homeostasis is fundamentally governed by the hepcidin–ferroportin regulatory axis, where hepcidin is the key regulator. Hepcidin deficiency could induce a few disorders, of which iron overload is the most representative phenotype. However, there was little investigation of the effects of hepcidin deficiency on bone metabolism. To this end, hepcidin-deficient (*Hamp1*$^{-/-}$) mice were employed to address this issue. Our results revealed that significant iron overload was induced in *Hamp1*$^{-/-}$ mice. Importantly, significant decreases of maximal loading and maximal bending stress were found in *Hamp1*$^{-/-}$ mice relative to wildtype (WT) mice. Moreover, the levels of the C-telopeptide of type I collagen (CTX-1) increased in *Hamp1*$^{-/-}$ mice. Therefore, hepcidin deficiency resulted in a marked reduction of bone load-bearing capacity likely through enhancing bone resorption, suggesting a direct correlation between hepcidin deficiency and bone loss. Targeting hepcidin or the pathway it modulates may thus represent a therapeutic for osteopenia or osteoporosis.



## 1. Introduction

According to the data of the World Health Organization, osteoporosis has become a global issue and an increasing threat to human health (Alexandre, 1995). Osteoporosis is a common disease characterized by low bone mineral mass, associated with a high incidence of bone fractures (Cooper and Aihie, 1995; Guggenbuhl et al., 2005). Bone metabolism is maintained through a kinetic balance between formation and resorption. Bone formation is performed by osteoblasts, while bone resorption is carried out by osteoclasts (Fohr et al., 2003; Mizuno et al., 1998; Suda et al., 1996). Disruption of this balance is likely associated with bone loss (Suda et al., 1996). There are a number of factors that may account for osteopenia or osteoporosis, such as estrogen deficiency and abnormal calcium metabolism (Chevalley et al., 1994; Kanis, 1994). Additionally, Delbarre and his colleagues reported that osteoporosis is a complication of hereditary hemochromatosis (HH) half a century ago (Delbarre, 1964). Since then, a number of clinical and experimental studies have documented that iron overload represents a risk factor responsible for bone loss, especially in patients suffering from HH (Guggenbuhl et al., 2011a), African hemosiderosis (Lorincz et al., 1974), thalassemia (Salama et al., 2006), sickle cell disease (Adams-Graves et al., 2013), and liver diseases (Diamond et al., 1990). A recent study described that 25% of HH patients were diagnosed with osteoporosis and 41% developed osteopenia (Valenti et al., 2009). HH is a genetic disorder, associated with iron overload in various organs (Fleming and Sly, 2002). In parallel to this finding, hemochromatosis (HFE)-deficient male mice, a mouse model of HH, developed osteoporosis with low bone mass (Guggenbuhl et al., 2005, 2011b). Meanwhile, numerous clinical studies suggested that patients with osteoporosis accompanied iron accumulation in bone (Jian et al., 2009). Together, iron homeostasis is closely coupled to bone metabolism, and excessive iron accumulation is recognized as a risk factor for osteoporosis (Haidar et al., 2011; Kudo et al., 2008; Tsay et al., 2010; Valenti et al., 2009).

Iron homeostasis is fundamentally governed by the hepcidin–ferroportin regulatory axis (Park et al., 2001). Hepcidin, a 25 amino acid peptide, is the master hormone in modulating systemic iron homeostasis (Ganz, 2011). Its transcription is stimulated by iron, inflammatory cytokines and bone morphogenetic proteins (BMPs), and repressed by signaling from anemia and hypoxia (Ganz, 2011). Hepcidin inhibits iron egress out of cells through binding and inducing degradation of its receptor ferroportin. Ferroportin, the only known

*Abbreviations:* WT, wildtype; CTX-1, C-telopeptide of type I collagen; HH, hemochromatosis; HFE, hemochromatosis; BMP, bone morphogenetic protein; PBS, phosphate-buffered saline; OD, optical density; ROS, reactive oxygen species.

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mammalian iron exporter is mainly expressed in epithelial cells in duodenum and macrophages (Jelic et al., 2013; Pinnix et al., 2010). Suppression of hepcidin expression or hepcidin deficiency would cause enhanced iron egress out of macrophages, leading to iron overload in various organs, associated with neoplasia, arthropathy and neurodegenerative diseases (Jelic et al., 2013; Pinnix et al., 2010).

However, there was still no investigation of the effects of hepcidin deficiency on bone metabolism. To this end, we used the hepcidin-deficient (hepcidin knockout, *Hamp1*$^{-/-}$) mice to study bone metabolism under the setting of disordered iron homeostasis. We overall demonstrated that iron overload due to hepcidin deficiency greatly undermined bone strength and bone load-bearing capacity. Thus, targeting hepcidin or the signaling it mediates may represent a novel therapeutic for osteoporosis.

## 2. Materials and methods

### 2.1. Animal experiment

*Hamp1*$^{-/-}$ mice were originally provided by Dr. Sophie Vaulont (Lesbordes-Brion et al., 2006) and currently with the C57BLK/6 background (Ramos et al., 2012). All mice were housed in the central animal facility of the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. Male *Hamp1*$^{-/-}$ mice and wildtype (WT) mice with the same genetic background were sacrificed at 8 weeks, 18 weeks and 30 weeks. Sera and organ specimens were collected. Tibias were removed and packed in gauze soaked with phosphate-buffered saline (PBS), and stored at −80 °C for future analyses.

### 2.2. Iron parameters

Serum iron concentration was determined with a serum iron detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Hepatic iron content was assessed as previously described (Liu et al., 2007, 2008). Briefly, the specimens of liver were digested by the acid solution for 72 h at 65 °C. Thereafter, chromagen working solution was added. Finally, absorption at 535 nm was determined with a plate reader (Thermo).

### 2.3. Assessments of bone metabolic markers

Serum osteocalcin and C-telopeptide of type I collagen (CTX-1) were assayed by ELISA (Rapidbio; RapidBio, West Hills, CA, USA), according to the manufacturer's instructions. Briefly, the samples and standards were added into 96-well plates, and then incubated for 30 min at 37 °C. After washing five times, HRP-conjugate reagent was added, followed by another round of incubation and washing. Optical density (OD) at 450 nm was measured after adding stop solution within 15 min. Finally, serum osteocalcin and CTX-1 concentrations were calculated according to the standard curves.

### 2.4. Bone biomechanical analyses

Biomechanical properties of tibias were determined by the three-point bending test using a universal material test machine (AG-1S; Shimadzu Co., Kyoto, Japan) at room temperature, as described in previous studies (Mattila et al., 1999; Yamasaki and Hagiwara, 2009). The distance between the supporting rods had a fixed length (L) of 12 mm. Load was applied at a constant deformation rate of 2 mm/min. The diaphysis of the tibia was loaded until fracture occurred, which provided measurement of yield and fracture parameters. Yield represents the point at which the bone ceases to behave elastically. Data were automatically recorded in a computer interfaced to the testing machine, and a typical load-deformation curve was created. The material properties of bone including the maximal loading and maximal bending stress were calculated according to the established formulas (Mattila et al., 1999; Yamasaki and Hagiwara, 2009). PBS was used to keep specimens moist during testing.

### 2.5. Statistical analysis

The SPSS Statistics 17.0 package was utilized to analyze the date. One-way analysis of variance (ANOVA) was applied to determine the mean differences among groups compared to the control. The difference between two groups was determined using the independent *t*-test. Data were shown in mean ± SD. $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Systemic iron overload in hepcidin-deficient mice

To look into iron concentrations of *Hamp1*$^{-/-}$ mice, we compared serum and tissue iron concentrations between hepcidin-deficient mice and WT mice of different ages. As shown in Fig. 1a, serum iron level was increased by approximately 25% in *Hamp1*$^{-/-}$ mice relative to WT mice at 8 weeks ($p < 0.05$). This difference became even greater when mice got older, as evidenced by over 40% and 50% increase of serum iron in *Hamp1*$^{-/-}$ mice compared to WT mice at 18 weeks and 30 weeks, respectively (Fig. 1a, $p < 0.05$). Moreover, there was a clear time-dependent increase of serum iron for *Hamp1*$^{-/-}$ mice from 8 weeks to 30 weeks (Fig. 1a, $p < 0.05$). However, the serum iron level remained consistent in WT mice over the time course from 8 weeks to 30 weeks (Fig. 1a, $P > 0.05$). Similarly, liver iron content was greatly increased in *Hamp1*$^{-/-}$ mice compared to WT mice for all ages tested (Fig. 1b, $p < 0.05$). There was an increase of liver iron concentrations in a time-dependent manner (Fig. 1b, $p < 0.05$), and hepatic iron concentrations were similar at different ages (Fig. 1b). Consistent with previous findings (Masaratana et al., 2011), our date revealed that hepcidin deficiency led to remarkable systemic iron overload, as reflected by increased serum iron and hepatic iron.

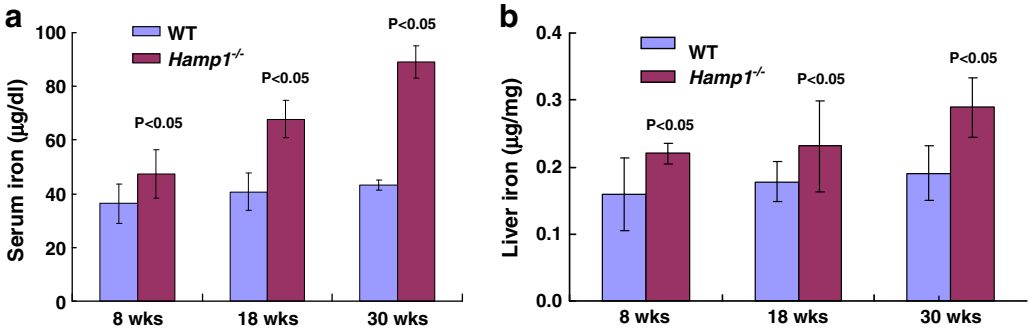


**Fig. 1.** Iron content in WT mice and *Hamp1*$^{-/-}$ mice at different ages. Serum iron (a) and hepatic iron (b) were shown (n = 4-5).