Endogenous n-3 polyunsaturated fatty acids (PUFAs) mitigate ovariectomy-induced bone loss by attenuating bone marrow adipogenesis in *FAT1* transgenic mice

Tian-yu Chen^{1,2,*}Zhong-min Zhang^{1,2,*}Xiao-chen Zheng^{1,2}Liang Wang^{1,2}Min-jun Huang^{1,2}Si Qin³Jian Chen^{1,2}Ping-lin Lai⁴Cheng-liang Yang^{1,2}Jia Liu^{1,2}Yi-fan Dai⁵Da-di Jin^{1,2}Xiao-chun Bai^{1,2,4}

¹Department of Orthopaedic, the Third Affiliated Hospital of Southern Medical University, Guangzhou, Guangdong, People's Republic of China; ²Academy of Orthopaedics, Guangdong Province, Guangzhou, Guangdong, People's Republic of China; ³Department of Dermatology and STD, Guangdong No.2 Provincial People's Hospital, Guangzhou, Guangdong, People's Republic of China; ⁴Department of Cell Biology, School of Basic Medical Science, Southern Medical University, Guangzhou, Guangdong, People's Republic of China; ⁵Center of Metabolic Disease Research, Nanjing Medical University, Jiangsu, People's Republic of China

*These authors contributed equally to this work

Correspondence: Xiao-chun Bai/Da-di Jin
Department of Cell Biology, School of Basic Medical Science, Southern Medical University, 1838 North Guangzhou Avenue, Guangzhou 510515, People's Republic of China
Tel +86 20 6164 8724
Fax +86 20 6164 8208
Email xiaochun_bai@163.com/baixc15@smu.edu.cn

Aim: To investigate the effect of endogenous n-3 polyunsaturated fatty acids (PUFAs) on bone marrow adipogenesis under osteoporosis conditions.

Methods: A mouse osteoporosis model overexpressing the *FAT1* gene from *Caenorhabditis elegans* and converting n-6 PUFAs to n-3 PUFAs endogenously was used.

Results: The mice presented significantly lower bone marrow adiposity (adipocyte volume/tissue volume, mean adipocyte number) but increased the bone parameters (bone mineral density, bone mineral content, bone volume/total volume) in the distal femoral metaphysis.

Conclusion: Endogenous n-3 PUFAs protect bone marrow adipogenesis, which provides a novel drug target.

Keywords: antiosteoporosis, n-3 PUFAs, bone marrow, adipogenesis

Introduction

Osteoporosis is a common clinical disease in postmenopausal women and aging populations, is caused by an imbalance between bone resorption and bone formation, and leads to osteoporotic fractures and other serious complications.¹ Age-induced osteoporosis is mainly associated with increased bone marrow adipocytes and bone loss.^{2,3} In osteoporotic bone, reduced bone formation is directly correlated with increasing adipose tissue.⁴

Osteoblasts and marrow adipocytes are both differentiated from a common precursor: bone marrow mesenchymal stem cells (BMSCs).⁵ An in vitro study has revealed that mesenchymal stem cells (MSCs) derived from postmenopausal women with osteoporosis are more likely to differentiate into adipocytes than osteoblasts, compared with cells isolated from healthy women.⁶ There is virtual redifferentiation plasticity in both osteoblasts and adipocytes. It has been demonstrated that fully differentiated osteoblasts from human MSCs presented capabilities of differentiation and transdifferentiation into adipocytes and vice versa.⁷ It also has been reported that the differentiation of MSCs into adipocytes or osteoblasts is competitively balanced within the bone marrow.⁸ Beresford and coworkers have shown the inverse relationship between the differentiation of adipocytic and osteogenic cells.⁹ Moreover, Clabaut et al proposed that MSC-derived adipocytes are able to induce MSC-derived osteoblasts to differentiate to an adipocyte phenotype within an in vitro coculture system.¹⁰ On the other hand, various studies have indicated that adipocytes can secrete factors, such as hormones, leptin, adiponectin, and fatty acids, which affect the prolifera-

tion, apoptosis, and function of neighboring cells.^{11,12} The evidence from the investigation conducted by Elbaz et al suggests an effect of lipotoxicity from marrow adipocytes on osteoblast function.¹³ In addition, cross talk between adipocytes and osteoclasts has been unearthed recently. It has been reported that preadipocytes have a stronger ability in supporting osteoclast-like cell formation without prostaglandin E2 than preosteoblasts, suggesting an emerging relationship between bone marrow-cell adipogenesis and osteoclastogenesis.¹⁴

The existing evidence delineates that bone marrow-fat accumulation may play a critical role in the onset of osteoporosis, owing to its potential effects on either the commitment of BMSC differentiation or the homeostasis of osteoclast-mediated bone resorption. Based on this notion, reversal bone marrow adipogenesis may be an effective therapeutic strategy for the prevention of age-related osteoporosis.

N-3 long-chain polyunsaturated fatty acids (PUFAs) are essential fatty acids for human beings, and include alpha-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid, which cannot be self-synthesized in mammals.¹⁵ During the past few decades, n-3 PUFAs have been supposed to be beneficial for prevention from bone loss in both in vitro and in vivo studies. However, the underlying mechanisms of this action have not yet been fully defined.^{16–22} A recent study has identified that inhibition of osteoclast differentiation and activation may be one of the mechanisms involved in preventing osteoporosis by n-3 PUFAs (n-6:n-3 = 1:1).²³

To the best of our knowledge, there are no reports regarding the roles and mechanisms of n-3 PUFAs on bone marrow adipogenesis. In the present study, by employing a transgenic (TG) mouse model that overexpresses the *FAT1* gene from *Caenorhabditis elegans* and converts n-6 PUFAs to n-3 PUFAs endogenously at a fixed ratio compared to other diet-formulated approaches, we aimed firstly to investigate the effect of endogenous n-3 PUFAs on bone marrow adipogenesis under osteoporosis conditions, which could give rise to a novel insight into current therapy for antiosteoporosis. Peroxisome proliferator-activated receptor gamma (PPAR γ) is an essential transcription factor in adipogenesis,²⁴ the expression and/or activity of which determines the commitment of BMSCs to osteoblasts or adipocyte lineages.²⁵ Runt-related transcription factor 2 (RUNX2) is a key regulator of osteoblast differentiation, playing an important role in bone formation,²⁶ which was used as the measure of adipogenesis/osteogenesis in the present study.

Materials and methods

Animal model and diet feeding

FAT1 TG mice and C57BL/6 wild-type (WT) control mice were donated by Professor Yifan Dai. Since the *FAT1* gene is a kind of heterozygote gene, breeder mice were mated with WT C57BL/6 mice to obtain female *FAT1* gene-positive C57BL/6 mice and *FAT1* gene-negative C57BL/6 mice screened by genotyping using a polymerase chain reaction kit (Takara, Dalian, People's Republic of China) (Figure 1). The amount of n-3 PUFAs and n-6 PUFAs in mouse tails were measured by gas-liquid chromatography (Figure 1). The mice were maintained in cages (two to four mice per cage) under controlled laboratory conditions: 12-hour light/dark cycle at 24°C, fed normal diets and tap water. At age 2 months, 40 mice (20 *FAT1* mice, 20 WT mice) with matched weight received sham or ovariectomy surgeries. Then they were divided into four groups (ten mice/group): group A, *FAT1* mice sham; group B, *FAT1* ovariectomized (OVX); group C, WT sham; group D, OVX. All animals were fed with standard high-fat diets (Research Diets, New Brunswick, NJ, USA) containing 44.9 kcal% fat, 35.1 kcal% carbohydrate, and 20 kcal% protein. When the mice were 5 months old, they were killed. The final body weight was measured and, bilateral femurs were excised. Then we stripped the soft tissue on bones and immersed them in 4% paraformaldehyde at 4°C for 24 hours.

This study was approved by the Animal Ethics Committee of Southern Medical University, and all experiment procedures strictly followed principles of the National Institutes of Health Guide.

Micro-CT analysis

Micro-computed tomography (CT) was performed on each bone and focused on distal femoral metaphysis using a ZKKS-MCT-Sharp-III scanner system (Caskaisheng,

Figure 1 (A and B) Genotyping. (A) PCR genotyping confirmation using *FAT1* fragment-specific primers indicated *FAT1* gene expression (lane 1) and wild-type (WT) gene expression (lane 2); lane 3 is the negative control. (B) N-6/n-3 ratio of *FAT1* mice (mean = 7.762) was significantly lower than WT mice (mean = 28.43). **Note:** * $P < 0.05$ versus *FAT1*.

Guangzhou, People's Republic of China). A small field was selected for scanning and was corrected for the CT value, a 70 kV scanning voltage, 30 W power, 429 μ A current, and 5 μ m scan thickness. The 3D-MED 3.0 (Institute of Automation, Chinese Academy of Sciences, Beijing, People's Republic of China) was used for 3-D knee reconstruction and image capture. Our analysis included various bone parameters: bone morphometry, bone mineral density (BMD; volumetric), total volume (TV), bone volume (BV), BV/TV, and trabecular number (Tb.N).

Bone marrow adiposity analysis

The samples of femur bone were processed for decalcification, as previously described,²⁸ and then routinely embedded in paraffin. Serial sections (4 μ m) were obtained from the sagittal plane of the femur condyle. Hematoxylin and eosin and toluidine blue staining were conducted to quantify proximal metaphyseal adipocyte parameters: adipocyte number (AD[#], per mm²), percent adipocyte volume per tissue volume (AV/TV). A uniform number of fields were screened in all sections by three individuals, starting three fields from the left end and three fields from the top endocortical surface,²⁹ excluding the adipocytes with disruption in the fields. To avoid any bias in the final analysis, all sections were read in a blinded way without knowledge of the groups (*FAT1* or WT). Pictures were obtained at 20 $\times$ magnifications using an Olympus (Tokyo, Japan) BX51RF stereomicroscope. All measurements were done at 20 $\times$ magnification using the Image-Pro Plus analysis software (Media Cybernetics, Rockville, MD, USA).

Immunofluorescence and immunohistochemistry analysis for PPAR γ and RUNX2 protein expression

The following primary antibodies were used: rabbit antimouse PPAR γ monoclonal antibody (Cell Signaling Technology, Beverly, MA, USA) and rabbit antimouse RUNX2 polyclonal antibody (Bioworld Technology, Louis Park, MN, USA). Tissue sections were incubated in sodium citrate buffer (0.01 M, pH 6.0) for 10 minutes to retrieve antigen after deparaffinization and rehydration. The next step was quenching the endogenous peroxidase with 3% hydrogen peroxide prepared in 100% ethanol for 5 minutes. Nonspecific binding was blocked by addition of goat serum for 1 hour at room temperature. Then, sections were incubated with primary antibodies – either anti-PPAR γ or anti-RUNX2 – overnight at 4°C. After being washed in

phosphate-buffered saline three times, sections were further incubated with biotinylated secondary antibody (antirabbit immunoglobulin G) for 30 minutes at room temperature. Sections were counterstained with hematoxylin. As negative control, nonimmune mouse immunoglobulin G was used as the primary antibody, and this gave uniformly negative results (data not shown). Pictures were obtained at 20 $\times$ magnification using an Olympus BX51RF stereomicroscope. The quantification of the level of these two proteins was analyzed by Image-Pro Plus analysis software at 20 $\times$ magnification.

Statistical analysis

Results were expressed as mean $\pm$ standard error of mean, and data were analyzed with the SPSS 13.0 software (IBM, Armonk, NY, USA) using one-way analysis of variance and least significant difference. Post hoc Tukey's tests were performed with SPSS software directly. *P*-values < 0.05 were considered statistically significant.

Results

The effect of endogenous n-3 PUFAs on body weight and abdominal adipose tissue in OVX-induced osteoporosis model

There was a difference in the weight of uterus between the OVX and sham groups, which identified the sound establishment of the OVX-induced osteoporosis model in mice. At the beginning of this study, all mice were well matched for age and weight. The values for body weight and abdominal adipose tissue between *FAT1* and WT OVX mice were different (*P* < 0.05) 3 months postoperatively, but there was no significant difference in body weight between *FAT1* and WT sham mice (*P* > 0.05). OVX groups showed higher levels of body weight compared with sham groups (Figure 2).

Effect of endogenous n-3 PUFAs on femur bone microstructure morphometries

BMD, BMC, BV/TV, and Tb.N in the OVX groups were lower in the femoral region than the sham group. BMD, BV/TV, and BMC in the distal femur of *FAT1* OVX mice were higher (*P* < 0.05) compared to WT OVX mice. However, the Tb.N in the WT OVX group mildly decreased (*P* > 0.05) compared with the *FAT1* OVX group. There was no statistical significance between WT and *FAT1* sham mice in femur bone microstructure morphometries (Figure 3).

Figure 2 (A–E) Gross examination and tissue weight. Three months after ovariectomy, there was increased body weight (**C**) and abdominal adipose tissue (**D**) in the ovariectomized (OVX) group compared to the sham group. However, the *FAT1* OVX group presented significant lower body weight (**A** and **C**) and abdominal adipose tissue (**B** and **D**) compared to the wild-type (WT) OVX group. The weight of uterus was significantly lower in the OVX group than the sham group (**E**).

Notes: * $P < 0.05$ versus WT OVX; * $P < 0.05$ versus *FAT1* OVX; # $P < 0.05$ versus *FAT1* OVX.

Effect of endogenous n-3 PUFAs on bone marrow adiposity

The adipocyte parameters in the WT and *FAT1* groups are described in Figure 4. There was increased AD[#] in the OVX group compared with the sham group ($P < 0.05$) for both WT and *FAT1* mice. Adipocyte parameters such as AV/TV and AD[#] from histological analyses confirmed extensive fat accumulation in the WT OVX group versus the *FAT1* OVX group ($P < 0.05$), but the adipocyte parameters between the *FAT1* and WT sham groups remained stable (Figure 4).

Effect of endogenous n-3 PUFAs on PPAR γ and RUNX2 expression in bone marrow

In agreement with immunofluorescence data, the intensity of positive staining for PPAR γ was enhanced by OVX significantly ($P < 0.05$), and the level of PPAR γ protein expression was lower in the *FAT1* OVX group compared with the WT OVX group ($P < 0.05$). There was no significant difference between WT and *FAT1* sham mice in the intensity of PPAR γ -positive staining.

In immunohistochemistry data, positive immunoreaction of RUNX2 appearing in osteoblasts was stronger in the sham group than the OVX group ($P < 0.05$). In the WT OVX group, RUNX2-positive staining was elevated compared with *FAT1* OVX mice ($P < 0.05$). There was no statistical significance between WT and *FAT1* sham mice in the level of RUNX2 protein expression (Figure 5).

Discussion

In the present study, the effect of endogenous n-3 PUFAs on bone marrow adipogenesis was investigated in a *FAT1* TG OVX model. Our data suggested that TG mice present significantly lower bone marrow adiposity (AV/TV, AD[#]) but increased bone parameters (BMD, BMC, BV/TV) in the distal femoral metaphysis.

Despite the fact that the intimate relationship between bone marrow adipogenesis and bone loss has been realized in osteoporosis pathogenesis,^{30,31} involving several studies on the potential effect of dietary or endogenous n-3 PUFAs on osteogenesis and osteoclastogenesis,^{32–34} the influence of endogenous n-3 PUFAs on adipogenesis in bone marrow is still poorly understood.

Figure 3 (A–E) Bone parameter assessment by micro-computed tomography scan. **(A)** Three-dimensional reconstructions of the distal femoral metaphysis of *FAT1* and wild-type (WT) mice by micro-CT scan. The *FAT1* ovariectomized (OVX) group exhibited more bone mass than the WT OVX group. **(B–D)** Quantitation of bone parameters of distal femur bone. The values of bone mineral density (BMD, **B**), bone mineral content (BMC, **C**), bone volume/total volume (BV/TV, **D**), and trabecular number (Tb.N) (**E**) in the OVX group were decreased compared with those in the sham group. The bone parameter (BMD, BMC, BV/TV) values of the *FAT1* OVX group were significantly higher than the WT OVX group, but the Tb.N in the *FAT1* OVX group only mildly increased than WT OVX group.

Notes: **(B–E)** * $P < 0.05$ versus *FAT1* OVX; **(B–E)** # $P < 0.05$ versus WT OVX; **(B–E)** • $P < 0.05$ versus *FAT1* OVX.

For better understanding of the effect of endogenous n-3 PUFAs, TG mice with *FAT1* gene overexpression were developed, which was able to show a much lower n-6:n-3 PUFA ratio of ~1:1 compared to WT mice with ratio of 20–30:1.²³ Therefore, we used *FAT1* transgenic mice in our study to investigate bone marrow adipogenesis under osteoporosis situations for the first time, taking advantage of its steady endogenous n-3 PUFA ratio.

Since adipogenesis and osteoblastogenesis from BMSCs are regulated by key transcription factors – PPAR γ and RUNX2, respectively – expression of same could determine the establishment of adipocytes or osteoblasts. In this study, we observed a significantly lower expression of PPAR γ but higher RUNX2 expression in bone marrow of TG mice when compared to WT mice, which confirmed the inhibitory effect of n-3 PUFAs on bone marrow adipogenesis theoretically.

With regard to the effect of fat on bone, peripheral adipose tissue and bone marrow should be taken into consideration. It has been suggested peripheral adipose tissue influences bone through stresses caused by mechanical loading.³⁵ Recent in vitro and in vivo studies have drawn a conclusion that lowering dietary the n-6:n-3 PUFA ratio is associated with lower intra-abdominal fat mass and lower area of adipocytes from retroperitoneal fat,³⁶ which suggests that n-3 PUFA additions

can inhibit the formation of peripheral adipocytes hypertrophy and hyperplasia. However, the effect of endogenous n-3 PUFAs on bone marrow adiposity and their possible mechanism needs further investigation due to underlying endocrine functions in bone, since adipocytes in bone marrow are an important source of adipokines that could have a direct lipotoxic effect on BMSCs and osteoblasts, which leads to bone loss.¹³ Our results revealed that endogenous n-3 PUFAs not only suppress the whole body weight of mice but also inhibit adiposity accumulation in bone marrow, preventing bone loss induced by ovariectomy in mice, indicating the potential effects of endogenous n-3 PUFAs on BMSCs, the unique precursors of bone marrow adipocytes. Future studies should look into the differentiation-modulation mechanism of BMSCs by endogenous n-3 PUFAs.

It has been suggested the current antiosteoporosis drugs, such as strontium ranelate and risedronate, which have been widely used in the prevention and treatment of osteoporosis in postmenopausal women,^{37,38} could inhibit bone loss partially by retarding bone marrow adipogenesis, with alteration of the RUNX2/PPAR γ balance during BMSC differentiation. This study on *FAT1* transgenic mice provides compelling evidence that bone marrow adipose is the critical therapeutic target of osteoporosis, and endogenous n-3 PUFAs are pro-

Figure 4 (A–C) Histological analysis of bone marrow adiposity. **(A)** Hematoxylin and eosin and toluidine blue staining for distal femur bone marrow. Images were taken at 200× magnification. There were fewer adipocytes in the *FAT1* OVX group than the wild-type (WT) ovariectomized (OVX) group. **(B)** Adipocyte number (AD#, per mm²) and **(C)** adipocyte volume/tissue volume (AV/TV) were significantly reduced in the *FAT1* OVX group. Three months after ovariectomy, the OVX group showed more bone marrow adiposity than the sham group ($P < 0.05$).

Notes: * $P < 0.05$ versus *FAT1* OVX; # $P < 0.05$ versus WT OVX; * $P < 0.05$ versus *FAT1* OVX. Scale bars, 100 μ m.

posed as a novel treatment in preventing postmenopausal osteoporosis.

Some hypotheses about the mechanisms by which n-3 PUFAs prevent bone mass loss associated with menopause have been proposed – enhancement of osteoblastogenesis and reduction of osteoclast activity – but these mechanisms

are not yet fully understood. Combined with their effect of alleviation of proinflammatory cytokines such as interleukin 6, interleukin 1, and tumor necrosis factor- α secreted by bone marrow adipocytes^{39,40} and the effect of the marrow microenvironment modulating osteoclastogenic activity and increasing bone resorption,^{41–45} endogenous n-3 PUFAs,

Figure 5 (A–C) Immunohistochemistry and counting of positively stained cells in bone marrow three months after ovariectomy. The black arrows indicate the runt-related transcription factor 2 (RUNX2) positively stained cells, and the red arrows indicate peroxisome proliferator-activated receptor gamma (PPAR γ) positively stained cells (200× magnification, scale bar = 100 μ m) **(A and C)**. The proportion of RUNX2-positive cells was significantly increased in the *FAT1* ovariectomized (OVX) group compared to wild-type (WT) mice. **(B and C)** The proportion of PPAR γ -positive cells was significantly reduced in the *FAT1* OVX group compared to WT mice.

Notes: * $P < 0.05$ versus *FAT1* OVX; # $P > 0.05$ versus *FAT1* sham; * $P < 0.05$ versus *FAT1* OVX; * $P > 0.05$ versus *FAT1* sham. Scale bars, 100 μ m.

which alleviated bone marrow adiposity in an osteoporosis animal model, may target bone marrow adipocytes by attenuating adipogenesis of BMSCs, subsequently increasing the osteoblastogenesis. However, the specific signaling pathway mechanism involved in this process should be further pursued.

In conclusion, in this study, we showed that the endogenous conversion of n-6 PUFA to n-3 PUFA and maintaining a lower ratio of n-6:n-3 PUFAs could attenuate adipogenesis within the bone marrow, which is associated with decreasing the level of PPAR γ and increasing the level of RUNX2 expression.

Conclusion

Our findings provide us with a more comprehensive understanding of the mechanism of action of n-3 PUFAs on promoting osteoblastogenesis and perhaps will lead to the development of a therapeutic approach for osteoporosis aimed at reversing adipocyte differentiation from BMSCs.

Disclosure

The authors report no conflicts of interest in this work.

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