Exposure to omega-3 fatty acids at early age accelerate bone growth and improve bone quality

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Abstract

Omega-3 fatty acids (FAs) are essential nutritional components that must be obtained from foods. Increasing evidence validate that omega-3 FAs are beneficial for bone health, and several mechanisms have been suggested to mediate their effects on bone, including alterations in calcium absorption and urinary calcium loss, prostaglandin synthesis, lipid oxidation, osteoblast formation and inhibition of osteoclastogenesis. However, to date, there is scant information regarding the effect of omega-3 FAs on bone development during the rapid growth phase. In this study we aim to evaluate the effect of exposure to high levels of omega-3 FAs on bone development and quality during prenatal and early postnatal period. For this purpose, we used the fat-1 transgenic mice that have the ability to convert omega-6 to omega-3 fatty acids and the ATDC5 chondrogenic cell line as models. We show that exposure to high concentrations of omega-3 FAs at a young age accelerates bone growth through alterations of the growth plate, associated with increased chondrocyte proliferation and differentiation. We further propose that those effects are mediated by the receptors G-protein coupled receptor 120 (GPR120) and hepatic nuclear factor 4α, which are expressed by chondrocytes in culture. Additionally, using a combined study on the structural and mechanical bone parameters, we show that high omega-3 levels contribute to superior trabecular and cortical structure, as well as to stiffer bones and improved bone quality. Most interestingly, the fat-1 model allowed us to demonstrate the role of maternal high omega-3 concentration on bone growth during the gestation and postnatal period.

Keywords: ω-3 Fatty acids; ω-3 Desaturase; Bone structure; Bone mechanics; Chondrocyte differentiation; Growth plate

1. Introduction

Adequate growth and intrinsic bone quality depend mainly on heredity, diet and physical activity. Nonetheless, total bone mass can be improved by modifications of the material properties of the tissue [1,2]. In recent years, it has become clear that proper nutrition and in particular consumption of certain foods directly affect bone material properties and are required to reach the full genetic potential during bone development.

Omega-3 (ω-3) is the common name for long-chain polyunsaturated fatty acids (LCPUFAs) that contain unsaturated carbon in the third position from their methyl terminal. ω-3 and ω-6 (another LCPUFA containing unsaturated carbon in the sixth position) originate from dietary α-linolenic acid and linoleic acid, respectively, two essential fatty acids [3] that are vital components of membrane phospholipids, as well as precursors for a range of metabolites [4,5].

These fatty acids are progressively desaturated and elongated by a shared enzyme system to form longer chain and more highly unsaturated FA, resulting in the formation of the ω-6 arachidonic acid [5] (n-6, 20:4) and ω-3 eicosapentaenoic (EPA) (n-3, 20:5) and docosahexaenoic acids (DHA) (n-3, 22:6). Mammals depend on dietary sources of long-chain ω-3 and ω-6 FA since they lack the enzymatic system synthesizing such fatty acids. In addition, mammalian cells cannot convert ω-6 to ω-3 fatty acids because they lack the converting enzyme named ω-3 desaturase.

In many nutritional studies, diet is commonly used to change the composition of nutrients in the tissues, creating inevitable differences between the various diets that could lead to inconsistent or conflicting results. This fact is particularly known for dietary studies in the field of lipids, in which fish oil, plant seeds and vegetable oil are used instead of pure ω-3 and ω-6 FA. However, these oils contain more bioactive components that can affect study results. Furthermore, the use of dietary supplements is also challenging because there is a possibility for interactions between nutrients and other components that exist inside the foods. Thus, there is a need to create a research model that is capable to revoke these confounding factors and can lead to an understanding of the health effects of specific nutrients such as ω-3. The FAT-1 mice were engineered to express the Caeonorhabditis elegans fat-1 gene that encodes the enzyme ω-3 desaturase. This enzyme, which does not exist in mammals, converts...
ω-6 to ω-3 FA and is expressed in various tissues [6]. Conversion of ω-6 to ω-3 FA leads to an increase in endogenous levels of ω-3 and a reduction of ω-6 FA in the organs and tissues of FAT-1 mice, without the need for supplementation in the diet.

Over the past years, it has become clear that ω-3 PUFA:s are beneficial for bone health [7]. Several mechanisms have been suggested to mediate the effects of dietary fats on bone, including alterations in calcium absorption and urinary calcium loss, prostaglandin synthesis, osteoblast formation and lipid oxidation [8,9]. In addition, ω-3 PUFA:s works synergistically with estrogen to exert a stimulatory effect on bone mineral deposition and an inhibitory effect on bone resorption. It was suggested that ω-3 FA may involve in down-regulation of osteostagogenesis or an up-regulation of osteoblastogenesis [9–11] and thus may play a beneficial role in preventing osteoporosis.

Most studies conducted to date have focused on providing ω-3 as a means of secondary prevention, after the development of bone disease, or in Sigma Aldrich Biocare (Santa Cruz, CA, USA). Digeoxigenin-11-UTP was purchased from Enzo (Mannheim, Germany). Digeoxigenin-11-UTP labeling mix, 4-nitroblue tetrazolium (nbt) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were purchased from Roche (Wiesbaden, Germany). Rabbit polyclonal anti-hepatic nuclear factor 4α (HNF4α) antibody was purchased from Abcam (Cambridge, MA, USA).

2. Materials and methods

2.1. Materials

Dubelco’s modified Eagle’s medium and Ham’s F-12, insulin–transferrin–sodium selenate and XTT proliferation assay were purchased from Biological Industries (Beit Haemek, Israel). TRI reagent, cis,5,8,11,14,17-ETA and cis,4,7,10,13,16,19-DHA were purchased from Sigma (USA). Digeoxigenin-11-UTP was purchased from Enzo (Mannheim, Germany). Digeoxigenin-RNA labeling mix, 4-nitroblue tetrazolium (nbt) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were purchased from Roche (Wiesbaden, Germany). Rabbit polyclonal anti-hepatic nuclear factor 4α (HNF4α) antibody was purchased from Abcam (Cambridge, MA, USA).

2.2. Animals

Male and female fat-1 mice (with the background of C57BL/6) were kindly received from J.X. Kang (Boston, MA, USA). For all analyses (unless described differently), heterozygous–heterozygous breeding was conducted, and heterozygous fat-1 offspring were selected (homozygous offspring are lethal). C57BL/6 mice at the matching age were used as control. All mice were housed under specific pathogen-free conditions in an environmentally controlled clean room and fed normal chow diet. All procedures were approved by the Hebrew University Animal Care Committee. At 3 weeks of age, 2 mm of tail sample was taken from fat-1 offspring for genotyping and FA composition analysis. Genotyping was performed by extraction and amplification of DNA (1 μg) with specific primers for fat-1 gene (F: ATATCTAGACAGGGTTAGG- TATTGTCGC; R: ATATCTAGACAGCATG CCGTTCTAGCA NN_001028390). The presence of the 1500-bp product was detected on ethidium bromide gel. Fatty acid analysis of tissue lipids was performed from extraction of tail as described by Kang et al. [4,17]. Briefly, 1 cm of mouse tail was snap-frozen in liquid nitrogen. Frozen tissues were separately ground to rough powder using a craser and pestle, followed by adding 1 ml of GC-grade hexane and 1 ml of 14% boron trifluoride in methanol reagent. The mixture was flushed with nitrogen for about 30 s, incubated for 1 h at 100°C and then cooled on ice for 5–10 min. After adding 1 ml of H2O, the extract was vortexed and centrifuged at 18,000g for 1 min. The upper phase, containing the methylated fatty acids, was concentrated under nitrogen. Fatty acid methyl esters were analyzed using an Agilent gas chromatograph (7890A) equipped with a flame ionization detector and capillary column (Agilent DB-23, 60 m, 0.25 mm, 0.25 μm). Samples were injected in pulsed split-less mode (pulsed pressure of 30 psi for 0.65 min). Conditions of analysis were the following: flow of carrier gas (hydrogen), 1 ml/min; temperature of injector, 270°C; temperature of detector, 250°C; temperature of column oven, 150°C (1 min); then ramped at 5°C/min to 230°C (held for 10 min). The concentrations of EPA ([n-3 ω-3]–20:5), α-linolenic acid ([n-3 ω-6]–18:3), arachidonic acid ([n-6 ω-6]–20:4) and linoleic acid ([n-6 ω-6]–18:2) were evaluated using authentic standards (Sigma-Aldrich, Rehop, Israel). After final identification, weight and tail length of fat-1 and control mice were measured at 4, 6, 8, 10, 12, 14 and 16 weeks of age (n=10 at each age).

2.3. Histological staining and in situ hybridization of growth plate sections

Tibial growth plates from 2-, 5-, 12- and 24-week-old mice were fixed overnight in 4% paraformaldehyde (Sigma, USA) at 4°C followed by 3 weeks of decalcification in 0.5 M EDTA pH 7.4. The samples were then dehydrated, cleared in Histo-Clear (Bar-Nao, Ramat-Gan, Israel) and embedded in Paraplast, and 5-μm sections were prepared. Masson’s trichrome staining was performed as described by Simsa-Mazeli et al. In brief: slides were stained in Weigert’s iron hematoxylin solution for 10min, followed by rinsing in running warm tap water for 10 min, and washed with distilled water [18]. Next, stain in Biebrich scarlet-acid fuchsin solution for 15 min and wash in Distilled water (DW). Incubate in phosphomolybdic–phosphostungic acid solution and transferred directly to anilin blue solution for 5min, followed by quick rinse in DW and 1% acetic acid solution for 2 min [19]. Hybridizations were performed as described previously. In brief: the sections were deparaffinized in xylene, rehydrated and digested with proteinase K. After digestion, slides were fixed in 10% formaldehyde, blocked in 0.2% glycine and rapidly dehydrated. The sections were then hybridized with digoxigenin-labeled antisense probes for Col II or X (probes were kindly provided by Dr. Elazar Zelzer, the Weizmann Institute of Science, Rehopov, Israel). Probes were detected using a polyclonal antidigoxigenin antibody attached to alkaline phosphatase (ALP) that, upon reaction with its substrate NBT and BCIP, produces a color response. Endogenous ALP was inhibited with levamisole [16,19,20]. In all hybridizations, no signal was observed with sense probes which were used as controls.

2.4. Measurement of growth plate width and chondrocytes number

The width of the whole growth plate and of the proliferative, prehypertrophic and hypertrophic zones was measured at 6 different points along the plate, averaged for each plate and then averaged with measurements from 10 other plate samples in each group. The number of cells per column at each zone was counted and averaged in slides from 10 different mice in each group. In each slide, 10 different columns were counted [16,19].

2.5. Micro-computed tomography (CT)

The region of proximal to mid-diaphysis of all tibiae (n=5) isolated from fat-1 and C57BL/6 mice at the ages of 2, 5, 12 and 24 weeks was scanned with a Skyscan 1174 X-ray computed microtomograph scanner (SkyScan, Aartselaar Belgium) with a CCD detector. Images were obtained using 50-kVp X-ray tube potential and 800-μm tube current. Specimens were scanned using a 0.25-mm aluminum filter. Integration time was 3500 ms, and isotropic voxel size was 8.6 μm. For each specimen, a series of 900 projection images was obtained with a rotation step of 0.4°, two-frame averaging, for a total 360° rotation. Flat field correction was performed at the beginning of each scan for a specific zoom and image format. A stack of two-dimensional (2D) X-ray shadow projections was reconstructed to obtain transverse images using NRecon software version 1.6.1.1 (SkyScan) and subjected to morphometric analysis using CTA software version 1.9.2.3 (SkyScan). During reconstruction, dynamic image range, postalignment value, beam hardening and ring-artifact reduction were optimized for each experimental set. Cortical analysis was performed on a standardized region of interest (ROI) in the mid-diaphysis equidistant from the ends of the bone, containing 150 slices, corresponding to 1.29 mm. The trabecular ROI consisted of 70 slices, extending distally from the end of the proximal growth plate of each bone and corresponding to 0.602 mm. Global grayscale thresholding levels were used for the cortical region and adaptive grayscale thresholding levels for the trabecular region. Three-dimensional (3D) images (CTM file format) were constructed from cortical and trabecular ROIs utilizing Marching Cubes 33 algorithm in CTVol software (SkyScan) [19,21].

2.6. Mechanical testing

Mechanical properties of tibiae isolated from fat-1 and C57BL/6 mice at the ages of 5, 12 and 24 weeks (n=6 for each group) were determined by three-point bending tests performed with a custom-made micromechanical testing device. The caudal aspect of each bone was placed on two supports with rounded profiles (0.5 mm diameter) such that the supports were located equidistant from the ends of the bone and at the maximum feasible distance from each other so that they contacted a reasonably tubular part of the diaphysis [19,22]. The optimal distance between the supports was found to be 8 mm. Each bone was loaded on its anterior aspect by a prong attached in series to a load cell and linear motor, such that the prong contacted the bone at the midpoint between the two supports. Monotonic loading was then conducted at a constant rate of 2 mm/min up to fracture. Force and displacement data were collected by the custom-made system at 10 Hz. The following whole-bone biomechanical parameters were derived from the load/displacement curves: area under the curve, ultimate load, failure load, whole bone stiffness and yield load.
2.7. Cell culture and differentiation conditions

ATDC5 cells were kindly provided by Dr. Galia Gat-Yablonski [23]. Cells were grown in Dulbecco’s modified Eagle’s/F-12 medium containing 5% fetal bovine serum, 10μg/ml insulin, 10μg/ml transferrin, and 3×10^-6 M sodium selenite under 5% CO2 [14]. For proliferation assays, cells were seeded and grown with either 5 μM DHA, 5 μM EPA, or 5 μM DHA + EPA dissolved in bovine serum albumin (BSA) or same volume of BSA dissolved in medium for 24 h. For differentiation experiments, cells were seeded at initial density of 50,000 cells/well in 12-well plates and incubated with or without 5–20 μM DHA + EPA dissolved in BSA or same volume of BSA dissolved in medium for 21 days. Differentiation markers were evaluated on days 7, 14 and 21. Cultures at least in triplicate were used for each test.

2.8. Nile red staining

For the detection of cytoplasmic lipid droplets, ATDC5 cells were cultured as described above, fixed and washed with phosphate-buffered saline. Fixed cells were stained with Nile red and incubated for 10 min in 37°C. Nile red fluorescence was viewed under fluorescent microscope using 450–500-nm exciter filter.

2.9. XTT cell proliferation assay

For XTT assay, cells were seeded in 96-well plates at a density of 5000 cells/well with either 5μM DHA, 5μM EPA or 5μM DHA + EPA dissolved in BSA or same volume of BSA dissolved in medium for 24 h. Assay was performed according to the manufacturer’s protocol. Product formation was measured by using an enzyme-linked immunosorbent assay reader spectrophotometer (ELX808 Ultra Microplate Reader, Bio-Tech Instruments, Inc.). The intensity of absorbance was measured at 450 nm.

2.10. Alcian blue and Alizarin red staining, and ALP activity in cell monolayer and cell lysate

Cells were fixed in 1% paraformaldehyde and stained with either 0.6% Alcian blue 8G X or with 0.5% Alizarin red. For ALP activity, cells were washed with alkaline buffer (pH 2.10). Alcian blue and Alizarin red staining, and ALP activity in cell monolayer and cell lysate were evaluated by Tukey Kramer honestly significant difference test and considered significant at P<0.05.

2.11. RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from ATDC5 cells using TRI reagent (Sigma, USA) according to the manufacturer’s protocol. Total RNA (1 μg) was reverse-transcribed using a high-capacity cDNA reverse-transcription kit (Applied Biosystems, Foster City, CA, USA). Relative quantitative real-time PCR was performed using platinum SYBR Green (Invitrogen Life Science, Israel) according to the manufacturer’s protocols, 1 μl of cDNA template and the gene specific primer sets:

18S (F) GTACACCCTTGACCAACC, (R) CATACC ATCCCCTGAG
Col II (F) GTAAGACCCTGGCAGCTTG, (R) GGTTGGTCGAAGGCTCT
Col X (F) CTCTACACGTGAGTGAA, (R) ACTCCCTGAAGCCTCACA, GPR120 (F) GCGAACGGAAAAATCGGC, (R) AAGTCACAAAAAACACATCCCATG,
HNF4α (F) GCTGCTGGCTCCATAA, (R) CTACGTCGTCTCAGCCCGAC

PCR was carried out in the ABI Prism 7300 system (Applied Biosystems). Relative quantification of the interesting gene was normalized to 18S housekeeping in the comparative Ct method. The results are presented as a mean±S.D. of triplicates runs from a representative experiment.

2.12. Western blot analysis

Total protein was extracted from ATDC5. Protein concentration was measured using a bicinchoninic acid protein assay reagent kit (Pierce Biotechnology). Lysates (30 μg protein) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis; transferred to nitrocellulose membranes; incubated overnight with either anti-α- protein coupled receptor (120 (GPR1) (sc-95105)). Hepatocyte nuclear factor 4 (HNF4α) (ab-41898) or β-actin (A-2066) antibodies at 4°C followed by incubation with peroxidase-conjugated secondary antibody; and detected with ECL.

2.13. Statistical analysis

All data are expressed as mean±S.D. The significance of differences between groups was determined using JMP 8.0 Statistical Discovery Software (SAS Institute 2000) by one-way analysis of variance. Differences between groups were further evaluated by Tukey-Kramer honestly significance difference test and considered significant at P<0.05.

3. Results

3.1. Fat-1 transgenic mice express the fat-1 gene and have high levels of ω-3 fatty acid and low ω-6:ω-3 ratio as well as higher body weight (BW)

Fat-1 transgenic mice heterologously express the C. elegans fat-1 gene, which encodes ω-3 fatty acid desaturase [17]. Homozygous fat-1 offspring were not viable suggesting that extremely high ω-3:ω-6 ratio is lethal [24]. Prior to each experiment, fat-1 offspring were identified by genotyping of the 1500-bp PCR product as described in the “Methods and materials” section. Fig. 1A presents a representative gel showing samples from wild type (w.t.) mice which do not express the fat-1 gene at lines 1, 5 and 6 compared with samples from fat-1 mice at lines 2, 3 and 4 (Fig. 1A).

Next, we characterized the fatty acid profile of w.t. and fat-1 mice using gas chromatograph. Fat-1 lipid profile was rich in ω-3 fatty acids (FA) compared to w.t. mice: the amount of EPA (n-3, 20:5) was 10 times higher in fat-1 mice compared to C57BL/6 mice, α-linolenic acid (n-3, 18:3) levels were almost 3 times higher, and total ω-3 FAs were 4 times higher in fat-1 mice (Fig. 1A). In contrast, ω-6 FAs were lower in fat-1 mice, arachidonic acid (n-6, 20:4) was 7 times lower in these mice, and total ω-6 was also lower (Fig. 1A). These differences are especially evident in the low ω-6:ω-3 ratio which is approximatley 2:1 in the fat-1 transgenic mice compared to 9.2:4.1 in w.t. mice (Fig. 1A), proving that ω-6 FAs are converted to ω-3 FAs in the transgenic mice.

To evaluate growth, we measured the BW of fat-1 transgenic mice compared with w.t. mice from the age of 4 to 16 weeks. Fat-1 female, but not male, had higher BW compared with w.t. mice. This was evident starting from 12 weeks of age (1B).

3.2. Fat-1 transgenic mice have increased trabecular bone volume fraction (BV/TV)

The architecture and morphology of the developing bones were studied by micro-CT scanning of tibiae from 2-, 5-, 12- and 24-week-old C57BL/6 and fat-1 mice. In w.t. animals, analysis of trabecular bone demonstrated decrease in all architectural parameters from 2 to 5 weeks of age. These include BV/TV, trabecular thickness (Th.B), trabecular number (Th.N) and trabecular separation (Th.Sp) (Fig. 1C). However, as the animals aged (5–24 weeks), the Th.B increased markedly, Th.Sp increased slightly, Th.N decreased slightly, and BV/TV did not change (Fig. 1C). This indicates that, as bone ages, there are less trabeculae per unit volume, but the average Th.B increases.

Comparison with the trabecular morphology of the fat-1 mice shows that at, 2 weeks of age, the transgenic mice are not different from the w.t. in all the trabecular bone characteristics (Fig. 1C). However, from 5 weeks, the fat-1 mice show improved trabecular bone manifested by significantly higher BV/TV and Th.B, and a trend towards thicker trabeculae, with the corresponding significant decrease in Th.Sp (Fig. 1C). The increased Th.B and BV/TV of fat-1 mice are also evident in the representative 2D and 3D (Fig. 1D) images of the bones. Red arrows indicate trabeculae. Increased trabecular volume is notable in fat-1 mice. These data suggest that the presence of ω-3 FA contributes to the process of bone development and optimizes characteristics of trabecular bone.

3.3. Fat-1 transgenic mice have increased cortical thickness (Ct.Th) and improved mechanical properties of the bone

The morphology and mineral density of the cortical bone, which contribute to the bone’s structural stability and strength, were studied by micro-CT scanning of tibiae from 2-, 5-, 12- and 24-
week-old C57BL/6 and fat-1 mice. In w.t. animals, analysis of cortical bone demonstrated a decrease in the total cross-sectional area inside the periosteal envelope (Tt.Ar) and medullary area (Ma.Ar) between 2 and 5 weeks, with no further changes until 24 weeks (Fig. 2A). The cortical cross-sectional area (Ct.Ar) and Ct.Th increased from week 2 to 24 (Fig. 2A), and so did the cortical area fraction (Ct.Ar/Tt.Ar) (Fig. 2A). Bone mineral density (BMD) was markedly increased only on week 24 compared to previous weeks (Fig. 2A). Comparison between fat-1 and w.t. mice demonstrates that fat-1 mice have smaller Tt.Ar and smaller Ma.Ar compared to the w.t. However, their Ct.Ar, Ct.Th and Ct.Ar/Tt.Ar are all higher (Fig. 2A), as also demonstrated by the bone’s 3D structure at all ages (Fig. 2B). The BMD, which reflects the bone mineral quantity per unit volume of bone material and significantly affects bone strength [25], was not different between fat-1 and w.t. mice, except for week 5 in which it was higher in the transgenic mice (Fig. 2A).

To evaluate whether the differences observed in trabecular and cortical morphology lead to changes in the mechanical properties of the bone, we performed three-point bending test [19,22] on tibiae isolated from mice at the ages of 5, 12 and 24 weeks. At 5 weeks, no differences were observed in the mechanical properties of bones from fat-1 transgenic mice and w.t. (Fig. 2C). This may have...
resulted from the fact that, at this age, differences are only starting to appear in the trabecular and cortical structure of fat-1 mice. However, as the animals aged, the bones of the fat-1 transgenic mice became significantly stronger, as noted by higher area under curve (a surrogate for the energy need for fracture), ultimate load, failure point, stiffness and yield point (Fig. 2C). These results point to the fact that ω-3 FAs improve the stiffness as well as plastic and elastic properties of the bone, thus improving its ability to bear load and making it less vulnerable to fractures.

3.4. High exposure to ω-3 FA in gestation and lactation improves cortical bone but damages trabecular bone architecture

Next, we examined whether high ω-3 during gestation and lactation period affects the offspring’s bone parameters. In order to examine this, we designed two types of breeding: the first was a fat-1 female with w.t. male and the second was w.t. female with fat-1 male (Fig. 3A). The offspring of these pairs were approximately half fat-1 (heterozygous) and half w.t. Offspring from the...
first pair (fat-1 mother and w.t. father) were exposed to high amounts of ω-3 FA during gestation and lactation period due to their fat-1 mother, as opposed to offspring from the second pair (w.t. mother and fat-1 father).

Tibiae from 2-week-old offspring of both groups were scanned by micro-CT. Cortical and trabecular bone parameters were examined, and two types of analyses were performed: the first analysis compared all the offspring (heterozygous fat-1 and w.t.) from the first breeding pair [referred to as mother fat-1 (MF)] to all offspring from the second pair [referred to as mother black (MB)]. The second analysis compared all fat-1 offspring from both pairs to all w.t. offspring from both pairs. These analyses aimed to evaluate whether the dominant effect in bone architecture characteristics in an early age is the genetic background of the fat-1 mice or the exposure to ω-3 fatty acids during gestation and lactation period.

Our results show that both w.t. and fat-1 offspring which were exposed to higher ω-3 during gestation and lactation (first group, MF) had superior cortical morphology demonstrated by higher Ct.Th, Ct.Ar, Ct.Ar/Tt.Ar, and Ma.Ar as well as BMD. (C) Trabecular bone morphological parameters were analyzed: BV/TV, Tb.N, Tb.Sp and Tb.Th. Results are shown as means (n=6)±S.E. Parameters which were found to differ significantly (P<.05) are marked by asterisks. That exposure to high maternal ω-3 levels during gestation and lactation has beneficial effects on the cortical bone, but negative influence on the trabecular component.

Results of the second analysis, comparing all fat-1 offspring from both pairs to all w.t. offspring from both pairs, showed no significant differences in all cortical and trabecular bone parameter (data not shown). This demonstrates that, at a very early age, the influence of maternal ω-3 on cortical bone is very dominant; it has a beneficial effect on the bone of both w.t. and fat-1 offspring of those mothers, even more than the influence of the endogenous ω-3 in fat-1 mice, which, at these stages, is not large enough to be observed. Furthermore, these results prove the dominance of the embryonic environment on bone development.

3.5. Fat-1 transgenic mice have accelerated growth, thicker growth plate and higher chondrocyte number

Contrary to BW which represents growth rate of the whole body including energy balance, tail length is a more specific indication for longitudinal bone growth. We measured tail length of fat-1 transgenic mice compared with w.t. from the age of 4 to 16 weeks and show that both fat-1 male and female have longer tail than w.t. mice (Fig. 4A), indicating that the presence of ω-3 results specifically in acceleration of bone elongation.

Next, we examined the mice’s growth plate as the origin of longitudinal bone growth. Tibial growth plates were isolated from 2-
5-, 12- and 24-week-old C57BL/6 and fat-1 mice, representing various stages of growth plate development (Fig. 4B). More detailed analysis was performed at 5 weeks of age, representing the fastest growing stage, in which the growth plate is most active (Fig. 4 C,D). At this stage, fat-1 transgenic mice had thicker tibial growth plates compared with w.t. (Fig. 4C) together with thicker proliferative,
prehypertrophic and hypertrophic zones (Fig. 4C). This was accompanied by higher number of chondrocytes in the proliferative and prehypertrophic zones (Fig. 4C). Fat-1 transgenic mice also showed stronger expression of two main ECM proteins — collagen type II (Fig. 4D), the major collagenous ECM protein of the proliferative zone, and collagen type X (Fig. 4D), the major collagenous ECM protein of the hypertrophic zone — suggesting increased matrix production in fat−1mice. Taken together, these results prove that the presence of ω−3 accelerates bone growth by affecting many aspects of growth plate development, including cell proliferation, initiation of hypertrophic differentiation and matrix production.

3.6. Omega-3 fatty acids, EPA and DHA increase chondrocyte proliferation and differentiation in vitro

ATDC5 cell line was chosen for this study because it undergoes cell proliferation, hypertrophy, synthesis and mineralization of extracellular matrix, as occur during longitudinal bone growth in vivo, thereby providing an excellent model for studying the molecular mechanisms underlying the effect of ω−3 FA on growth plate development [14–16]. Cells were treated with EPA and DHA as indicated in the “Methods and materials” section.

To validate the extent of penetration of the FA into the cells, Nile red staining was used. This selective fluorescent stain is an indicator for the amount of intracellular lipid droplets [26]; thus, higher fluorescent staining indicates higher amount of intracellular lipid droplets. Our results, showing higher fluorescence in EPA+DHA-treated cells compared with control or vehicle (BSA in EtOH), validate the incorporation of EPA and DHA into the cell (Fig. 5A).

Next, we examined the effect of ω−3 on chondrocyte proliferation by XTT assay [16]. The cells were cultured for 24 h in media containing 5 μM of DHA, EPA or DHA+EPA. Treatment with DHA or EPA, but not both together, increased XTT cleavage, indicating that ω−3 FAs increase ATDC5 proliferation (Fig. 5A).

ATDC5 cells cultured in the presence of insulin for long periods first proliferate and then differentiate and become hypertrophic, in a similar pattern to the growth plate’s chondrocytes [14,27]. We cultured ATDC5 cells for 21 days with or without 5–20 μM DHA + EPA. On days 7, 14 and 21, the cell’s differentiation pattern was determined by several markers: Alcian blue staining for proteoglycan synthesis, Alizarin red staining for matrix mineralization and ALP activity in cell monolayer as well as in cell lysate (Fig. 5B). All markers confirmed the chondrogenic and hypertrophic differentiation of the cells during the culture period (data not shown). As soon as day 7, DHA+EPA treatment increased Alcian blue and Alizarin red staining, as well as ALP activity (Fig. 5B). In addition, we examined the expression of collagen type II, a marker for chondrocytes in the proliferative zone, and aggrecan, the most abundant ECM protein in the growth plate, in the first 14 days of culture (Fig. 5B). This period is parallel to the proliferative phase of the growth plate [16]. We show that DHA+EPA treatment increases the expression of these two genes (Fig. 5B). Starting from day 14, the cells undergo a process of hypertrophic differentiation manifested by the increased expression of collagen type X. DHA+EPA treatment induced a significant increase in the expression of this hypertrophic marker at these stages (Fig. 5B).

Taken together, our results indicate that prolonged exposure to ω−3 FA increases ATDC5 differentiation.

3.7. GPR120 and HNF4α are expressed in ATDC5 chondrocytes in a differentiation-dependent manner and are affected by ω−3

To further understand the mechanisms by which ω−3 FAs affect chondrocytes proliferation and differentiation, we examined two ω−3 receptors, GPR120 and HNF4α, as possible mediators of ω−3 in these processes.

GPR120 functions as a receptor for PUFA and is abundantly expressed in the intestine, adipose tissue and proinflammatory macrophages [28,29]. In addition, it was also found in osteoblasts and osteoclasts [30] but was never shown to be expressed in chondrocytes. Here, we show for the first time that GPR120 is expressed in chondrocytes at all stages of differentiation at both mRNA (Fig. 5C upper panel) and protein levels (Fig. 5C middle panel), with the highest expression on day 14 when the cells start their hypertrophic differentiation. In this day and on day 7, treatment with DHA+EPA decreased the expression of this receptor, but on day 21, when the cells are fully differentiated, DHA+EPA treatment highly up-regulated the expression GPR120 (Fig. 5C bottom panel).

HNF4α is a nuclear receptor expressed in the liver, kidney, pancreas [31] and intestine in which it plays an important role in the differentiation of intestinal epithelium [32]. Binding of long-chain fatty acids to the receptor can either activate or inhibit its transcriptional activity [33]. We show here, for the first time, the expression of HNF4α mRNA (Fig. 5C middle panel) and protein (Fig. 5D middle panel) at all stages of chondrocytes differentiation. The expression of the mRNA and protein was fairly constant throughout the different stages of differentiation, besides small reduction in mRNA level on day 14 (Fig. 5C middle panel). DHA+EPA treatment induced decreased expression of the receptor on days 14 and 21 (Fig. 5D bottom panel).

Taken together, these results clearly show that ω−3 FAs are involved in the differentiation process in chondrocytes and indicate that this may be mediated through the receptors GPR120 and HNF4α; however, further investigation is required in order to elucidate the specific mechanisms.

4. Discussion

In this study, we demonstrate that exposure to ω−3 FA at young ages accelerates bone growth, alters the structure of the growth plate, improves trabecular and cortical morphology, and is beneficial for bone quality.

For our in vivo studies, we used fat-1 transgenic mice. These mice express fat-1 gene that encodes the enzyme ω−3 desaturase, converting ω-6 to ω-3 FA, a gene that is normally expressed in the roundworm C. elegans but not in mammals. The conversion of ω-6 to ω-3 FA leads to an increase in endogenous levels of ω-3 and a reduction of ω-6 FA in the organs and tissues of fat-1 mice, without the need for supplementation in the diet [17]. Lau et al. [34] found that the fat-1 gene resulted in changes in the n-6/n-3 ratio in both femoral and vertebral bones. In a previous work, Lau et al. found that...
femora from fat-1 mice had greater bone mineral content and biomechanical strength properties compared to w.t. mice [7]. In addition, they showed that lower ω-3/ω-6 PUFA ratio is associated with stronger vertebrae and higher BMD [35]. Banu et al. reported that fat-1 mice had higher cortical and trabecular bone mass compared to w.t. after ovariectomy [36]. The innovation of our study is the use of animals at different and earlier ages — 2, 5, 12 and 24 weeks — in order to examine the process of bone development at different stages of life, including gestation and lactation. In addition, we used a variety of methods to study structural and mechanical features of the bones: histological analysis, micro-CT and three-point bending. Moreover, we also studied the growth plates of fat-1 mice and extended our research to examining mechanisms underlying ω-3 effect on chondrocytes by using an in vitro model. This study is the first to treat ATDC5 with ω-3 and monitor the effect of these FA on chondrocytes in terms of their effect on proliferation and differentiation of growth plate chondrocytes at all stages of differentiation.

We report herein that fat-1 mice exhibit accelerated bone growth, which is evident by longer tail length of both male and female fat-1 mice. This accelerated growth rate is a result of thicker growth plates with thicker proliferative, prehypertrophic and hypertrophic zones, together with higher number of proliferative and prehypertrophic chondrocytes and stronger expression of the main extracellular matrix (ECM) proteins: collagen type II and collagen type X. These effects of ω-3 are also evident in vitro: treatment of chondrocytes in the proliferative phase with ω-3 FA increased chondrocytes proliferation, and prolonged exposure to ω-3 increased hypertrophic differentiation, proteoglycan synthesis, matrix mineralization, ALP activity and the expression of collagen type II, aggrecan and collagen type X. Taken together, these results demonstrate the beneficial effect of ω-3 on all the parameters that determine bone elongation, i.e., chondrocytes proliferation, differentiation and ECM production, thereby accelerating bone growth.

In this work, we show for the first time that two receptors for ω-3 — GPR120 and HNF4α — are expressed in ATDC5 chondrocytes. The expression of GPR120 depends on the differentiation stage of the cells; it peaks on day 14, which represents cells in the onset of hypertrophic differentiation, but dramatically drops on day 21 when the cells are fully differentiated. The expression of HNF4α is rather constant throughout differentiation but show a trend which is opposite to GPR120 — it is slightly but significantly down-regulated on day 14. These results suggest that ω-3 pathway, through GPR120 and HNF4α, participates in the normal process of chondrocytes differentiation and point to the possibility that these two receptors play opposite roles during this process. This suggestion is further strengthened by the fact that ω-3 differentially affects the expression of GPR120 and HNF4α: while GPR120 is down-regulated by ω-3 at the beginning of differentiation and highly up-regulated in hypertrophic cells (when normally it is in its lowest), HNF4α is down-regulated in hypertrophic cells. The involvement of GPR120 in cell differentiation process was shown before in adipocyte; GPR120 mRNA is increased during adipocyte differentiation, and down-regulation of the receptor resulted in inhibition of this process, suggesting that GPR120 has an important role in adipogenesis by functioning as a factor that facilitates maturation of adipocyte differentiation [28]. HNF4α was shown to inhibit proliferation of human embryonic kidney cells, rat pancreatic beta cells and liver hepatocytes by different mechanism including down-regulation of cancer genes, promitogenic genes and inducing cell cycle arrest [18,37,38]. It is possible that, in chondrocytes too, these receptors participate in regulation of proliferation and differentiation. Nevertheless, further investigation, which is beyond the scope of this paper, is needed for further understanding of their role in these processes.

Fat-1 mice have increased cortical and trabecular bone mass; the Ct.Th and BMD as well as trabecular number and volume were increased in the tibiae from the fat-1 mice compared with the w.t. As shown by us and others, C57BL/6 mice have very few trabeculae in proximal tibia [19,39]; therefore, our findings demonstrating such a large increase in BV/TV (up to 15% increase at 5 weeks of age) and Tb.N in the fat-1 mice are very significant. This influence of ω-3 can be explained by two mechanisms: (a) ω-3 LCPUFAs stimulate osteoblasts differentiation by increasing the expression of parathyroid hormone and insulin-like growth factor 1 [40], thus increasing bone formation. (b) ω-3 LCPUFAs inhibit osteoclastogenesis by reducing NF-κB expression and modulating RANKL signaling [41], thus reducing bone resorption.

The improved architecture led to superior mechanical properties manifested by higher area under the curve (and thus the energy required for fracture), ultimate load, failure point, whole-bone stiffness and yield load. Stiffness is a measure of the resistance of the entire bone to bending under the applied load in the elastic zone. The elevated BMD of fat-1 mice is one of the reasons for the higher stiffness observed. Moreover, the bones from fat-1 mice had superior mechanical properties manifested by higher yield and failure loads and higher ultimate load, suggesting that their bones are more resistant to fractures than those of the w.t. These changes demonstrate that high levels of ω-3 have beneficial effect on bone development. Importantly, we show significant influence to prenatal exposure to high levels of ω-3 during gestation, as well as exposure through the mother’s milk during lactation. These offspring had improved cortical parameters at very early ages compared to w.t. but also compared to fat-1 mice which had no maternal exposure to ω-3. Interestingly, at this age (2 weeks), high ω-3 had negative influence on the trabecular bone. To the best of our knowledge, no previous study has investigated the effect of ω-3 on the developing bone at such an early age.

Bone mineral density is one of the major predictors of osteoporotic fractures in both the elderly and children. It is determined by the amount of bone gained in early life (peak bone mass) and subsequent bone loss [42]. In recent years, the emerging approach to prevent osteoporosis is to maximize peak bone mass during the growth stage by using dietary approaches and encouraging exercise [43]. Proper nutrition and exercise are required in order to achieve the full genetic potential of peak bone mass [44]. Furthermore, fetal and early life periods are critical for the development and programming of metabolic systems, including the skeleton. Here we demonstrate the contribution of high ω-3 levels to bone elongation and quality during pregnancy and early stages of skeleton development. It was shown before that dietary ω-6:ω-3 ratio during prenatal period have long-term effects on cortical bone parameters in adult rats [45]. Taken together, we conclude that consumption of ω-3 by pregnant mothers and children will lead to improved skeleton quality at young age and may be beneficial not only early in life but also in adulthood when it can help preventing bone deterioration and osteoporosis.

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