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# Brown but not white adipose cells synthesize omega-3 docosahexaenoic acid in culture

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ABSTRACT

Adipose tissue is a complex endocrine organ which coordinates several crucial biological functions including fatty acid metabolism, glucose metabolism, energy homeostasis, and immune function. Brown adipose tissue (BAT) is most abundant in young infants during the brain growth spurt when demands for omega-3 docosahexaenoic acid (DHA, 22:6n-3) is greatest for brain structure. Our aim was to characterize relative biosynthesis of omega-3 long chain polyunsaturated fatty acids (LCPUFA) from precursors in cultured white (WAT) and brown (BAT) cells and study relevant gene expression. Mouse WAT and BAT cells were grown in regular DMEM media to confluence, and differentiation was induced. At days 0 and 8 cells were treated with albumin bound d5-18:3n-3 (d5-ALA) and analyzed 24 h later. d5-ALA increased cellular eicosapentaenoic acid (EPA, 20:5n-3) and docosapentaenoic acid (DPA, 22:5n-3) in undifferentiated BAT cells, whereas differentiated BAT cells accumulated 20:4n-3, EPA and DPA. DHA as a fraction of total omega-3 LCPUFA was greatest in differentiated BAT cells compared to undifferentiated cells. Undifferentiated WAT cells accumulated EPA, whereas differentiated cells accumulated DPA. WAT accumulated trace newly synthesized DHA. *Zic1* a classical brown marker and *Prdm16* a key driver of brown fat cell fate are expressed only in BAT cells. *Ppargc1a* is 15 fold higher in differentiated BAT cells. We conclude that in differentiated adipose cells accumulating fat, BAT cells but not WAT cells synthesize DHA, supporting the hypothesis that BAT is a net producer of DHA.



## 1. Introduction

Adipose tissue depots are major metabolic organs, now recognized as white adipose tissue (WAT) and brown adipose tissue (BAT) [1,2]. WAT is the main energy reservoir in mammals where the lipids are stored in the form of TAGs (triacylglycerols), whereas BAT is a specialized thermogenic organ which is best known for its capacity to burn glucose and fat to produce heat. WAT is heterogeneous and contains most white adipocytes [3]. White adipocytes mainly consist of a single large cytoplasmic lipid droplet and possess only a few mitochondria, while brown adipocytes contain multiple lipid droplets that are packed with mitochondria [4,5]. Recently, brown adipocyte-like cells, commonly known as beige/brite cells which express *Ucp1* and are of multilocular morphology were identified in WAT depots in response to cold and hormonal stimuli [6,7]. Increased brite fat mass is associated with significant improvements in glucose and lipid homeostasis [7].

The brain growth spurt occurs perinatally in mammals [8]. In humans, it bridges in utero and postnatal life continuing to age 2 [9]. The brain growth spurt is the time of most intense accumulation of omega-3 (n-3) docosahexaenoic acid (DHA, 22:6n-3) and omega-6 (n-6) arachidonic acid (ARA, 20:4n-6), which together constitute more than 25% of whole brain fatty acids at birth [10]. Both DHA and ARA are at least in part obtained by endogenous synthesis from precursors alpha-linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6), in utero transferred across the placenta and postnatally in breast milk or infant formula. Whole body measurements show the relative timing of greatest synthesis is perinatally though synthesis is unable to maintain ARA levels comparable to those at birth when no ARA is in formula even with high LA levels, or even with formula ARA levels above that of most breastmilks [11]. Brain DHA levels are well known to be compromised in infants fed formulas without DHA [12]. These data collectively suggest that the endogenous biosynthetic capacity for de novo ARA and DHA synthesis is limited, on average, in human infants.

At birth, the normal term human infant is born with much greater WAT tissue than other mammals or primates. WAT appears

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to be an important storage organ for supplying DHA to the neonatal brain in the first months of life [13]. The brain of mammals contains no adipose tissue and only trace triglyceride. Physically, interscapular BAT depots are among the closest adipose depots to the brain in infants. Their importance as thermogenic organs has recently been highlighted [14]. Tissue of high energy production tend to have high DHA levels [15,16], and we have recently presented evidence of the key endogenous long chain polyunsaturated fatty acids (LCPUFA) synthetic desaturase enzymes residing in mitochondria [17,18]. We hypothesize that mitochondria-rich BAT is a net producer of DHA and possibly ARA for export to the brain. As an initial test of this hypothesis, we looked the relative biosynthesis of omega-3 LCPUFA from precursors in cultured white (WAT) and brown (BAT) cells, as well as expression of genes important to LCPUFA synthesis.

## 2. Materials and methods

### 2.1. Cell Lines

The mouse white preadipocyte 3T3-L1 and mouse SV40T-immortalized brown adipocyte cell lines are gifts from Dr. Ling Qi, Cornell University and Dr. Johannes Klein, University of Lübeck, respectively.

### 2.2. Cell culture and sample preparation

WAT and BAT cells were grown in DMEM media containing FBS (10% FBS for 3T3-L1 and 20% FBS for Brown adipocytes) and 1% penicillin/streptomycin in a humidified environment at 37 °C with 5% $CO_2$. At confluence WAT and BAT cells were subjected to differentiate by adding induction media. After 48 h induction media is replaced by differentiation media and the cells were allowed to grow untill day 8. Details of the culturing conditions are presented in Supplementary table 1.

### 2.3. Oil Red O Staining

Oil Red O (ORO) staining was used to monitor lipid accumulation in undifferentiated (Day 0) and differentiated (Day 8) WAT and BAT cells. Briefly, cultured cells were washed thrice using PBS solution and were fixed with 10% formalin for 20 min. After fixation, cells were stained with 0.5% ORO solution for 30 min at room temperature. After ORO staining, ORO solution was removed and cell nucleus was stained by hematoxylin for 10 min. The stained adipocytes were washed carefully with PBS and were visualized under Olympus BX50 fluorescence microscope at 40× magnification at the BRC imaging facility, Cornell University.

### 2.4. d5-18:3 n-3 dosing

WAT and BAT cells on day 0 (i.e before adding induction media, undifferentiated) and day 8 (differentiated) were incubated with 50 μM of albumin bound d5-18:3n-3 for 24 h. After 24 h, the incubated cells were washed twice with 1× PBS and harvested using trypsin for gene expression and fatty acid analysis studies.

### 2.5. RNA isolation and cDNA synthesis

Total RNA was isolated from WAT and BAT cells using RNeasy Mini kit (Qiagen, MD). The concentration and quality of RNA was measured by using a microspectrophotometer (NanoDrop 2000, Thermo Scientific). Total RNA (1 μg) was reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, NY) as per manufacturer's instructions. The resulting cDNA was used as template for semi-quantitative real time PCR and quantitative real time PCR (qRT-PCR) reactions.

### 2.6. Semiquantitative and quantitative RT-PCR

Gene specific primers were designed using PrimerQuest software from Integrated DNA Technologies (Coralville, IA) and were ordered from the same vendor. All the gene specific primer sequences and conditions are presented in Supplementary table 2. Semiquantitative RT-PCR amplification reactions were run on a gradient thermal cycler (Eppendorf, NY) using EmeraldAmp GT PCR Master Mix (Clontech, CA). PCR products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide and bands visualized under UV light. *Gapdh* was used as control.

Quantitative RT-PCR analysis was carried-out using LightCycler 480 instrument (Roche, Madison, WI) and SYBR Green Master Mix (Roche, Madison, WI). The expression levels of *Fads1, Fads2*, *Ppargc-1a* and *Pdk4* transcripts were measured and the data was normalized using the geometric mean of reference control genes *Gapdh* and *β-actin*. All reactions were run in triplicate and performed with initial denaturation at 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 65 °C for 20 s, 72 °C for 10 s, and a final extension at 72 °C for 5 min. Relative quantification was carried out by using the $2^{-\Delta\Delta CT}$ method.

### 2.7. Fatty acid extraction and analysis

Harvested cell pellets from WAT and BAT cells were used for fatty acid extraction and analysis. Fatty acid methyl esters (FAME) were prepared using a modified one-step digestion and methylation method [19]. FAME mixtures were analyzed with routine methods described elsewhere. Briefly, FAME mixtures were analyzed quantitatively with a HP 5980 gas chromatograph (GC) using an equal weight external standard to calculate response factors [20]. FAME structural identity was verified by mass spectrometry [21]. Deuterium-labeled fatty acid analyses were performed by GC coupled to a Varian Saturn 2000 ion trap MS, also as described elsewhere [18].

### 2.8. Chemicals

Growth media, FBS and reagents for cell culture work were obtained from Life Technologies (NY), Corning (MA), Sigma-Aldrich (MO) and Thermo Fisher Scientific (MA). Fatty acid d5-18:3n-3 was purchased from Cambridge Isotope Laboratories (Cambridge, MA). Solvents for lipid extraction were HPLC grade from Sigma-Aldrich (St. Louis, MO) and Burdick & Jackson (Muskegon, MI).

### 2.9. Statistical analysis

Data was analyzed using Microsoft Excel (2010) software. All data in figures are presented as mean ± SD. Student's *t*-test was used to compare the differences between Day 0 and Day 8. $P < 0.05$ was set as statistical significance threshold.

## 3. Results

### 3.1. Lipid accumulation observed by Oil Red O staining

As the hallmark of adipogenesis is accumulation of lipid droplets in adipocytes, we monitored lipid droplets accumulation in undifferentiated and differentiated WAT and BAT cells. As shown in Fig. 1, neither BAT nor WAT cells on day 0 (undifferentiated) have lipid droplets, whereas on day 8 (differentiated) both cell types