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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.

Keywords

brown adipose tissue; white adipose tissue; omega-3 long chain polyunsaturated fatty acids; undifferentiated; differentiated

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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 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 therefore 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% CO₂. At confluence WAT and BAT cells were subjected to differentiate by adding induction media. After 48 hours induction media is replaced by differentiation media and the cells were allowed to grow till 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 minutes. After fixation, cells were stained with 0.5% ORO solution for 30 minutes at room temperature. After ORO staining, ORO solution was removed and cell nucleus was stained by hematoxylin for 10 minutes. 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 d5-18:3n-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 hours (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 2^{-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 $\pm$ 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 figure 1, neither BAT nor WAT cells on day 0 (undifferentiated) have lipid droplets, whereas on day 8 (differentiated) both cell types show significant amounts of lipid droplet accumulation. BAT cells have a greater number of lipid droplets than WAT consistent with previous observations.

3.2 Gene Markers Specifically Expressed in BAT

We have measured the expression levels of well-known BAT specific markers by semi-quantitative real time PCR (Figure 2A). The *Ucp1* which mediates thermogenesis and drives ATP synthesis is moderately expressed only in BAT cells at day 8, no expression is detected on day 0. *Prdm16*, a zinc finger transcription factor and a key driver of brown fat cell fate and *Zic1*, a member of zinc finger family considered to be a classical brown marker are expressed in both day 0 and day 8 BAT Cells. None of the three genes (*Ucp1*, *Prdm16* and *Zic1*) are expressed in WAT cells.

3.3 Gene Markers Expressed In Both WAT and BAT

We have measured the expression levels of uncoupling protein 2 (*Ucp2*), *Tle3* which is known to participate in adipocyte differentiation, *Tmem26* and *Shox2* that are annotated as brite markers, *Ppargc1a*, also known as *pgc-1alpha* a cold-inducible co-activator of nuclear receptors, known to stimulate mitochondrial biogenesis and respiration and *Erap1* which localizes to endoplasmic reticulum. All these genes are expressed in both WAT and BAT cells at day 0 and day 8, however, expression of *Ppargc1a* and *Erap1* are feeble in WAT and BAT cells on day 0 (Figure 2B). *Ppargc1a* is strongly expressed in differentiated (Day 8) BAT cells.

As *Ppargc1a* expression was found to be highly variable we measured its expression by quantitative real time PCR (qRT-PCR; Figure 3A). By qRT-PCR analysis we found *Ppargc1a* expression to be 15 fold higher in differentiated BAT cells (Day 8) compared to undifferentiated (Day 0) cells, no differences were found in WAT. As BAT cells are known to be rich in mitochondria, we measured expression of *Pdk4* which encodes a mitochondrial protein by qRT-PCR (Figure 3B). *Pdk4* is significantly upregulated in differentiated BAT cells, whereas, it is significantly downregulated in differentiated WAT cells.

3.4 Expression levels of *Fads1* and *Fads2*

We measured the expression levels of *Fads1* and *Fads2* critical enzymes involved in the biosynthesis of LCPUFA by qRT-PCR. The *Fads1* expression levels were found to be significantly lower in differentiated (Day 8) WAT and BAT cells, when compared to undifferentiated (Day 0) cells (Figure 4A), whereas, *Fads2* is significantly lower only in differentiated (Day 8) BAT cells (Figure 4B).

3.5 d5-18:3 dosing

Synthesis of omega-3 LCPUFA was investigated with isotopically labeled d5-18:3n-3 using BAT and WAT cells (Figure 5A and 5B). Differentiated BAT and WAT incubated with 50 uM d5-18:3n-3 for 24 h had 1.5%,w/w and 2.6%,w/w of fatty acids as d5-18:3n-3, respectively. Incubation of undifferentiated BAT cells with 50 uM of d5-18:3n-3 for 24 h resulted in accumulation of eicosapentaenoic acid (EPA, 20:5n-3) and docosapentaenoic acid (DPA, 22:5n-3), whereas differentiated cells accumulate 20:4n-3, EPA, DPA and DHA (Figure 5A). Incubation of WAT cells with 50 uM of d5-18:3n-3 for 24 h resulted in accumulation of EPA in undifferentiated cells, whereas differentiated cells accumulate

mostly DPA. Very little DHA is found in undifferentiated or differentiated WAT cells (Figure 5B).

4. Discussion

Our data show that differentiated BAT and WAT cells accumulating fat in culture both desaturate and elongate 18:3n-3 to omega-3 LCPUFA, and that their distribution depends on adipose type. Moreover, differences in omega-3 LCPUFA synthesis were observed between undifferentiated and differentiated cells. Undifferentiated BAT cells accumulate EPA and DPA with small but detectable DHA synthesis and no detection of 20:4n-3 intermediate; in contrast, differentiated cells accumulate more DHA as a fraction of total omega-3 LCPUFA, and accumulate other intermediates 20:4n-3, EPA and DPA. We found differences in *de novo* LCPUFA synthesis between BAT and WAT cells. Differentiated WAT cells accumulate only DPA, with no change in DHA, whereas, BAT cells show more DHA synthesis as they both accumulate total fat (Figure 1).

Surprisingly, *Fads1* was significantly downregulated in differentiated WAT and BAT cells, whereas, *Fads2* was downregulated in BAT cells. Recent data demonstrate that differentiated white adipocytes when treated with ALA showed no significant changes in *Fads1* and *Fads2* expression levels, though the mean levels of both *Fads1* and *Fads2* trended downward [22]. Several fatty acids of chain length 16, 18, 20, 22 and 24 carbons are substrates for the *Fads2* gene product and they compete among themselves to access *Fads2* [18, 23]. Front end desaturases are well known to be ER proteins. Expression of *Erap1*, a class of proteases localized to the ER [24], was highly expressed in differentiated BAT, poorly expressed in WAT cells.

Several recent studies have indicated classical brown fat cells and beige fat cells originate from completely different cell lineage [25, 26]. Our Oil Red O staining data shows clear lipid droplet accumulation in differentiated WAT and BAT cells, with numerous smaller lipid droplets in BAT cells. Numerous studies show molecular mRNA markers that are different between classical BAT and Beige cells [26, 27]. Consensus about the origins of fat cells and their gene expression signatures are still evolving. Expression of all five well studied BAT signature genes (*Zic1*, *Ucp1*, *Prdm16*, *Ppargc1a* and *Pdk4*) in our BAT cells shows that they are classical brown cells.

Our gene expression data indicates existence of BAT specific molecular characteristics. In only BAT cells *Zic1* and *Prdm16* are expressed in undifferentiated and differentiated cells, we could find expression of *Ucp1* only in differentiated cells. Our results are in agreement with previous studies which showed *Zic1* as classical brown marker [6, 27]. *Zic1* is a member of the ZIC family of C2H2-type zinc finger proteins, which are involved in a variety of developmental processes, including neurogenesis [28, 29]. It is abundantly expressed during development, and is highly enriched in cerebella and neural tube [30]. *Zic1* ablation in mice caused cerebellar defects [30]. In mice *Zic1* expression is limited to interscapular BAT (iBAT), whereas, it is virtually absent in mouse beige adipose tissue [31]. Similarly, in human immunofluorescence for ZIC1 showed distinct ZIC1 staining in iBAT, whereas, supraclavicular and periaxillary BAT showed faint staining patterns [32]. The

presence of BAT in human infants and young mammals has long been known, however, its existence in adult human has been not well established [33, 34]. Recently, using 18F fluorodeoxyglucose positron emission tomography (FDG PET) imaging existence of BAT in adult human has been shown [34-36].

PRDM16 a 140 kDa zinc-finger protein containing PR-(PRD1-BF-1-RIZ1 homologous) domain has been shown to drive brown fat cell fate, from white fat precursors which express *Pparg* [37, 38]. Earlier it has been shown that *Prdm16* is highly expressed in mouse BAT and in depots that are more prone to beiging [37, 39]. *Prdm16* knockdown caused ablation of thermogenic characteristics of brown cells, by decreasing thermogenic gene expression and increasing expression of muscle specific genes [37, 40]. *Ppargc1a*, is a coactivator of transcription factors which regulates hepatic gluconeogenesis, mitochondrial biogenesis and oxidation metabolism and muscle fiber-type switching [41] and is 15 fold higher in differentiated BAT cells. Earlier it has been shown that *Ppargc1a* as a central transcriptional effector of adrenergic activation induces *Ucp1* expression in adipocytes, however, BAT develops normally without *Ppargc1a* [27, 41-43]. *PPARGC1A*, directly activates *PDK4* expression in skeletal muscle, leading to a reduction in glucose oxidation rates [44]. *Pdk4* is 2 fold upregulated in differentiated BAT cells and significantly downregulated in differentiated WAT cells. Earlier it has been shown that *Pdk4* is highly expressed in mouse BAT than in WAT [45].

Uncoupling protein 2 (*Ucp2*) is a mitochondrial anion carrier protein and has been shown to be highly expressed in WAT [46]. The transcription of *Ucp2* gene is regulated by several factors, including fatty acids. ALA supplementation to white adipocytes caused 5.4 fold upregulation of *Ucp2* [46]. In our study *Ucp2* is highly expressed in both BAT and WAT cells. Transducing-like enhancer of split 3 (*Tle3*), is highly expressed in both BAT and WAT cells. *Tle3*, a groucho family member, acts as a white-selective Ppar- γ cofactor and competes with *Prdm16* for Ppar- γ binding and enforces an opposing transcriptional program. It has been shown in mouse that increased expression of *Tle3* in BAT caused phenotypic switch from brown to white [47-49]. *Tmem26* and *Shox2* identified as beige specific markers in rodents [25, 50] are abundantly expressed in both BAT and WAT cells.

In conclusion, our results show that both WAT and BAT synthesize omega-3 LCPUFA. Differentiated BAT synthesizes more DHA than WAT, supporting the hypothesis that it may serve as a source of DHA for the developing brain. BAT is most abundance during the intense period of the human brain growth spurt, suggesting BAT may be a net producer of DHA during the period of most intense DHA requirements. Well studied BAT signature genes are expressed in our BAT cells, including *Zic1* a classical brown marker and *Prdm16* a key driver of brown fat cell fate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- [1]. Greenberg AS, Obin MS. Obesity and the role of adipose tissue in inflammation and metabolism. *Am J Clin Nutr.* 2006; 83:461S–465S. [PubMed: 16470013]
- [2]. Park A, Kim WK, Bae KH. Distinction of white, beige and brown adipocytes derived from mesenchymal stem cells. *World J Stem Cells.* 2014; 6:33–42. [PubMed: 24567786]
- [3]. Feng B, Zhang T, Xu H. Human adipose dynamics and metabolic health. *Ann N Y Acad Sci.* 2013; 1281:160–177. [PubMed: 23317303]
- [4]. Seale P. Transcriptional Regulatory Circuits Controlling Brown Fat Development and Activation. *Diabetes.* 2015; 64:2369–2375. [PubMed: 26050669]
- [5]. Betz MJ, Enerback S. Human Brown Adipose Tissue: What We Have Learned So Far. *Diabetes.* 2015; 64:2352–2360. [PubMed: 26050667]
- [6]. Petrovic N, Walden TB, Shabalina IG, Timmons JA, Cannon B, Nedergaard J. Chronic peroxisome proliferator-activated receptor gamma (PPARgamma) activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes. *J Biol Chem.* 2010; 285:7153–7164. [PubMed: 20028987]
- [7]. Kajimura S, Spiegelman BM, Seale P. Brown and Beige Fat: Physiological Roles beyond Heat Generation. *Cell Metab.* 2015; 22:546–559. [PubMed: 26445512]
- [8]. Dobbing J, Sands J. Comparative aspects of the brain growth spurt. *Early Hum Dev.* 1979; 3:79–83. [PubMed: 118862]
- [9]. Martinez M. Tissue levels of polyunsaturated fatty acids during early human development. *J Pediatr.* 1992; 120:S129–138. [PubMed: 1532827]
- [10]. Diao GY, Hsieh AT, Sarkadi-Nagy EA, Wijendran V, Nathanielsz PW, Brenna JT. The influence of long chain polyunsaturate supplementation on docosahexaenoic acid and arachidonic acid in baboon neonate central nervous system. *BMC Med.* 2005; 3:11. [PubMed: 15975147]
- [11]. Carnielli VP, Simonato M, Verlato G, Luijendijk I, De Curtis M, Sauer PJ, Cogo PE. Synthesis of long-chain polyunsaturated fatty acids in preterm newborns fed formula with long-chain polyunsaturated fatty acids. *Am J Clin Nutr.* 2007; 86:1323–1330. [PubMed: 17991642]
- [12]. Brenna JT. Long chain polyunsaturated fatty acids and the preterm infant. A case study in developmentally sensitive nutrient needs in the USA. *Am J Clin Nutr.* 2015 in press.
- [13]. Cunnane SC, Francescutti V, Brenna JT, Crawford MA. Breast-fed infants achieve a higher rate of brain and whole body docosahexaenoate accumulation than formula-fed infants not consuming dietary docosahexaenoate. *Lipids.* 2000; 35:105–111. [PubMed: 10695931]
- [14]. Lidell ME, Betz MJ, Enerback S. Two types of brown adipose tissue in humans. *Adipocyte.* 2014; 3:63–66. [PubMed: 24575372]
- [15]. Brenna JT, Diao GY. The influence of dietary docosahexaenoic acid and arachidonic acid on central nervous system polyunsaturated fatty acid composition. *Prostaglandins Leukot Essent Fatty Acids.* 2007; 77:247–250. [PubMed: 18023566]
- [16]. Infante JP, Kirwan RC, Brenna JT. High levels of docosahexaenoic acid (22:6n-3)-containing phospholipids in high-frequency contraction muscles of hummingbirds and rattlesnakes. *Comp Biochem Physiol B Biochem Mol Biol.* 2001; 130:291–298. [PubMed: 11567891]
- [17]. Park WJ, Kothapalli KS, Reardon HT, Lawrence P, Qian SB, Brenna JT. A novel FADS1 isoform potentiates FADS2-mediated production of eicosanoid precursor fatty acids. *J Lipid Res.* 2012
- [18]. Park HG, Park WJ, Kothapalli KS, Brenna JT. The fatty acid desaturase 2 (FADS2) gene product catalyzes Delta4 desaturation to yield n-3 docosahexaenoic acid and n-6 docosapentaenoic acid in human cells. *FASEB J.* 2015; 29:3911–3919. [PubMed: 26065859]

- [19]. Garces R, Mancha M. One-step lipid extraction and fatty acid methyl esters preparation from fresh plant tissues. *Anal Biochem.* 1993; 211:139–143. [PubMed: 8323025]
- [20]. Zhou Y, Nijland M, Miller M, Ford S, Nathanielsz PW, Brenna JT. The influence of maternal early to mid-gestation nutrient restriction on long chain polyunsaturated fatty acids in fetal sheep. *Lipids.* 2008; 43:525–531. [PubMed: 18481131]
- [21]. Van Pelt CK, Brenna JT. Acetonitrile chemical ionization tandem mass spectrometry to locate double bonds in polyunsaturated fatty acid methyl esters. *Anal Chem.* 1999; 71:1981–1989. [PubMed: 10361497]
- [22]. Ralston JC, Matravadia S, Gaudio N, Holloway GP, Mutch DM. Polyunsaturated fatty acid regulation of adipocyte FADS1 and FADS2 expression and function. *Obesity (Silver Spring).* 2015; 23:725–728. [PubMed: 25755223]
- [23]. Park WJ, Kothapalli KS, Lawrence P, Tyburczy C, Brenna JT. An alternate pathway to long-chain polyunsaturates: the FADS2 gene product Delta8-desaturates 20:2n-6 and 20:3n-3. *J Lipid Res.* 2009; 50:1195–1202. [PubMed: 19202133]
- [24]. Hattori A, Tsujimoto M. Endoplasmic reticulum aminopeptidases: biochemistry, physiology and pathology. *J Biochem.* 2013; 154:219–228. [PubMed: 23946506]
- [25]. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, Khandekar M, Virtanen KA, Nuutila P, Schaart G, Huang K, Tu H, van Marken Lichtenbelt WD, Hoeks J, Enerback S, Schrauwen P, Spiegelman BM. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell.* 2012; 150:366–376. [PubMed: 22796012]
- [26]. Cohen P, Spiegelman BM. Brown and Beige Fat: Molecular Parts of a Thermogenic Machine. *Diabetes.* 2015; 64:2346–2351. [PubMed: 26050670]
- [27]. Harms M, Seale P. Brown and beige fat: development, function and therapeutic potential. *Nat Med.* 2013; 19:1252–1263. [PubMed: 24100998]
- [28]. Zhong J, Chen S, Xue M, Du Q, Cai J, Jin H, Si J, Wang L. ZIC1 modulates cell-cycle distributions and cell migration through regulation of sonic hedgehog, PI(3)K and MAPK signaling pathways in gastric cancer. *BMC Cancer.* 2012; 12:290. [PubMed: 22799764]
- [29]. Merzdorf CS. Emerging roles for zic genes in early development. *Dev Dyn.* 2007; 236:922–940. [PubMed: 17330889]
- [30]. Aruga J, Minowa O, Yaginuma H, Kuno J, Nagai T, Noda T, Mikoshiba K. Mouse Zic1 is involved in cerebellar development. *J Neurosci.* 1998; 18:284–293. [PubMed: 9412507]
- [31]. Walden TB, Hansen IR, Timmons JA, Cannon B, Nedergaard J. Recruited vs. nonrecruited molecular signatures of brown, “brite,” and white adipose tissues. *Am J Physiol Endocrinol Metab.* 2012; 302:E19–31. [PubMed: 21828341]
- [32]. Lidell ME, Betz MJ, Dahlqvist Leinhard O, Heglind M, Elander L, Slawik M, Mussack T, Nilsson D, Romu T, Nuutila P, Virtanen KA, Beuschlein F, Persson A, Borga M, Enerback S. Evidence for two types of brown adipose tissue in humans. *Nat Med.* 2013; 19:631–634. [PubMed: 23603813]
- [33]. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev.* 2004; 84:277–359. [PubMed: 14715917]
- [34]. Nedergaard J, Bengtsson T, Cannon B. Unexpected evidence for active brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab.* 2007; 293:E444–452. [PubMed: 17473055]
- [35]. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, Taittonen M, Laine J, Savisto NJ, Enerback S, Nuutila P. Functional brown adipose tissue in healthy adults. *N Engl J Med.* 2009; 360:1518–1525. [PubMed: 19357407]
- [36]. Hany TF, Gharehpapagh E, Kamel EM, Buck A, Himms-Hagen J, von Schulthess GK. Brown adipose tissue: a factor to consider in symmetrical tracer uptake in the neck and upper chest region. *Eur J Nucl Med Mol Imaging.* 2002; 29:1393–1398. [PubMed: 12271425]
- [37]. Seale P, Kajimura S, Yang W, Chin S, Rohas LM, Uldry M, Tavernier G, Langin D, Spiegelman BM. Transcriptional control of brown fat determination by PRDM16. *Cell Metab.* 2007; 6:38–54. [PubMed: 17618855]
- [38]. Kajimura S, Seale P, Tomaru T, Erdjument-Bromage H, Cooper MP, Ruas JL, Chin S, Tempst P, Lazar MA, Spiegelman BM. Regulation of the brown and white fat gene programs through a PRDM16/CtBP transcriptional complex. *Genes Dev.* 2008; 22:1397–1409. [PubMed: 18483224]

- [39]. Seale P, Conroe HM, Estall J, Kajimura S, Frontini A, Ishibashi J, Cohen P, Cinti S, Spiegelman BM. Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice. *J Clin Invest*. 2011; 121:96–105. [PubMed: 21123942]
- [40]. Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, Scime A, Devarakonda S, Conroe HM, Erdjument-Bromage H, Tempst P, Rudnicki MA, Beier DR, Spiegelman BM. PRDM16 controls a brown fat/skeletal muscle switch. *Nature*. 2008; 454:961–967. [PubMed: 18719582]
- [41]. Lin J, Wu PH, Tarr PT, Lindenberg KS, St-Pierre J, Zhang CY, Mootha VK, Jager S, Vianna CR, Reznick RM, Cui L, Manieri M, Donovan MX, Wu Z, Cooper MP, Fan MC, Rohas LM, Zavacki AM, Cinti S, Shulman GI, Lowell BB, Krainc D, Spiegelman BM. Defects in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1alpha null mice. *Cell*. 2004; 119:121–135. [PubMed: 15454086]
- [42]. Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell*. 1998; 92:829–839. [PubMed: 9529258]
- [43]. Tiraby C, Tavernier G, Lefort C, Larrouy D, Bouillaud F, Ricquier D, Langin D. Acquisition of brown fat cell features by human white adipocytes. *J Biol Chem*. 2003; 278:33370–33376. [PubMed: 12807871]
- [44]. Wende AR, Huss JM, Schaeffer PJ, Giguere V, Kelly DP. PGC-1alpha coactivates PDK4 gene expression via the orphan nuclear receptor ERRalpha: a mechanism for transcriptional control of muscle glucose metabolism. *Mol Cell Biol*. 2005; 25:10684–10694. [PubMed: 16314495]
- [45]. Hao Q, Yadav R, Basse AL, Petersen S, Sonne SB, Rasmussen S, Zhu Q, Lu Z, Wang J, Audouze K, Gupta R, Madsen L, Kristiansen K, Hansen JB. Transcriptome profiling of brown adipose tissue during cold exposure reveals extensive regulation of glucose metabolism. *Am J Physiol Endocrinol Metab*. 2015; 308:E380–392. [PubMed: 25516548]
- [46]. Reilly JM, Thompson MP. Dietary fatty acids Up-regulate the expression of UCP2 in 3T3-L1 preadipocytes. *Biochem Biophys Res Commun*. 2000; 277:541–545. [PubMed: 11061990]
- [47]. Villanueva CJ, Waki H, Godio C, Nielsen R, Chou WL, Vargas L, Wroblewski K, Schmedt C, Chao LC, Boyadjian R, Mandrup S, Hevener A, Saez E, Tontonoz P. TLE3 is a dual-function transcriptional coregulator of adipogenesis. *Cell Metab*. 2011; 13:413–427. [PubMed: 21459326]
- [48]. Villanueva CJ, Vergnes L, Wang J, Drew BG, Hong C, Tu Y, Hu Y, Peng X, Xu F, Saez E, Wroblewski K, Hevener AL, Reue K, Fong LG, Young SG, Tontonoz P. Adipose subtype-selective recruitment of TLE3 or Prdm16 by PPARgamma specifies lipid storage versus thermogenic gene programs. *Cell Metab*. 2013; 17:423–435. [PubMed: 23473036]
- [49]. Rosen ED, Spiegelman BM. What we talk about when we talk about fat. *Cell*. 2014; 156:20–44. [PubMed: 24439368]
- [50]. Servera M, Lopez N, Serra F, Palou A. Expression of “brown-in-white” adipocyte biomarkers shows gender differences and the influence of early dietary exposure. *Genes Nutr*. 2014; 9:372. [PubMed: 24292948]

Figure 1. Oil Red O staining of undifferentiated and differentiated WAT and BAT cells
No lipid droplets were detected in undifferentiated BAT and WAT cells (day 0) but both differentiated BAT and WAT cells (day 8) accumulated lipid droplets. As expected, BAT cells had more lipid droplets than WAT.

Figure 2. mRNA expression levels of gene markers in WAT and BAT cells

Images of semi-quantitative real time PCR products run on 2% agarose gels. Gapdh was used as reference gene. (A) mRNA expression of gene markers specifically expressed in BAT cells. *Ucp1* only expressed in differentiated BAT (day 8). *Prdm16* and *Zic1* were expressed in both undifferentiated (day 0) and differentiated BAT (day 8). WAT cells did not express *Ucp1*, *Prdm16* and *Zic1*. (B) mRNA expression of gene markers detected in both WAT and BAT cells. *Ucp2*, *Tle3*, *Tmem26*, *Shox2*, *Pparg1a*, *Erp1* are all expressed

in WAT and BAT cells. *Ppargc1a* and *Erap1* are strongly expressed in differentiated BAT cells.

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Figure 3. mRNA expression levels of *Ppargc1a* and *Pdk4* tested by quantitative real time PCR (qRT-PCR)

Data was normalized using the geometric mean of *Gapdh* and β -*actin*, and presented as mean $\pm$ SD. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$. (A) Left: BAT *Ppargc1a* expression level; Right: WAT *Ppargc1a* expression level. *Ppargc1a* was expressed 15 fold higher in differentiated BAT cells (day 8) than undifferentiated cells (day 0); no differences were detected in WAT. (B) Left: BAT *Pdk4* expression level; Right: WAT *Pdk4* expression level. Compared to undifferentiated cells, differentiated BAT cells had significantly higher expression of *Pdk4*, whereas, differentiated WAT cells had significantly lower expression.

A

B

Figure 4. mRNA expression levels of *Fads1* and *Fads2* tested by quantitative real time PCR (qRT-PCR)

Data was normalized using the geometric mean of *Gapdh* and β -*actin*, and presented as mean $\pm$ SD. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$. (A) Left: BAT *Fads1* expression level; Right: WAT *Fads1* expression level. *Fads1* was significantly downregulated in differentiated BAT and WAT cells (day 8) compared to undifferentiated BAT and WAT cells (day 0). (B) Left: BAT *Fads2* expression level; Right: WAT *Fads2* expression level. *Fads2* expression in differentiated BAT cells was only forty percent of that in undifferentiated BAT cells. No difference in *Fads2* expression was found in WAT.

Figure 5. *De novo* biosynthesis of omega-3 LCPUFA from d5-18:3n-3 in cultured BAT and WAT cells

Cells were treated with isotopically labeled d5-18:3n-3 and omega-3 LCPUFA biosynthesis monitored. The relative amount of *de novo* LCPUFA biosynthesis was shown as a ratio of d5-product/d5-n3C₂₀₋₂₂. (A) BAT cells LCPUFA biosynthesis. Undifferentiated BAT cells (day 0) mainly accumulated EPA (20:5n-3) and DPA (22:5n-3) whereas differentiated BAT cells (day 8) accumulated 20:4n-3, EPA and DPA. No labeled 20:4n-3 was detected in undifferentiated BAT. Differentiated BAT synthesize more DHA. (B) WAT cells LCPUFA

biosynthesis. Undifferentiated WAT cells mainly accumulated EPA whereas differentiated WAT cells accumulated DPA. Very little DHA was synthesized in both undifferentiated and differentiated WAT cells.

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