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Hepcidin is an endogenous protective factor for osteoporosis by reducing iron levels

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Abstract

Postmenopausal osteoporosis is a global health issue. Although a lack of estrogen is considered the major reason for postmenopausal osteoporosis, other factors might also contribute the etiology of the disease. In previous reports, we and others proposed that iron accumulation after menopause accelerates osteoporosis, and here, we genetically modified the expression of an endogenous hormone, hepcidin, to modulate iron status in a mouse model. Our results show that hepcidin levels negatively correlate with bone loss in both knockout and overexpression (with ovariectomy) murine models. In addition, iron overload enhances reactive oxygen species (ROS) activity and attenuates the functions of primary osteoblasts, while iron depletion could reverse this phenomenon through inhibiting the functions of primary osteoclasts. Therefore, our results provide more evidence of the 'iron accumulation' hypothesis, which suggests that high iron levels are risk factors for osteoporosis, and the 'Huang's hypothesis' that hepcidin is a potential drug target for the prevention of postmenopausal osteoporosis.

Key Words

- ▶ hepcidin
- ▶ osteoporosis
- ▶ oxidative stress
- ▶ bone metabolism
- ▶ iron metabolism

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Introduction

Hepcidin is a small peptide hormone that is synthesized and secreted by the liver and regulates iron metabolism (Zhao *et al.* 2013). Hepcidin acts by binding to the ferroportin iron exporter, which triggers its internalization and intracellular degradation (Qian *et al.* 2015). In the presence of hepcidin, enterocytes release less iron into the portal system, which downregulates iron uptake throughout the intestine. Similarly, hepcidin negatively regulates macrophage iron export. Xu and coworkers reported that hepcidin, liver and bone iron all increase weightlessness in mice, through increasing osteoclast activity and decreasing osteogenic activity and bone

mass. However, the loss of bone mass was more serious upon siRNA interference of hepcidin expression (Xu *et al.* 2017). With respect to the mechanism, hepcidin promotes the differentiation and mineralization of osteoblasts through L-type Ca²⁺ channels and regulates osteoblasts through insulin receptor substrate 1, FPN, BMP6, bone morphogenetic protein 2, smad mothers against decapentaplegic and mitogen-activated protein kinase P38 signaling pathways to promote bone formation (Ma *et al.* 2010, Xu *et al.* 2011, Li *et al.* 2012, Huading *et al.* 2015, Liu *et al.* 2018). Our previous studies have found that serum ferritin and liver iron contents were significantly higher

in hepcidin-knockout mice than in wild-type mice. In hepcidin-knockout mice, the BMP/Smad pathway was also repressed, resulting in decreased osteoblast function and bone mass (Shen *et al.* 2014).

Menopause is a naturally occurring phenomenon that affects most women above the approximate age of 52 years. The menopause-associated physiological exhaustion of ovarian function increases reproductive, cardiovascular and mental impairments and cancer (Pluchino *et al.* 2011, Cutler *et al.* 2015, Gong *et al.* 2016, Honour 2018). Furthermore, the musculoskeletal system is also deeply affected by menopause (Cauley 2015, Sullivan *et al.* 2015). Deficiency of estrogen accelerates bone resorption, resulting in type I osteoporosis. Furthermore, serum ferritin increases 2- to 3-fold during this period, due to the cessation of iron loss with decreased menstrual bleeding. Therefore, iron accumulation is very common in postmenopausal women (Huang *et al.* 2013). Iron is an essential trace element for many metabolic process; however, like most transition metals, iron accumulation is toxic and an independent risk factor for osteoporosis (Weinberg 2006, Messner *et al.* 2009, Jeney 2017). Previous studies have found that iron accumulation stimulates osteoclast activity and accelerates bone loss in mice (Xiao *et al.* 2015). Similar observations were found in postmenopausal women, who ultimately developed osteoporosis and bone fractures (Huang *et al.* 2013). It has been reported that desferrioxamine and deferasirox, which are iron-chelating agents that have been used in research and in patients with hematonosis, promote osteoblast differentiation and reduce osteoporosis fracture risk, although these medicines have been observed to cause side effects during long-term treatment (Qu *et al.* 2008, Messa *et al.* 2010).

In this study, the hepcidin knockout and conditional overexpression mouse models were used to investigate the effects of hepcidin deficiency and overexpression on bone mineral density, proliferation, osteoblast and osteoclast differentiation and activity. Interestingly, we detected a change in reactive oxygen species (ROS) activity that furthers our understanding of the influences of hepcidin on iron metabolism and bone remodeling.

Materials and methods

Experimental animal models

All transgenic mice used in our experiments were provided as generous gifts from the Cambridge-Soochow University Genome Resource Center (CAM-SU GRC), including wild-type (WT), knockout (Hamp $-/-$), ovariectomized

(WT OVX) and hepcidin conditional overexpression (Hamp OVX). The mice were bred and maintained on a C57Bl/6J background, in a specific pathogen-free (SPF) laboratory in Soochow University, where the temperature was controlled at 25°C. The relative humidity was adjusted to 45–55%, and mice were maintained in 12 h-light/12 h-darkness cycles with free access to food and weakly acidic tap water. The mice were fed a normal diet with low iron, and trace elements in the diet did not differ significantly between cohorts. Hamp OVX mice were ovariectomized at 8 weeks of age to produce osteoporosis models. After 1 week, tamoxifen (Sigma) was used to induce hepcidin overexpression, and WT and WT OVX mice were not sensitive to tamoxifen-induced hepcidin overexpression. All mice were intraperitoneally injected with 0.1 mL of a 10 mg/mL solution of tamoxifen for 5 days (Indra *et al.* 1999), and 8 weeks after the experiment, mice were killed and specimens were collected.

Serum hepcidin, serum ferritin, and bone turnover assays

Serum samples were collected and analyzed for hepcidin (Elabscience, TX, USA), serum ferritin (Abcam), osteogenic marker (P1NP) and osteoclast marker (CTX) (Immunodiagnostic Systems Ltd, UK) levels by enzyme-linked immunosorbent assays (ELISA). All ELISA assays were performed according to manufacturer instructions.

Bone specimen processing

The muscle and soft tissue around the bilateral femur of the mice were removed, and the integrity of the distal femur was preserved. Specimens were fixed in a 10% formalin solution for 48 h. The left femur was used for micro-CT detection of bone mass, and the right bone specimens were washed and soaked in distilled water for 24 h to prevent iron contamination. Washed samples were dehydrated in 75, 85, 95 and 100% ethanol for 24 h, respectively, vitrified by dimethylbenzene and embedded in methyl methacrylate (MMA). Sections (6 mm thick) were cut dry on a heavy-duty sliding microtome equipped with 40° tungsten carbide knives (Leica SM2500). Some of the sections were stained by Prussian blue for 24 h to visualize ferric iron deposits, and other sections underwent HE staining to investigate bone microstructure.

Micro-CT scanning

The left femur specimens were scanned from the mid-diaphyses of femurs and trabecular bones and from distal

femurs with a Skyscan 1176 micro-computed tomography machine (Skyscan, Belgium) at 9 μ m resolution, 50 kV, 500 μ A and 0.5° rotation step. Analyses indices included bone mineral density (BMD), percent bone volume (BV/TV), trabecular number (TB. N), trabecular thickness (TB. Th) and trabecular separation (Th. Sp).

Primary osteoblast (OB) and osteoclast (OC) cell proliferation assays

Primary osteoblasts were extracted from newborn mice calvarias and osteoclasts were obtained from 12-week-old mice femora. Cell proliferation was analyzed using the Cell Counting kit 8 (Dojindo, Japan). Briefly, cells were detached and seeded into 96-well plates, at a density of 5×10^3 cells/well. After 24 h, cells were treated with ferric ammonium citrate (FAC) at various concentrations (0, 25, 50, 100, or 200 μ M, at 100 μ L/well) or desferrioxamine (DFO) (0, 10, 20, 30, or 50 μ M, at 100 μ L/well) for another 24 h. The optical density (OD) of each well was measured at 450 nm, using a BioTek microplate reader.

Alizarin red and alkaline phosphatase assays

OBs were treated with FAC and DFO for three days, after which cells were stained for alkaline phosphatase (ALP) (Beyotime Biotechnology, Shanghai, China) following manufacturer instructions. For alizarin red staining, cells were treated according to the experimental design described below, and after 14 days, cells were fixed and exposed to Alizarin Red-S solution (Beyotime Biotechnology) for 30 min, and mineralized nodules were photographed. Experimentally, cultured cells were seeded into 12-well plates and treated with FAC and DFO. After 10 days, cells were lysed with cell lysis buffer and centrifuged at 250 g for 5 min. Aliquots of supernatant were collected, and ALP activity and protein concentration were measured using ALP (Jiancheng, China) and BCA protein assay (Beyotime Biotechnology) kits, respectively. The OD was measured at a wavelength of 520 nm, using a BioTek microplate reader.

Tartrate-resistant acid phosphatase staining and pit formation assay

To estimate the number of differentiated osteoclasts within the bone marrow cell population, cells were stained with tartrate-resistant acid phosphatase (TRAP). Briefly, cells were fixed in 3.7% formaldehyde for 10 min and then incubated for 60 min at 37°C in the dark with a solution containing sodium nitrite, Fast Garnet GBC, acetate,

naphthol AS-BI phosphoric acid and tartrate from the Leukocyte Acid Phosphatase Assay kit (Sigma), following manufacturer instructions. Trap-positive multinucleated cells (MNCs) containing three or more nuclei were scored using light microscopy. The data are expressed as the mean $\pm$ s.d.

OCs were detached from the flask using a trypsin/EDTA solution and resuspended as a uniform, single cell suspension. Cells were re-plated into bone slices at a density of 2.5×10^4 cells/well. α -MEM with 10% FBS and 100 ng/mL RANKL was added to each well. Plates were incubated at 37°C in a humidified atmosphere with 5% CO₂ for four days. Cells were removed from bone slices on day four and stained by 0.1% toluidine blue for 30 min. Individual pits were examined in dry wells using a light microscope. The pit areas and object area/total area ratios were measured using Image-Pro Plus, version 6.0 software (Media Cybernetics).

Quantitative RT-PCR analysis

Total RNA was extracted from OB and OC cells and reverse-transcribed into cDNA using a reverse transcription kit (Invitrogen), following manufacturer instructions. The polymerase chain reaction was performed with a total reaction volume of 10 μ L: 2 $\times$ Taq PCR mix 5 μ L, nuclease-free water 4 μ L, cDNA 0.5 μ L, forward or reverse primers 0.5 μ L. PCR conditions: 95°C for 30 s, 95°C for 30 s and 60°C for 20 s, for 40 cycles. The primer sequence is shown in Table 1.

DCFH fluorescent probe analysis of reactive oxygen species in primary osteoblasts

OBs were detached and seeded into 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were cultured in serum-free medium. Seven days later, we added 10 μ M DCFH-DA and incubated for 30 min. The OD of DCFH-DA was measured at a wavelength of 525 nm, and the fluorescence spectra of FITC were observed under a fluorescence microscope.

Statistical analysis

All results are presented as mean $\pm$ standard deviation (s.d.). Statistical analyses were performed using SPSS 19.0 Software. The differences between experimental groups were evaluated using one-way ANOVA with *post hoc* Tukey's multiple comparisons test. $P < 0.05$ was considered statistically significant.

Table 1 Primers used for quantitative RT-PCR.

Gene	Primers (Forward/Reverse)
<i>RUNX 2</i>	(F) 5'-AATTCTCTGTGCTCCGTGCTG-3' (R) 5'-TCGTTGAACCTGGCTACTTGG-3'
<i>SP7</i>	(F) 5'-AGGAGGCACAAAGAAGCCATAC-3' (R) 5'-GATGCCTGCCTTGACACGAGC-3'
<i>BGLAP</i>	(F) 5'-GGACCATCTTCTGCTCACTG-3' (R) 5'-GTTCACTACCTTATTGCCCTCTG-3'
<i>Axin2</i>	(F) 5'-AGTGTGAGGTCCACGGAAC-3' (R) 5'-TGGCTGGTGCAAAGACATAG-3'
<i>OPG</i>	(F) 5'-CCTTGCCCTGACCACTCTTAT-3' (R) 5'-CGCCCTTCTCACAACCTAC-3'
<i>Sod1</i>	(F) 5'-TAACGAGATGCCACCAGA-3' (R) 5'-TGTATAACGCCACGGCAAA-3'
<i>CTK</i>	(F) 5'-GGTTCACATTATCAGGTGACA-3' (R) 5'-GAGCGAAGGGTCAGTCAG-3'
<i>MMP9</i>	(F) 5'-TCCAGTACCAAGACAAAG-3' (R) 5'-TTGCACTGCACGGTTGAA-3'
<i>PTK2β</i>	(F) 5'-CGTCTTCTCTCCACATCC-3' (R) 5'-TTGAGCCGTGTAAAGTGGG-3'
<i>TRAP</i>	(F) 5'-TACCTGTGTGGACATGACC-3' (R) 5'-CAGATCCATAGTGAAACCGC-3'
<i>CTSK</i>	(F) 5'-GCCGTGGCGTTATACATACA-3' (R) 5'-CTTCAATACGTGCAGCAGA-3'
β -actin	(F) 5'-TCCTGTGGCATCCACGAAACT-3' (R) 5'-GAAGCATTTGCGGTGGACGAT-3'

Results

Hepcidin-knockout mice have a more severe osteoporosis phenotype

Hepcidin is an endogenous hormone that decreases circulating iron levels *in vivo* (Zhao *et al.* 2013, Qian *et al.* 2015). To study the effects of endogenous hepcidin on osteoporosis, we first investigated Hamp-knockout ($-/-$) mice. Serum ferritin, a classic and standard marker of iron storage, was readily detected by ELISA and found to be significantly higher in the Hamp $-/-$ mice (Fig. 1A). We next examined serum alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP), which are markers for the activities of osteoblasts and osteoclasts, respectively. Serum ALP levels significantly decreased (Fig. 1B) and TRAP levels increased (Fig. 1C) in Hamp $-/-$ mice, and bone tissue mRNA levels of *Runx2* and *Trap5b* showed similar changes (Fig. 1D and E). To directly evaluate the impact of Hamp knockout on bone structure and metabolism, we carried out micro-CT analyses of the distal femoral trabecular bone. As shown in Fig. 1F, there was significantly less bone mass in Hamp $-/-$ mice, compared to WT mice. Together, these results suggest that loss of hepcidin leads to accelerated osteoporosis in mice.

Figure 1

Detrimental effects caused by hepcidin deficiency. Male hepcidin-knockout mice were bred to 7 months of age ($n=4$). Resulting increase of serum ferritin (A), serum TRAP (C), *Trap5b* (E) and bone loss (F) and decrease of serum ALP (B) and *Runx2* (D). The bar graph represents mean $\pm$ s.d. The asterisks (*, **) indicate significant differences at $*P<0.05$, $**P<0.01$.

Overexpression of hepcidin in animal models ameliorates osteoporosis processes

We next overexpressed hepcidin *in vivo*, by generating a double transgenic mouse line (Fig. 2A). In this double transgenic line, expression of hepcidin is driven by a liver-specific albumin promoter controlled by CRE-ERT, which only turns on in the presence of tamoxifen (Fig. 2B).

Based on our results with Hamp-knockout mice, we speculated that overexpression of hepcidin may have protective roles against osteoporosis processes. To test this hypothesis, we created murine osteoporosis models by removing the ovaries (ovariectomized OVX models) from both WT and transgenic female mice. All mice received two doses of tamoxifen 1 week before surgery (see Methods section for details). As shown in Fig. 2C, the transgenic lines had higher levels of serum hepcidin after treatment with tamoxifen and significantly lower serum ferritin levels (Fig. 2D). No obvious defects or health problems were observed for both the wild-type and transgenic groups (Fig. 2E).

Next, we sought to investigate osteoporosis processes in transgenic mouse models. It has previously been reported that OVX leads to dramatic bone loss in wild-type animals (Huang *et al.* 2013). However, the phenotype is relatively mild in the transgenic mouse group, as reflected by micro CT and HE staining (Fig. 3A, B, C, D, E, F and G). Furthermore,

Figure 2

Generation of hepcidin overexpression mouse models. Female mice received i.p. injections of tamoxifen with or without ovariectomy for 5 days ($n=6$). (A and B) Schematic of transgenic strategy that overexpressed hepcidin after drug administration. Increased Serum hepcidin (C) and decreased serum ferritin (D) after tamoxifen treatment in transgenic mice. No difference in body weight was observed among the different murine groups (E). The bar graph represents mean $\pm$ s.d. The asterisks (*) indicate significant differences at $*P<0.05$.

serum ALP and PINP levels significantly increased, while TRAP and CTX (C-terminal telopeptide of type 1 collagen) levels decreased in the transgenic mice (Fig. 3H, I, J and K). We also tested bone tissue mRNA levels of multiple genes involved in both osteogenesis and break-down processes. As shown in Fig. 3E, the expression of *Sp7*, *Bglap* and *Runx2*, which play important roles in osteoblasts, were significantly higher in transgenic mice. In contrast, the expression of *Ctk*, *Mmp9*, *Ptk2b*, *Trap* and *Ctsk* in osteoclasts were lower (Fig. 3M). In summary, our results suggest that WT mice, after OVX, experience active osteoporosis processes, which can be ameliorated by hepcidin.

Iron overload enhances ROS activities and undermines the functions of primary osteoblasts

Next, we explored the mechanisms through which hepcidin regulate osteoporosis. Because there is no evidence that hepcidin directly affects osteoblasts or osteoclasts, and the primary function of hepcidin is to decrease endogenous circulating iron levels, it is likely that hepcidin regulates osteoporosis through modulating circulating iron levels. Thus, we set up an *in vitro* primary osteoblasts culture model that mimics Hamp KO and overexpression phenotypes. We treated cells with either FAC, to simulate iron overload, or DFO, an iron chelating agent that is widely used in the clinic for treating diseases associated with iron overload, to decrease exogenous circulating iron levels.

We found that neither FAC nor DFO-effected cell viability at the experimental doses investigated herein (Fig. 4A and B). Interestingly, cells that were treated with FAC treatment had significantly lower ALP activities than non-treated control cells, and this effect was largely

rescued by DFO (Fig. 4C and D). Alizarin red staining showed that FAC-inhibited osteoblast mineralization, which was reversed in the presence of DFO (Fig. 4E and F). We also quantified mRNA levels of the genes related to osteogenesis. As shown in Fig. 5A, B, C, D and E, mRNA levels of *Runx2*(5A), *Sp7*(5B), *Bglap*(5C), *Axin2*(5D) and β -catenin(5E) in primary osteoblasts decreased after FAC treatment, and this decrease was partially rescued by DFO. Moreover, *Sod1* expression was significantly induced upon FAC treatment, and this elevation was reversed by DFO (Fig. 5F), which suggests that cells treated with FAC suffer greater ROS stress than other cell groups examined herein (Fig. 5G and H). Taken together, our *in vitro* cell-based assays mimic *in vivo* Hamp KO and overexpression models and suggest that increased ROS, caused by iron overload, is a contributing factor for osteoporosis.

Iron depletion attenuates ROS activities and inhibits the functions of primary osteoclasts

We also examined the effects of FAC and DFO on primary osteoclasts (Fig. 6). Interestingly, cells that received FAC treatment displayed significantly higher TRAP activities than nontreated cells, and this effect was largely reversed by DFO (Fig. 6C and D). Pit formation assays showed that FAC enhances osteoclast bone resorption activity, which was also downregulated in the presence of DFO (Fig. 6). We examined primary osteoclast relative mRNA levels (Fig. 7), and mRNA levels of *Ctk*(7A), *Mmp9*(7B), *Ptk2b*(7C), *Ctsk*(7D), *Trap*(7E) and *Sod1* (7F) increased after FAC treatment, and these increases were partially reversed by DFO. The increase in *Sod1* expression suggests that cells treated with FAC suffer greater ROS stress than other cells examined herein (Fig. 7G and 7H).

Figure 3

Protective effects of hepcidin against bone loss. Micro-CT (A) and HE staining (B) were used to detect bone microstructure of WT, WT OVX and Hamp OVX mice. Compared with WT OVX, the trabecular BMD (C), mean ratio of trabecular bone volume vs total volume, BV/TV (D), trabecular number, Tb.N (E), trabecular thickness, Tb.Th (G), were higher, and trabecular separation, Tb.Sp (F), was lower in Hamp OVX mice. Serum levels of bone formation markers, such as alkaline phosphatase (ALP) (H), have no differences and procollagen type 1 N-terminal propeptide (P1NP) (I) decreased. Serum levels of the bone resorption markers tartrate-resistant acid phosphatase (TRAP) (J) and C-terminal telopeptide of type 1 collagen (CTX) (K) decreased significantly. Bone formation (*Sp7*, *Bglap* and *Runx2*) and resorption (*Ctk*, *Mmp9*, *Ptk2b*, *Trap* and *Ctsk*)-related genes all decreased. The bar graph represents means $\pm$ s.d. The asterisks (*, **) indicate significant differences at $P < 0.05$, $P < 0.01$.

Discussion

Since iron is an independent risk factor for osteoporosis, iron reduction treatment methods may help prevent osteoporosis (Liu *et al.* 2006, Weinberg 2006, Chen *et al.* 2015, Jeney 2017). Hepcidin is an iron-reducing parahormone that is synthesized in and secreted by

Figure 4

Relative biological function of OBs. Cell viability at different concentrations of ferric ammonium citrate (FAC) (A) and desferrioxamine (DFO) (B). ALP staining of osteoblasts (OBs) (C) and the level of ALP activity in OBs (D). Alizarin red staining of OBs (E) and the level of mineralized nodules (F). The bar graph represents means $\pm$ s.d. The asterisks (*, **, ***) indicate significant differences at $P < 0.05$, $P < 0.01$, $P < 0.001$.

the liver and a key regulator of iron homeostasis *in vivo* (Zhao *et al.* 2013, Qian *et al.* 2015). We hypothesize that hepcidin is an intrinsic protective factor against osteoporosis, and previous studies by us and others have shown that hepcidin deficiencies impair osteoblast function in zebrafish and mice, inhibit bone formation and decrease bone mass (Shen *et al.* 2014, Sun *et al.* 2014, Huang 2015). Few studies have investigated the effects of hepcidin overexpression on bone mass in mice, and in this study, we examined the effects of hepcidin on bone mass in murine hepcidin knockout and overexpression models.

Our results demonstrate that iron content increases in hepcidin-knockout mice and that in these mice, bone formation activity is inhibited, osteoclasts undergo activation and bone mass decreases. In comparison with the WT control group, the bone mass of the WT OVX mice decreases significantly, and the bone microstructure appears sparser. After tamoxifen-induced hepcidin overexpression, serum hepcidin levels in Hamp OVX mice significantly increase, serum ferritin levels decrease and serum bone turnover markers CTX and CTK, and osteoclast-related marker genes *Ptk2b*, *Mmo9*, *Ctsk* and

Figure 5

Bone formation gene expression and ROS level of OBs. Quantitative real-time polymerase chain reaction (RT-PCR) analysis of the expression of bone formation markers including *Runx2* (A), *Sp7* (B), *Bglap* (C), *Axin2* (D), β -catenin (E) and *Sod1* (F). The effect of FAC on the generation of reactive oxygen species (ROS) in OBs in the presence and absence of DFO (G) and OBs ROS level (H). The bar graph represents means $\pm$ s.d. The asterisks (*, **, ***) indicate significant differences at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Trap, undergo downregulation. Hepcidin deficiency increases circulating iron levels *in vivo* and promotes bone loss in ovariectomized mice (Shen *et al.* 2014, Sun *et al.* 2014). Furthermore, hepcidin overexpression reduces circulating iron levels and retards bone loss,

which coincide with Huang's hypothesis (Ma & Xu 2010, Kodama *et al.* 2015, Jiang *et al.* 2016).

To further investigate the effects of hepcidin on bone metabolism, we made use of FAC and DFO to simulate hepcidin deficiency and overexpression conditions in cultured osteoblasts and osteoclasts. Our results show that the activity of osteoblasts decreases with the increase of FAC or DFO concentrations. Notably, the use of DFO to reduce iron levels resulted in upregulation of ALP activity, mineralization and osteogenesis-related gene levels, which correspondingly decrease upon iron accumulation. To further investigate these changes, we examined intracellular ROS levels and found that ROS levels increase significantly in iron-accumulating environments and decrease upon DFO intervention. Almeida *et al.* reported that upregulation of ROS levels competitively inhibits the downstream Tcf/Lef transcription of β -catenin, affects osteogenesis through Wnt signaling and results in decreased bone mass (Almeida *et al.* 2007).

We also found that osteoclast activity, bone resorption capacity and osteoclast-related genes were upregulated with increasing FAC concentration, and conversely, DFO inhibited osteoclast abilities. We also examined changes in ROS levels in osteoclast, and found that in this context, DFO also downregulated ROS levels. Previous studies have confirmed that iron enhances ROS levels through intracellular and extracellular fenton, activates the NF- κ B signaling pathway, raises the transcription level of osteoclast differentiation-related genes, promotes bone resorption and ultimately leads to osteoporosis (Jia *et al.* 2012).

In our study, we validated the hypothesis that hepcidin is an intrinsic protective factor for osteoporosis. Hepcidin deficiency increases circulating iron levels and leads to more severe bone loss in mice, while hepcidin overexpression downregulates circulating iron levels and inhibits bone loss. A possible mechanism for these

Figure 6

Relative biological function of OCs. TRAP staining of osteoclasts (OCs) (A) and the number of TRAP-positive OCs (B). Pit formation of OCs (C) and the area of pits (D). The bar graph represents mean $\pm$ s.d. The asterisks (*, **) indicate significant differences at $*P < 0.05$, $**P < 0.01$.

Figure 7

Bone resorption genes expression and ROS level of OCs. Quantitative real-time polymerase chain reaction (RT-PCR) analysis of the expression of bone resorption markers including *Ctk* (7A), *Mmp9* (7B), *Ptk2b* (7C), *Ctsk* (7D), *Trap* (7E) and *Sod1* (7F). The effect of FAC on the generation of reactive oxygen species (ROS) in OCs in the presence and absence of DFO (G) and OCs ROS level (H). The bar graph represents means $\pm$ s.d. The asterisks (*, **, ***) indicate significant differences at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

observations could involve hepcidin inhibition of iron and intracellular ROS levels, which increases bone formation and decreases bone resorption and results in elevated bone mass. Compared with traditional osteoporosis therapies, such as calcium and vitamin D supplements, for inhibiting osteoclast activity or promoting osteoblast bone formation function, this novel iron reduction method seems suitable for postmenopausal osteoporosis (Xu & Huang 2013, Song *et al.* 2014, Sirichakwal *et al.* 2015, Miller *et al.* 2016). It has been reported that postmenopausal women often suffer from iron accumulation, and traditional treatment has often been ineffective for such patients. The use of an iron chelator will be an ideal therapy for significantly increasing bone mineral density (BMD) in patients with hematonosis, whose serum ferritin levels reach iron overload (>1000 ng/mL) (Poggi *et al.* 2016). Moreover, osteoblast are inhibited by high iron levels, and this inhibition is reversed upon treatment with iron chelators

or hepcidin (Li *et al.* 2012, Baschant *et al.* 2016). However, ferritin levels, which are 2–3 times higher than normal values of 12–150 ng/mL in postmenopausal patients with osteoporosis, do not reach iron overload but rather reflect iron accumulation (Fucharoen & Paiboonsukwong 2015). Therefore, unlike iron chelators, hepcidin has a more moderate effect towards reducing iron, which involves feedback regulation of blood iron concentrations. When iron levels are high, absorption of iron is inhibited, and the absorbed iron is stored in organs, and when iron levels are low, the iron that was absorbed by the intestine is released. Furthermore, hepcidin, as an endogenous iron-reducing hormone, is a promising drug target for postmenopausal osteoporosis, and targeting hepcidin will likely present fewer side effects than chelating drugs, which are associated with hyperpigmentation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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