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Iron deficiency anemia's effect on bone formation in zebrafish mutant

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A B S T R A C T

Iron is one of the essential elements of life. Iron metabolism is related to bone metabolism. Previous studies have confirmed that iron overload is a risk factor for osteoporosis. But the correlation between iron deficiency and bone metabolism remains unclear. Ferroportin 1 is identified as a cellular iron exporter and required for normal iron cycling. In zebrafish, the mutant of ferroportin 1 gene (*fpn1*), *weh*^{tp85c} exhibited the defective iron transport, leading to developing severe hypochromic anemia. We used *weh*^{tp85c} as a model for investigating iron deficiency and bone metabolism. In this study, we examined the morphology of the developing cartilage and vertebrae of the *Weh*^{tp85} compared to the wild type siblings by staining the larvae with alcian blue for cartilage and alizarin red for the bone. In addition, we evaluated the expression patterns of the marker genes of bone development and cell signaling in bone formation. Our results showed that *weh*^{tp85c} mutant larvae exhibited the defects in bone formation, revealing by decreases in the number of calcified vertebrae along with decreased expression of osteoblast novel genes: *alpl*, *runx2a* and *col1a1a* and BMPs signaling genes in osteoblast differentiation: *bmp2a* and *bmp2b*. Our data suggest that iron deficiency anemia affects bone formation, potentially through the BMPs signaling pathway in zebrafish.

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1. Introduction

Bone is a dynamic connective tissue, which constantly remodels itself to accommodate growth and mechanical loads, and to maintain mineral homeostasis. Bone metabolism is influenced by some trace elements in the body, such as calcium, phosphorus, magnesium, etc.

Iron is essential for growth and proliferation of cells because it act as a protein cofactor for hemoglobin synthesis, DNA synthesis and mitochondrial respiration. The regulation of iron content is important since iron overload prompt the generation of reactive oxygen species which damage DNA and proteins, while iron deficiency leads to cell cycle arrest even cell death [1,2]. Dysregulation of iron homeostasis may cause hematological, metabolic and neurological diseases.

In recent years, the correlation between bone metabolism and iron is being a hotspot of research. Weinberg [3] firstly proposed that iron overload was a risk factor for osteoporosis in 2006. Kim et al. [4] conducted a 3-year retrospective longitudinal study and demonstrated that iron overload is related to osteoporosis in healthy adults, especially in postmenopausal women. Further more, several previous studies have proved that iron overload promoted osteoclast differentiation and bone reabsorption [5,6]. Iron overload has been recognized as an independent risk factor for osteoporosis.

However, besides iron overload, abnormal iron homeostasis also includes iron deficiency. Iron deficiency is one of widespread diseases in human beings with major consequences for human health and socioeconomic development. Globally, 1.5–1.7 billion people suffered by iron deficiency anemia, approximately 24.8% of the world population, while the highest prevalence is in preschool-age children (47.4%, 283–303 million children) [7]. Previous studies have proved that iron deficiency, even in absence of anemia, adversely affects the physical growth and cognitive performance of

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children [8]. It can also affect the immune status of human beings [9]. In terms of bone metabolism, it has been reported that iron deficiency diet can affect bone formation [10]. However, the molecular mechanism of the iron deficiency effect on bone metabolism still remains unclear.

Severe iron deficiency anemia has a negative effect on bone metabolism [11–13]. However, the relationship between iron deficiency and bone loss remains unclear. The zebrafish model is rapidly gaining prominence in the study of many aspects of biological and medical research. For example, zebrafish can serve as a good model for studying the development of the skeletal system due to its rapid bone development, simple bone structure, external embryonic development, and body transparence. The latter allows for easy observations of bone morphology in live embryos or larvea [14–16]. In addition, in the early stage of zebrafish bone development (<20 dpf), osteoclast has not yet appeared while only osteoblast and bone formation exist [17]. Zebrafish mutant, *weh*^{tp85}may provide a useful tool for studies of diseases related to pathological bone formation, such as osteoporosis or osteomalacia.

Weh^{tp85c} is the autosomal recessive zebrafish mutants of ferroportin 1 gene (*fpn1*), in which homozygotes develop severe anemia within 48 h after fertilization and dies between 10 and 14 days of age [18]. *Fpn1* is the only non-heme iron exporter with function in iron efflux, which is mainly expressed in duodenal mature enterocytes, reticuloendothelial macrophages, placental syncytiotrophoblast cells and hepatocytes [2]. Dietary iron exists mainly in form of ferric (Fe^{3+}) and is reduced to ferrous (Fe^{2+}) by duodenal cytochrome B (Dcyt B) prior to transport. The transport of non-heme iron across the apical membrane occurs via the divalent metal transporter 1 (DMT1). Cytosolic iron in intestinal enterocytes can be either stored in ferritin or exported into plasma by the basolateral iron exporter FPN1 [19]. In the plasma, iron binds with transferrin and is imported into erythroid precursors and other cells via transferrin-receptor-mediated endocytosis. Macrophages also play an important role in iron delivery to plasma transferrin through phagocytosis of senescent red blood cell, heme catabolism and recycling of iron [20].

In *Weh*^{tp85c} mutant the hypochromia is caused by inadequate circulatory iron levels, although the erythroid cells are fully capable of haemoglobinization. Therefore, *weh*^{tp85c} is one of the suitable models of iron deficiency anemia.

In this study, we examined the morphology of developing cartilage and vertebrae of the *Weh*^{tp85c} larvae by staining the fish with alcian blue and alizarin red, respectively. In addition, we evaluated the gene expression patterns of bone development markers (e.g., *alpl*, *runx2a*, *col1a1a* and *sox9b*) and genes involved in cellular signaling during bone development, such as *bmp2a* and *bmp2b*. Our investigation may provide a basis for the research of diseases related to pathological bone metabolism, such as iron deficiency anemia.

2. Materials and methods

2.1. Animals

Weh^{tp85c} Heterozygous zebrafish used in this study were donated by Professor Zhou Yi (Division of Hematology/Oncology, Children's Hospital and Dana-Farber Cancer Institute and Harvard Medical School, Howard Hughes Medical Institute, Boston, Massachusetts 02115 USA). Breeding colonies were kept in 28.5 °C with a 10/14 h dark/light cycle and staged by hours post-fertilization (hpf) or days post-fertilization (dpf). Protocols for experimental procedures were according to NIH guidelines.

2.2. Mutant homozygotes screening

Ten female and twenty male (4–6 months old) heterozygotes were used for mating to generate the F1 fish, which contain *weh*^{tp85c} homozygotes, heterozygotes and wild-type siblings. The *weh*^{tp85c} homozygotes were identified in a morphologic screen for defects in circulating erythroid cells at 48 hpf [18]. All of the *weh*^{tp85c} heterozygous zebrafish were identified by gene sequencing.

2.3. O-dianisidine staining

The expression of the hemoglobin can be demonstrated by the O-dianisidine staining method. The zebrafish at 48 hpf were dechorionated and stained with O-dianisidine solution (0.6 mg/ml), sodium acetate (0.01 M, pH4.5), H_2O_2 (0.65%), ethanol (40%) in the dark for 15 min [21]. The O-dianisidine solution was removed and phosphate-buffered saline (PBS) was added to stop the reaction. The embryos were observed under the microscope. Brown precipitate is formed when the heme in the hemoglobin reacts with the O-dianisidine solution. Changes in the intensity of brown precipitate can be considered as a semi-quantitative estimation of erythroid development in zebrafish embryos.

2.4. Alizarin red S and alcian blue staining

Bone and cartilage were stained by alcian blue (CAS No: 33864-99-2) and alizarin red S (CAS No: 130-22-3, Sigma-Aldrich, Diegem, Belgium) respectively on fixed specimen [22].

2.5. Quantitative RT-PCR

Total RNA of larvae were isolated using Trizol (Ambion, Austin, TX; 15596-018), and cDNA was generated by reverse-transcription using reverse transcriptase (Invitrogen, Carlsbad, CA; C28025-014). Expression of *alpl*, *runx2a*, *col1a1a*, *sox9b*, *catenin*, *bmp2a* and *bmp2b* was examined by quantitative real-time PCR. Quantitative PCR was conducted on an ABI StepOnePlusTM real-time PCR system with SYBR Premix Ex Taq reagents (TaKaRa, Shiga, Japan): one cycle of 95 °C for 1 min and 40 cycles of 94 °C for 10 s, 59 °C for 30 s, followed by melt curve analysis. Relative expression was calculated using the formula $2^{-\triangle\triangle CT}$. Primers used for PCR are showed in Table 1.

3. Results

3.1. Defect in hemoglobin synthesis of *weh*^{tp85c} homozygotes

Weh^{tp85c} homozygotes were screened and identified by gene sequencing. Wild-type siblings were selected as control. Use O-dianisidine staining to detect the hemoglobin-containing cells. The mutation of the *fpn1* gene in *weh*^{tp85c} homozygotes displayed the synthesis of hemoglobin reduced significantly, resulting in

Table 1
Primer sequences.

mRNA	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
runx2a	GACTCCGACCTCACGACAA	CGTCCCGTCAGGAACATC
alpl	CAGGCAAATCAGTGGGAATC	TTGGGCATGTCTGCATCA
col1a1a	CAGGAGCCCAGTGTTGAG	AGCCACCAGACATCTGAGGA
sox9b	GATCGGACAGCGAGACCCC	TCGTTCAGCAGTCTCCAGAGTT
catenin	AGGTGTTGTCAGTGTGCTCC	CCATGCCCTCCTGTTTGGTG
bmp2a	CGGCTTCTGAGCATGTTTGG	CGGATCTTCTGTAGATTCATCATGG
bmp2b	GATCTCGCGCTGTCACTTTTG	TGATCAGTCAGTTCCGGAGGA

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