Articles

Increased intracellular iron and mineralization of cultured hFOB 1.19 cells following hepcidin activation through ferroportin-1

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ABSTRACT

الأهداف: دراسة أهمية بروتين الهيبسيدين (Hepcidin) في التأثير على إستقلاب العظام وما إذا كان له دور في هذه العملية.

الطريقة: أجريت هذه الدراسة في معمل الطب الإشعاعي والصحة العامة بجامعة سوكو، ومعمل المستشفى الثاني التابع لجامعة سوكو، زوتشو، الصين وذلك خلال الفترة من سبتمبر 2009م إلى يوليو 2010م. تم التقصي عن تواجد الفيروبورتين – 1 (Fpn-1) بواسطة أحد أنواع التفاعل التسلسلي المبلمر وذلك باستخدام أنزيم الترانزكريبتيز (reverse transcriptase–PCR). لقد قمنا باختيار الخلايا البشرية العظمية الجنينية (hFOB 1.19) من أجل التجربة ومن ثم تم تقسيمها إلى أربع مجموعات وهي كالتالي: مجموعة التحكم وتم وضعها في الماء المقطر فقط، والمجموعات التي تم علاجها بما يعادل 25 نانومول/لتر، و50 نانومول/لتر، و100 نانومول/لتر من الهيبسيدين، وتم قياس درجة تركيز الفلوروسين التي تمثل تركيز الحديد داخل الخلايا البشرية العظمية الجنينية وذلك بواسطة التنظير المسحي باستخدام الليزر متشارك البؤرة. قمنا باعتماد مقايسة إم تي تي من أجل تقييم التكاثر الخلوي (MTT assay)، واختبار فون كوسا (Von kossa staining) من أجل تحديد درجة التمعدن في هذه الخلايا.

النتائج: أشارت النتائج إلى زيادة معدلات الفيروبورتين في الخلايا البشرية العظمية الجنينية حيث قامت المعدلات المختلفة من الهيبسيدين (25 نانومول/لتر، و50 نانومول/لتر، و100 نانومول/لتر) بخفض تركيز الفلرورسين وذلك بسبب تأثير الجرعات المناسبة من الهيبسيدين على زيادة تركيز الحديد وعمليات التمعدن داخل هذه الخلايا ($p<0.05$) من دون التأثير على تكاثرها ($p>0.05$).

خاتمة: أظهرت الدراسة مدى تأثير الهيبسيدين على الفيروبورتين في الخلايا البشرية العظمية الجنينية، كما أنها أظهرت أهميته ودوره في عملية إستقلاب العظام وذلك من خلال المحافظة على توازن الحديد والكالسيوم داخل الخلايا.

Objectives: To address whether hepcidin functions in bone metabolism.

Methods: This study was carried out in the Laboratory of Radiation Medicine and Public Health of Soochow University, and the Laboratory of the Second Affiliated Hospital of Soochow University, Suzhou, China, from September 2009 to July 2010. The positive expression of ferroportin-1 (Fpn-1) was detected by reverse transcriptase-polymerase chain reaction. After the treatment with distilled water (control group) and hepcidin (25noml/L, 50noml/L, 100noml/L), the fluorescence intensity related to intracellular iron concentration of a human fetal osteoblast cell line (hFOB 1.19) was measured by a confocal laser scanning microscope. A 3-(4,5- dimethylthiazol-2-yl) -2-5-diphenyltetrazolium bromide assay, and Von Kossa staining was performed to evaluate cell proliferation and mineralization in cultured hFOB 1.19 cells.

Results: This study revealed a high level expression of Fpn-1 in hFOB 1.19. On the basis of which, it was found that 25noml/L, 50noml/L, 100noml/L hepcidin could promote the fluorescence intensity related to intracellular iron concentration and mineralization in hFOB 1.19 in a dose-dependent manner ($p<0.05$), but hepcidin had no effect on FOB 1.19 proliferation ($p>0.05$).

Conclusion: The hepcidin-ferroportin signal pathway may function in the osteoblast cell line of hFOB 1.19 cells. It is also suggested that cross-talk between iron and calcium homeostasis may play a role in bone metabolism in responding to hepcidin activation.

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Iron is an indispensable cofactor for enzymes involved in many physiological processes such as cell proliferation, respiration, folate metabolism, and DNA synthesis. Iron homeostasis is crucial in maintaining a normal life in evolutionarily higher species. Both excess and less 'free' iron can have toxic or pathologic effects. Ferrous iron participates in 'Fenton chemistry' to catalyze the conversion of hydrogen peroxide to highly reactive hydroxyl radicals that damage DNA, proteins, and lipids. A low level of serum iron is a diagnostic parameter of anemia.[1] In addition to the abnormalities in uptake and absorption in the intestinal tract, iron homeostasis is also closely related to bone metabolism. The well-known bone metabolism disease, osteoporosis, is not only associated with calcium dysfunctioning but is also attributed to iron metabolic abnormality. The metabolic disorders of iron have major impacts on bone mineralization, and the formation or development of osteoporosis.[2-4] Serum ferritin has a direct correlation with bone mineral density in the skull of young women.[5] In patients with hypoferritinemia, serum iron and transferrin affect the normal process of bone mineralization.[6] Diet iron overload is followed by observable bone lesions, suggesting that iron metabolism is somehow associated with a metabolic disorder of bone.[7,8] Total iron in the body affects the dynamic balance of bone metabolism, as well as the process of bone mineralization. Osteoporosis is a remarkably frequent complication of iron loading conditions such as thalassemia, sicklemia, African siderosis, hemochromatosis, smoking, alcoholism, HIV infection, and cessation of menstruation.[2] The metal suppresses osteoblast formation of bone and may also stimulate osteoclast resorption of bone. Iron also inhibits anterior pituitary synthesis of gonadotrophs. This, in turn, results in depressed formation of gonadal hormones. The tendency of iron-loaded persons to become osteoporotic may be enhanced by gonadal hormone deficiency because of the inhibition of anterior pituitary synthesis of gonadotrophs brought about by excess iron.[2] Iron binding agents that could specifically withhold excess skeletal iron (and be excreted as the iron chelate) might have therapeutic utility.[9] Hepcidin is a 25-amino acid disulfide-rich peptide synthesized in the liver. Hepcidin acts as a systemic iron-regulatory hormone by regulating iron transport from iron-exporting tissues into plasma. Any factors that change the expression of hepcidin can alter the balance of iron, resulting in iron metabolism-related diseases.[10,11] Some studies have shown that hepcidin might have a role in the mineralization of bone and osteoporosis. In an animal model of experimental osteoporosis, significant changes in the expression level of hepcidin were reported.[12] Furthermore, factors that have significant impacts on osteoporosis such as interleukin (IL)-6, IL-1, and bone morphegeneic protein-6 (BMP-6) play important regulatory roles in hepcidin expression in the liver.[13] In the present study, we employed a human fetal osteoblast cell line (hFOB 1.19) to address the effect of hepcidin on iron homeostasis at a cellular level.

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Methods. This study was carried out in the Laboratory of Radiation Medicine and Public Health of Soochow University, and the Laboratory of the Second Affiliated Hospital of Soochow University, Suzhou, China, from September 2009 to July 2010. An immortalized human fetal osteoblast cell line (hFOB 1.19, Institute of Biochemistry and Cell Biology of Shanghai) was used for the cell culture study. This cell line is temperature sensitive, where cells proliferate at 34°C and differentiate between 38°C and 39°C. Cells were maintained in Dulbecco's modified Eagle's medium without phenol red, 10% fetal bovine serum, and 3% G418 disulfate solution. Cells were fed every 48-72 hours. Hepcidin (Peptide Institute, Osaka, Japan) was dissolved in double-distilled water. Iron fluorescence staining reagent (Phen Green FL, Molecular Probes, Eugene, Oregon) was dissolved in dimethylsulphoxide (DMSO) in a final concentration of 2 umol/L.

The determination of intracellular fluorescence intensity of iron. Confluent cells were digested with trypsin, then plated in 12 flat-bottomed well plates, each of which had circular coverslips at the bottom covered with a density of $2x10^4$ cells (soaking with absolute ethyl alcohol after cleaning). After incubation for 24 hours, the culture media were replaced before adding various concentrations of hepcidin. Distilled water was added to the control group (A0) and different concentrations of hepcidin were added to the experimental groups, 25 nmol/L for group A1, 50 nmol/L for group A2, and 100 nmol/L for group A3. Triplicates were used in this study. The cells were then incubated for another 20 hours, washed twice with 10 mmol/L phosphate-buffered saline (PBS, pH 7.2) and incubated with Phen Green FL at 34^0C in a humidified atmosphere containing 5% CO_2 for 30 minutes. Next, the circular coverslip was taken out, washed twice with 10 mmol/L PBS to remove the unbound fluorescent indicator, then incubated with the culture medium for another 15 minutes. A confocal laser scanning microscope (CLSM) was used to measure

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the green fluorescence of Phen Green FL when excited at 492 nm and emitted at 517 nm.

Reverse transcriptase-polymerase chain reaction. Total RNA was isolated from the hFOB 1.19 cells using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcriptase-polymerase chain reaction (RT-PCR) of the mRNA was performed with a reverse transcription kit from Promega (Madison, WI, USA) and Taq polymerase (Madison, WI, USA). Ferroportin-1 expressed in the spleen was used as a positive control group. The Primer sets are shown in **Table 1**, and the PCR was performed with a DNA thermal cycler (Eppendorf), as shown in **Table 2**. The amount of PCR products in ethidium bromide-stained gels was determined by Quantify One Software (Bio-Rad, Hercules, CA, USA) after a digital capture of the gel image on a Gel Doc 2000 gel documentation system (Bio-Rad).[14]

Influence of hepcidin on cell proliferation. The influence of hepcidin on cell proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl) -2-5-diphenyltetrazolium bromide (MTT) assay. Cells were plated in a 12 flat-bottomed well plate with a density of 2x104 cells per well. Hepcidin was added after incubation for 24 hours. The final concentrations of hepcidin were 0 nmol/L (B0), 25 nmol/L (B1), 50 nmol/L (B2), 100 nmol/L (B3). The cells were then incubated at 34^0C in a humidified atmosphere containing 5% CO_2 for 3 days. The cells were washed twice 4 hours before the end of incubation with phosphate buffer solution (PBS), and then incubated with 0.5 mg/m1 MTT for the last 4 hours. The medium was then decanted with formazan salts and afterwards dissolved with 200µ1 DMSO. The absorbance was determined at 570 nm using an enzyme linked immunosorbent assay (ELISA1) reader (BIO-TEK Instruments, Highland Park Box 998, Winooski, USA).

Von Kossa staining for mineralization. Calcified nodules secreted from the osteoblasts were demonstrated by von Kossa staining. The cells were seeded into 24-well plates with a density of $2x10^5$ cells per well and cultured for 24 hours, at which point the test agents were added to the wells. The final concentrations of hepcidin were 0 nmol/L (C0), 50 nmol/L (C1), 100 nmol/L (C2). Each medium was replaced every 3 days with fresh test agents. Fourteen days after the end of incubation, cell cultures were washed twice with PBS, fixed with 0.5 ml solution composed of formalin: methanol:H_20 (1:1:1.5) in each well for one minute at room temperature, and then washed 3 times with double-distilled water. These fixed cells were placed in a 5% silver nitrate solution for 15 minutes and kept in ultraviolet for one hour, then washed 3 times with PBS and deoxidized with 5% sodium thiosulfate ($Na_2S_2O_3$). After being counterstained by 1% neutral red for one minute, the stained calcified nodules that appeared black were identified with light microscopy.

Statistical analysis. Data were expressed as mean ± standard deviation (SD), and one-way Analysis of Variance was performed for statistical analysis using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) Version 13.0 for windows; $p<0.05$ was considered statistically significant.

Results.

Quantitative assay of the intracellular fluorescence intensity of iron. The hFOB 1.19 cells in culture showed typical spindle and polygon shape. When these hFOB 1.19 cells were exposed to various concentrations of hepcidin and incubated for 20 hours, the fluorescence intensity representing intracellular iron concentrations in the presence of hepcidin was significantly lower than those in the absence of hepcidin. When the concentrations of hepcidin were within the range of 25-100 nmol/L, the fluorescence intensity of iron ions in hFOB 1.19 decreased with the increase of hepcidin concentration (**Figures 1 & 2**). This dose-

Table 1 - Primer sets of polymerase chain reaction (PCR) and length.

Gene	Primer sets of PCR	Length (bp)
FPN-1	5'-CAATGACGCCTGCAAACAGCAG-3' (forward)	283
	5'-TTCGGACAGG TCT- GATCTCAGG-3'	
ß-actin	5'TCC TGT GGC ATC CAC GAA ACT-3' (forward)	314
	5'-GAA GCA TTT GCG GTG GAC GAT -3'	

Table 2 - Gene reverse transcriptase-polymerase chain reaction augmentation conditions.

Gene	Denaturation	Renaturation	Extension	Number of circle
FPN-1	94^0C/30 seconds	56^0C/30 seconds	72^0C/30 seconds	30
ß-actin	94^0C/30 seconds	55^0C/30 seconds	72^0C/30 seconds	25

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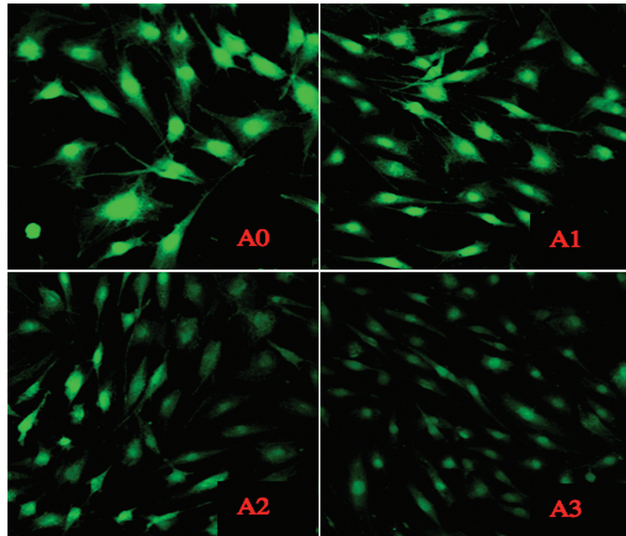

Figure 1 - The fluorescence intensity related to intracellular iron concentration in hFOB 1.19 (original magnification ×40). A0: hFOB 1.19 cells incubated without hepcidin. A1: Cells incubated with hepcidin at 25 nmol/L. A2: Cells incubated with hepcidin at 50 nmol/L. A3: Cells incubated with hepcidin at 100 nmol/L.

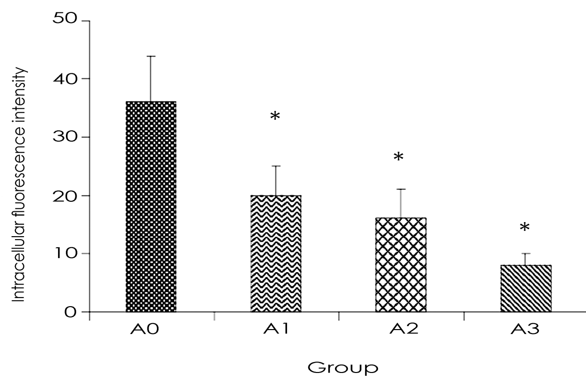

Figure 2 - The fluorescence intensity related to intracellular iron concentration in different experimental groups. *P<0.05, compared with the control group without hepcidin. A0, A1, A2, and A3 represented cells incubated at 0, 25, 50, and 100 nmol/L of hepcidin.

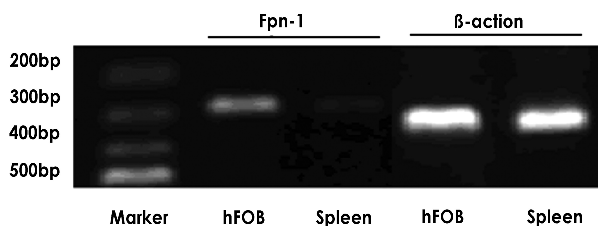

Figure 3 - Representative agarose gel photograph showing the positive expression of Fpn-1 in spleen tissue and osteoblasts. The positive polymerase chain reaction product with the size of 283 bp was observed in extract from cultured hFOB 1.19 cells and the control of spleen.

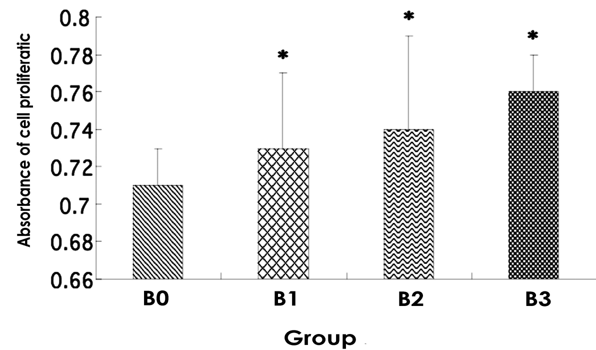

Figure 4 - The MTT represented the activity of cell proliferation of hFOB 1.19. *P>0.05, compared with the control group without hepcidin. B0, B1, B2, and B3 represented cells incubated at 0, 25, 50, and 100 nmol/L of hepcidin.

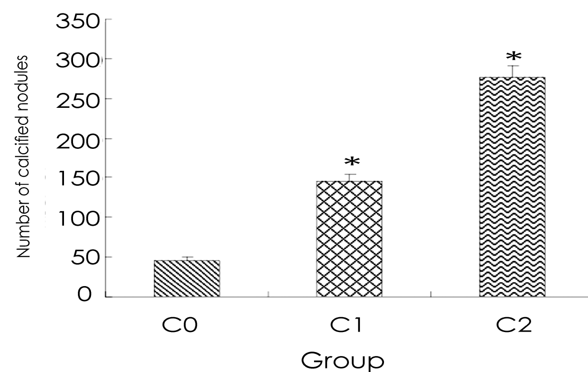

Figure 5 - The number of calcified nodules of different groups. *P<0.01, compared with the control group without hepcidin. C0, C1, and C3 represented cells incubated at 0, 50 and 100nmol/L of hepcidin.

dependent pattern of hepcidin increasing intracellular iron ions in hFOB 1.19 indicated that this effect directly resulted from the hepcidin activation.

The RT-PCR analysis. The mRNA expression of the Fpn-1gene in the cultured cells and in the spleen was detected using RT-PCR. The ß-actin mRNA level was analyzed in the same sample as a reference gene (**Figure** 3). Ferroportin-1 gene expression was detected in hFOB 1.19 cells in the present study. Furthermore, we demonstrated that the expressed Fpn-1 functioned well.

Hepcidin had no effect on cell proliferation. While hFOB 1.19 cells were exposed to various concentrations of hepcidin for 3 days, hepcidin showed no stimulatory effect on the cell viability within the concentrations of 25-100 nmol/L (**Figure** 4). Although this in vitro result cannot exclude the possible in vivo effect of promoting cell proliferation of hepcidin, it could however be indirect evidence to support the significant influence

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of hepcidin activation on cellular calcium and ion homeostasis as being cell proliferation independent.

Hepcidin increased mineralization of hFOB 1.19 cells. The calcified nodules appeared black through Von Kossa staining. The calcified nodules were unequal in size, dispersed as points and on top of the cell layer. Hepcidin stimulated the formation of calcified nodules and with the concentration increasing, the number of calcified nodules increased and mixed together and more lamellar formed (**Figure 5**).

Discussion. The present study observed an interesting result that hepcidin could increase the mineralization of hFOB 1.19 cells, which is osteoporosis relevant and its underlying mechanisms warrant further investigation. The reason that hepcidin could stimulate the formation of calcified nodules may be through the increased intracellular concentration of iron. The present study has shown that hepcidin can increase cellular iron through Fpn-1 in hFOB 1.19 cells. Iron is essential for normal collagen synthesis as it is required as a cofactor for prolyl-hydroxylase, and it increases the activity of this enzyme. Increased iron might enhance gene transcription or increase mRNA expression for formation of procollagen chains, leading to the promotion of the synthesis of subunits of collagen and/or enhanced conversion to the active tetramer. Iron homeostasis in mammals is regulated at the level of intestinal absorption, it is just the fact that iron homeostasis is regulated at the level of intestinal absorption and everyday iron excretion is little. But whether iron excretion could affect absorption need to be further explored. Currently, it has been widely accepted that hepcidin is the master regulator of systemic iron homeostasis, responsible for the coordination of the acquisition, use, and storage of iron.[16,17] Produced primarily by hepatocytes, hepcidin acts as a negative regulator of iron entry into plasma by inducing ferroportin internalization and degradation after binding it, which is an iron transporter present on cells of the intestinal duodenum, macrophages, and the placenta.[15] In human osteoblastic cell lines, it has been demonstrated that 17b-estradiol specifically increased BMP-6 protein production 6-fold, suggesting that some of the skeletal effects of estrogen on bone and cartilage may be mediated by increased production of BMP-6 secreted by osteoblasts and their precursors.[18] BMP-6 is an endogenous regulator of hepcidin expression and iron metabolism in vivo as well.[19] It is well known that BMP-6 has a major function in bone metabolism and therefore the potential link/cascade between the BMP-6-hepcidin-bone axis might exist. It has been also well documented that the hemojuvelin receptor is a BMP co-receptor, which initially connected iron and BMP signaling. Therefore, the connection between iron and BMP-signaling in osteoblasts may exist. In the present study, we observed both functional and morphological changes following the activation of the hepcidin-Fpn-1 pathway in hFOB 1.19 cells. Our data do not directly indicate, but may indirectly suggest a linkage between iron homeostasis and osteoporosis in responding to the activation of the hepcidin-Fpn-1 signal pathway. However, if hepcidin-induced ferroportin-1 integration and degradation occur in osteoblasts, the suggested effect on iron homeostasis mediated by hepcidin would be less important in bone metabolism and its potential association with osteoporosis may be less crucial. Our in vitro results prompted us to perform in vivo study in an osteoporosis animal model, which is undergoing, and some promising data from an osteoporosis rat has observed the alteration of hepcidin expression levels.

In conclusion, the present study identified a high level expression of Fpn-1 in hFOB 1.19 cells, and a regulatory effect on increasing intracellular iron concentrations by hepcidin through Fpn-1. Additionally, the increased mineralization of hFOB 1.19 cells with hepcidin activation may indicate an interaction between iron and calcium homeostasis in bone metabolism. Combined with the observation that the osteoporosis rat model had altered hepcidin expression, the present data may imply the possible involvement of hepcidin in bone metabolism, and may potentially play a role in the pathogenesis of osteoporosis.

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