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Hepcidin inhibition on the effect of osteogenesis in zebrafish

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ABSTRACT

Iron overload, as a risk factor for osteoporosis, can result in the up-regulation of *Hepcidin*, and *Hepcidin* knockout mice display defects in their bone microarchitecture. However, the molecular and genetic mechanisms underlying *Hepcidin* deficiency-derived bone loss remain unclear. Here, we show that *hepcidin* knockdown in zebrafish using morpholinos leads to iron overload. Furthermore, a mineralization delay is observed in osteoblast cells in *hepcidin* morphants, and these defects could be partially restored with microinjection of *hepcidin* mRNA. Quantitative real-time PCR analyses revealed the osteoblast-specific genes *alp*, *runx2a, runx2b,* and *sp7* in morphants are down-regulated. Furthermore, we confirmed qRT-PCR results by i*n situ* hybridization and found down-regulated genes related to osteoblast function in *hepcidin* morphants. Most importantly, we revealed that *hepcidin* was capable of removing whole-body iron which facilitated larval recovery from the reductions in bone formation and osteogenesis induced by iron overload.

1. Introduction

As a peptide hormone expressed in the liver [1], HEPCIDIN plays an essential role in iron absorption and iron delivery by binding to the iron transporter Ferroportin, which results in the internalization and degradation of this transporter [2]. However, *Hepcidin* mutations are associated with hemochromatosis and impaired *Hepcidin* expression, leading to iron overload [3]. Iron overload is a risk factor for osteoporosis, especially postmenopausal osteoporosis [4–6], and osteoporosis is regarded as a complication of iron overload-related diseases [7,8]. Bone loss accelerated by iron overload has also been observed in healthy postmenopausal women and middle-aged men [9].

Various animal models have shown that iron-induced osteoclastic activity plays a crucial role in iron overload [10,11]. Although it has been shown that excess iron could inhibit osteoblast activity and mineralization in vitro for bone formation [12], recent limited *in vivo* studies regarding the effect of iron overload on osteoblast function and bone formation are still controversial [10].

Zebrafish are an ideal vertebrate model for studying bone formation [13]. Given their short generation time and optically transparent embryos, zebrafish provide an excellent system for dissecting the bone metabolism [13,14]. Zebrafish larvae at 48–72 h post-fertilization (hpf) are an excellent model for examining the expression of *hepcidin*; at this stage, the larvae do not ingest food and are free of inflammatory stimuli, reducing variables that may affect *hepcidin* expression [15,16].

In this study, we first developed an iron-overload model in zebrafish by morpholino technology to demonstrate that the effect of excess iron on osteoblast calcification in vitro could have an effect on osteogenesis *in vivo* and then further investigated the therapeutic role of hepcidin in iron overload-induced inhibition of bone formation.

2. Methods

All animal care and experiments involving animals were conducted in accordance with the institutional Ethical Guidelines for Animal Experiments, and all fish experimental procedures were approved by the Committee on Animal Use and Care of Soochow University.

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2.1. Zebrafish maintenance and embryo production

Wild-type (WT) zebrafish were maintained in 3 L of modular aquaria with continuous water exchange at 28.5 °C under 14 h light/10 h dark cycles in the Soochow University Zebrafish Research Facility. All adult fish were fed live brine shrimps or flakes three times a day.

2.2. Microinjection of hepcidin MO

Antisense MO oligonucleotides against *hepcidin* were designed and synthesized by Gene-Tools (http://www.gene-tools.com/). an SPLMO that blocks the splicing of the second intron and two corresponding control MOs, each with five-nucleotide mismatches, were used (MO are sequences shown in Fig. 2A and C). The effectiveness of the SPLMO was examined by RT-PCR analysis (Fig. 2B).

2.3. Whole-mount larvae staining for iron

The larvae were collected at 4 dpf after fixation in 4% paraformaldehyde-PBS and dehydrated in methanol. The embryos were subsequently incubated in 2.5% potassium ferrocyanide/0.25 M HCl (no. HT20-1 KT; Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature. The embryos were rinsed three times in phosphate-buffered Tween (PBT), followed by incubation in 0.3% H_2O_2 in methanol for 20 min at room temperature. The embryos were rinsed twice in PBT and incubated in DAB substrate for 5–7 min at room temperature. The DAB substrate was generated by dissolving one tablet of Sigma Fast 3,3-DAB tetrahydrochloride with cobalt chloride enhancer (no. D-0426, Sigma-Aldrich) in 5 mL of water, which results in a dark blue or black stain. Photomicrographs of the iron-stained embryos were obtained using an M165FC stereomicroscope (Leica) with a 3.3RTV camera (QIMAGING).

2.4. Iron measurement by ICP-MS

The metal content in zebrafish larvae was measured using ICP-MS as described previously[23]. The larvae ($n = 20$ each with three replications) were collected at 4 dpf after treatment as previously described. The collected larvae were washed five times in iron-free E3 medium and then transferred into centrifuge tubes (15.0 mL). The samples were then digested with 70% HNO_3 (Tama Chemicals, Japan) in a microwave oven for 4 h. To measure the iron content, the digested samples were analysed using an Agilent 7500cx ICP/MS system (Agilent Technologies, USA) equipped with a G3160B I-AS integrated autosampler.

2.5. Rescue experiments

Capped *hepcidin* mRNAs were transcribed in vitro using the mMESSAGE mMACHINE SP6 kit (Invitrogen). Approximately 600 ng of capped mRNA of *hepcidin* was injected into one- or two-cell embryos. The 4 dpf larvae were collected for the experiments.

2.6. RNA isolation, cDNA synthesis, and qRT-PCR

More than 20 larvae with the same genotype at 4 dpf were pipetted into one RNA-free 1.5 mL-centrifuge tube. TRIzol (Invitrogen, CA, USA) was used to isolate the RNA from the WT or morphant larvae. Superscript III Reverse Transcriptase (Invitrogen, CA, USA) was used to generate cDNA through reverse transcription. Each genotype was collected three times simultaneously. Quantitative real-time PCR (qRT-PCR) was carried out with the ABI StepOne Plus™ system using SYBR® Premix Ex Taq™ (Takara, Japan) using the following thermal profile: 95 °C for 3 min; 95 °C for 10 s; and 58 °C for 30 s for 40 cycles. The primers for *alp*, *runx2a*, *runx2b*, *ferroportin*, *bmp2a*, and *sp7* are listed in Supplementary Table S1. Relative mRNA expression levels were quantified using the comparative Ct (ΔCt) method and expressed as $2^{-(\Delta\Delta Ct)}$. The calculation was carried out using Microsoft Office Excel. Each PCR assay was conducted using three biological samples.

2.7. Whole-mount in situ hybridization

Larvae were collected at 4 dpf and fixed in 4% PFA overnight. *In situ* hybridization was conducted as described previously [17]. The probe sequences of *runx2a* and *sp7* are listed in Supplementary Table S1.

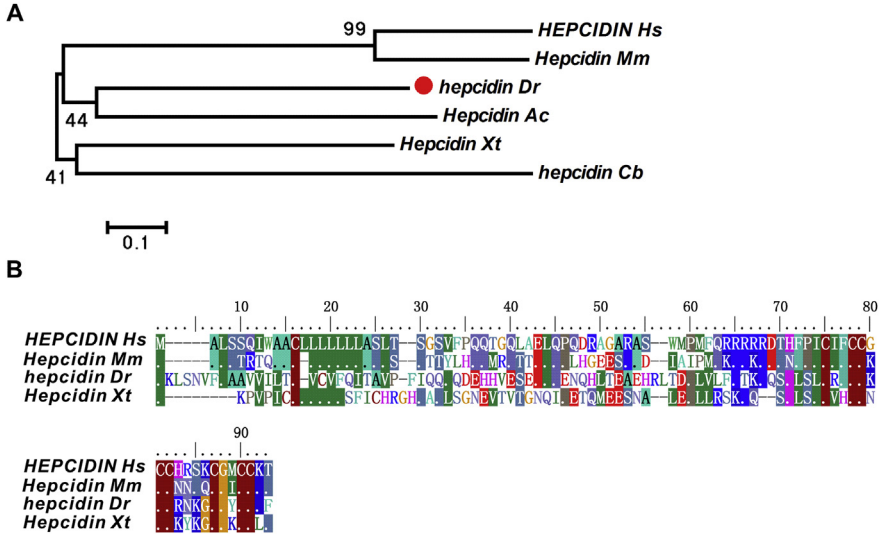

Fig. 1. Phylogenetic tree and amino acid sequence of HEPCIDIN proteins. . (A) Phylogenetic trees of HEPCIDIN proteins were constructed using the neighbour-joining (NJ) method and the maximum-likelihood method using MEGA6.06 [32]. The number of bootstrap replications was 1000. The numbers indicate the support value. *Dr*, *Danio rerio*; *Hs*, *Homo sapiens*; *Mm*, *Mus musculus*; *Xt*, *Xenopus tropicalis* and Ac, *Anolis carolinensis*. (B) Multiple alignment of the deduced partial amino acid sequence of HEPCIDIN with those of other species using Clustal X 1.83. Conserved residues are marked with a coloured frame. *Dr*, *Danio rerio*; *Hs*, *Homo sapiens*; *Mm*, *Mus musculus* and *Xt*, *Xenopus tropicalis*.