HHS Public Access

Author manuscript

Biochim Biophys Acta Mol Cell Biol Lipids. Author manuscript; available in PMC 2019 December 01.

Published in final edited form as:

Biochim Biophys Acta Mol Cell Biol Lipids. 2018 December ; 1863(12): 1523–1531. doi:10.1016/j.bbalip.2018.09.004.

Transcriptomic and MicroRNA Analyses of Gene Networks Regulated by Eicosapentaenoic Acid in Brown Adipose Tissue of Diet-Induced Obese Mice

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Abstract

Brown adipose tissue (BAT) dissipates chemical energy as heat via thermogenesis and protects against obesity by increasing energy expenditure. However, regulation of BAT by dietary factors remains largely unexplored at the mechanistic level. We investigated the effect of eicosapentaenoic acid (EPA) on BAT metabolism. Male C57BL/6J (B6) mice fed either a high-fat diet (HF, 45% kcal fat) or HF diet supplemented with EPA (HF-EPA, 6.75% kcal EPA) were used for 11 weeks. RNA sequencing (RNA-Seq) and microRNA (miRNA) profiling were performed on RNA from BAT using Illumina HiSeq and miSeq respectively. We conducted pathway analyses using ingenuity pathway analysis software (IPA®) and validated some genes and miRNAs using qPCR. We identified 479 genes that were differentially expressed (2-fold change, n=3, $p \leq 0.05$) in BAT from HF compared to HF-EPA. Genes negatively correlated with thermogenesis such as hypoxia Inducible factor 1 alpha subunit inhibitor (Hif1αn), was downregulated by EPA. Pathways related to thermogenesis such as peroxisome proliferator-activated receptor (PPAR) were upregulated by EPA while pathways associated with obesity and inflammation such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) were downregulated by EPA. MiRNA profiling identified nine and six miRNAs that were upregulated and downregulated by EPA, respectively (log2 fold change > 1.25, n=3, $P \leq 0.05$). Key regulatory miRNAs were involved in thermogenesis, such as miR-455–3p and miR-129–5p were validated using qPCR. In conclusion, the depth of

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transcriptomic and miRNA profiling revealed novel mRNA-miRNA interaction networks in BAT which are involved in thermogenesis which regulated by EPA.

Keywords

Brown adipose tissue; microRNA; obesity; omega 3 fatty acids; RNA sequencing

1. Introduction

Obesity is a major metabolic disease that has reached global epidemic proportions. It increases the risk of obesity-associated comorbidities including type 2 diabetes, cardiovascular diseases, and cancer [1]. White adipose tissue (WAT), includes both subcutaneous adipose tissue (SAT) and intra-abdominal visceral adipose tissue (VAT), which are depots for major fat storage in obesity [2]. On the other hand, brown adipose tissue (BAT) which is another adipose depot, acts as an anti-obesity target due to its thermogenic function [3]. BAT is further classified into classical, (uncoupling protein 1 positive; (UCP-1^+); found in the interscapular and perirenal depots and non-classical, “beige” or “brite” fat which are also UCP-1^+, but found in WAT depots [4].

BAT induces non-shivering thermogenesis by producing heat which is activated by cold, exercise, therapeutic and pharmacological approaches [5]. Cold temperature stimulates sympathetic nervous system (SNS) to activate β-adrenergic function which in turn increases lipolysis of triglycerides (TG) leading to increased release of fatty acids and fuel substrates for thermogenesis [6]. BAT also consumes circulating glucose and lipids for its thermogenic function making it a valuable anti-obesity target [7]. Recent research indicates that activation of thermogenesis leads to a higher weight loss in individuals with obesity, which has spiked interest in identifying potential pharmacological and dietary approaches to activate BAT [3].

While energy restriction is the main dietary approach to prevent and treat obesity, bioactive compounds such as fish oil which contains long-chain ω−3 polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (22:6n-3, DHA) and eicosapentaenoic acid (20:5n-3, EPA) have potential anti-obesity effects [8]. These fatty acids have established roles as anti-inflammatory, cardioprotective and triglyceride-lowering agents [9]. Moreover, our laboratory has reported that feeding a high-fat (HF) diet enriched with EPA to mice significantly reduced body weight, fat mass and glucose intolerance while improving insulin sensitivity and inflammation (to levels comparable to low-fat fed mice) [10]. Additionally, we recently reported that EPA increased levels of several classical thermogenic biomarkers at both gene and protein levels in BAT of mice fed EPA and in clonal brown adipocytes [11].

Recent studies also demonstrate the role of microRNAs (miRNAs) to regulate BAT [12]. However, to our knowledge, no studies have conducted a global search for mRNAs and miRNAs pairs which coordinately mediate dietary regulation of BAT. We hypothesize that EPA regulates novel genes and miRNAs to mediate BAT regulation. For these purposes, we employed RNA sequencing (RNA-Seq), which is an unbiased and integrated approach to identify differential regulation of BAT genes, along with small RNA-Seq to detect miRNA

profiles. We also identified integrated pathways and validated few targets to gain further insight into mechanisms mediating metabolic effects of EPA.

2. Materia and Methods:

2.1. Animals and Experimental Design:

The experimental groups used in this study have been previously described in detail [10]. Briefly, male B6 mice aged 5–6 weeks were fed a HF (45% kcal from fat) or a HF diet supplemented with 6.57% kcal EPA for 11 weeks (HF-EPA) [10]. BAT from interscapular depot was carefully dissected and weighed for subsequent analyses [10]. These protocols were all approved by the Institutional Animal Care and Use Committee at the University of Tennessee, Knoxville and Texas Tech University.

2.2. RNA Extraction and cDNA Library Preparation

Approximately 70 mg of frozen BAT was homogenized in QIAzol® (QIAGEN, Redwood City, CA, USA). RNA was isolated according to manufacturer's protocol using RNeasy Mini Kit (QIAGEN, Redwood City, CA, USA) and quantified using Nanodrop, (Thermo Scientific, Waltham, MA, USA). Quality of the RNA samples was assessed using Agilent 2200 Tape station (Agilent Technologies, Santa Clara, CA, USA). RNA integrity numbers (RIN) for the samples ranged from 7.2 to 8.1. cDNA libraries were made using Illumina's TruSeq RNA sample preparation kit v2 (Illumina Inc., San Diego, CA, USA) for 2–4 μg of RNA per sample. Briefly, the poly-A containing mRNA was purified, fragmented from total RNA and double stranded cDNA was made. cDNA was recovered using Agencourt, AMPure XP beads (Beckman Coulter, IN, USA). The sample identifier adapters were ligated to the ends of cDNA using 3' adenylation and adapters ligation. We used specific index for each sample to facilitate multiplexing then adaptor ligated fragments were purified by using AMPure XP beads and amplified with polymerase chain reaction (PCR). Validation and identification of mid insert size of cDNA libraries was performed using Agilent 2200 Tape station. Libraries were quantified using Qubit 2.0 fluorimeter (Invitrogen, Life Technologies Waltham, MA, USA). Prior to loading into HiSeq rapid flow cell, the cDNA libraries were diluted, and denatured.

2.3. mRNA and miRNA Sequencing Strategy

For transcriptomic studies, paired end sequencing was performed using Illumina HiSeq 2500 (Illumina, San Diego, CA, USA) with a 108 bp read length at the Center for Biotechnology and Genomics Core Facility at Texas Tech University to study gene expression. For miRNA profiling, Illumina small RNA protocol (Illumina, Inc., San Diego, CA, USA) was used for libraries preparation and sequencing, then the Illumina Genome Analyzer NextSeq 500 (Illumina, Inc., San Diego, CA, USA) was used to perform sequencing at Department of Molecular and Cell Biology, Baylor College of Medicine, Houston.

2.4. Quality Control (QC) and reads alignment

For gene expression, FastQC v0.10.1 High Throughput Sequence QC report (Version 0.11.2) [13] was used to check the quality of raw sequence reads. The average base call quality Q score based on fastqc data was 38 (Supplementary Figure S1). The reads were mapped to the

Mus musculus genome (GRCm38.p4) using Qseq® software Version 12 (DNASTAR Madison, WI). For miRNA expression, the Gunaratne Next Generation pipeline was used to identify miRNA expression profiles [14]. Fastqc data on quality control for small RNA sequencing are provided in supplementary figure S2. In the normalization process, counts of each unique read of miRNA were normalized to total usable reads and then 40 counts were added. Normalized expression values of genes and miRNAs were presented as reads per kilo base per million mapped reads (RPKM).

2.5. Data availability

RNA sequencing data for HF and HF-EPA were submitted in the BioProject at NCBI under PRJNA353387 individual accession numbers for HF Sequence Read Archive (SRA) submission: BAT1 (SAMN05717642), BAT2 (SAMN05717643), and BAT3 (SAMN05717644). For HF-EPA SRA submission: BAT-EPA1 (SAMN05991740), BAT-EPA2 (SAMN05991741), and BAT-EPA3 (SAMN05991742). The small RNA-Seq data were submitted to Gene Expression Omnibus (GEO) data repository (http://www.ncbi.nlm.nih.gov/projects/geo/) under accession number GSE85101 and GSE99506 for BAT HF and BAT HF-EPA respectively.

2.6. Quantitative real-time polymerase chain reaction (q-PCR) validation

RNA was isolated from BAT using RNeasy kit (QIAGEN, Redwood City, CA, USA) followed by cDNA synthesis using the iScript kit (Bio-Rad Laboratories, Inc. CA.USA) and TaqMan® Advanced miRNA cDNA Synthesis Kit (Life Technologies Corporation, Pleasanton, CA, USA) for genes and miRNAs respectively. Quantitative polymerase chain reaction (qPCR) was performed using BioRad CFX-96 real time PCR detection system (Bio-Rad Laboratories, Inc. CA.USA). 18S and miR-191–5p was used as housekeeping control for genes and microRNAs respectively.

2.7. Statistical analyses:

Differentially expressed (DE) genes between HF (control) and HF-EPA groups were identified as those with 2 or more-fold change at 95% confidence using Moderated t-test with false discovery rate (FDR) of 5% (using Benjamini & Hochberg test) with QSeq software. $P \leq 0.05$ was considered significantly different based on Student's t-test. The Ingenuity Pathways Analysis (IPA®, QIAGEN Redwood City, CA, USA; www.qiagen.com/ingenuity) software was used for analysis of target pathways, genes and networks. We used RPKM 0.3 or more in both HF and HF-EPA as the threshold for mRNA expression [15]. In the canonical pathway analyses, Fischer's exact test p values indicates the significance of enrichment of pathways by DE genes and the Z score to determine whether the canonical pathway is activated or inhibited based on the DE genes in the dataset. Z score ≤ -2 is inhibited and ≥ 2 is activated. For miRNAs, a cut off of log2 fold change > 1.25 was used to determine differentially regulated ones that were DE between HF vs. HF-EPA, RPKM $\geq$ 3.76. Also, we submitted differentially expressed miRNAs with log2 fold change > 1.25 to canonical pathways using the grow function in IPA®. For gene and miRNA validation, results are presented as means ± SEM and the ΔΔCt method was used to determine mRNA expression and fold changes. Three to five replicates were used for each dietary or treatment groups.

3. Results

We have previously reported significant reductions in body weight, insulin resistance, and body fat in HF-EPA and low fat (LF) group compared to HF fed mice [10]. The mean final body weights of the HF, HF-EPA, and LF fed mice were 40.4 ± 1.2 (g), 35.9 ± 0.9 (g), and 31.7 ± 1.0 (g) respectively (Supplementary Figure S3A), ($P \leq 0.05$). Mean BAT weights of the HF, HF-EPA, and LF groups were 0.28 ± 0.02 (g), 0.18 ± 0.02 (g), and 0.2± 0.02 (g) respectively (Supplementary Figure S3B), ($P \leq 0.05$). No significant differences were observed in food intake between the HF, HF-EPA, and LF groups (Supplementary Figure S3C) [10]. Furthermore, hematoxylin and eosin stained histological sections of BAT (HF, HF-EPA, and LF) showed reduced lipid content in HF-EPA compared to HF; while HF-EPA was comparable with LF (supplementary Figure 4).

3.1. RNA sequencing data Quality

BAT from HF and HF-EPA groups were used for Illumina paired-end sequencing for whole transcriptome mRNA sequencing. Total sequences per sample were about 24 million paired-end reads of 108 bp length. For small RNA sequencing, an average of 13.8 million sequences reads per sample was mapped to the *Mus musculus* genome (build mm 10) and a total of 307 miRNAs were identified in BAT.

3.2. Analyses of gene and miRNA differences between two diets

Using Qseq software analysis, genes with RPKM cut off ≥0.3 was filtered to avoid poorly expressed genes. HF and HF-EPA groups expressed a total of 11686 and 12054 mRNAs genes respectively; among these 11667 were common genes that existed between HF and HF-EPA as shown by venn diagram (Figure 1.A). Hence, only 19 and 387 genes, were found to be exclusively expressed in HF or HF-EPA groups respectively. Out of 11667 common genes identified, 479 of them were differentially expressed (DE) between HF and HF-EPA with a criterion of 2-fold change and significance of $p \leq 0.05$ (Supplementary Table 1). Out of the 479 DE genes, 31 were upregulated while 448 were downregulated with EPA respectively (Figure 1.A).

In the miRNA analyses, a total of 307 miRNAs were expressed in both groups with 267 common miRNAs, out of which, 290 and 284 miRNAs were expressed in HF and HF-EPA respectively. Using an RPKM cut off ≥ 3.76, we identified 23 and 17 miRNAs that were expressed only in HF or HF-EPA respectively. Next, using the Gunaratne Next Generation pipeline, we identified 15 miRNAs that were DE with a log2 fold change > 1.25 ($P \leq 0.05$). Out of the 15 miRNAs DE, 9 were upregulated and 6 were downregulated with EPA respectively (Figure 1.B). Based on the DE expression of genes and miRNAs, we used hierarchical clustering (HC) to determine the similarity in expression level in different treatment groups (HF and HF-EPA), which is represented by specific colors (Figure 1.C and 1.D).

3.3. Top up and downregulated mRNAs and miRNAs by EPA

In this study, we identified several DE genes by EPA using Qseq software in BAT (Table 1). Among these, Serine/threonine-protein kinase (*Sgk2*) gene which is associated with

thermogenesis [16], was significantly upregulated (2.11 fold change) by EPA compared to HF diet. Also, significant increases of Paraoxonase 1 (*Pon1*) gene (2.11 fold change), which has antioxidant properties [17] was observed with EPA. Top downregulated genes by EPA (Table 1) included genes negatively associated with thermogenesis such as translocation-associated Notch protein TAN-1(Notch1) [18], hypoxia Inducible factor 1 alpha subunit inhibitor (Hif1αn) [19], adenosine A1 receptor (Adora1) [20], nuclear receptor corepressor 2 (Ncor2) [21], early growth response 1 *(Egr1) [22]*, insulin like growth factor 2 (Igf2) [23], transforming growth factor receptor 3 *(Tgfβr3),* and smad family member 3 (*Smad3)* [24]. Genes associated with obesity, and type 2 diabetes such as CREB binding protein *(Crebbp),* inhibin beta B *(Inhbb),* Sp1 transcription factor *(Sp1*), caspase 7 *(Casp7),* and forkhead box O1 *(Foxo1)* were also downregulated [25–27]. Additionally, genes associated with inflammation, Jun proto-oncogene *(Jun*) [28], and lipid metabolism, Glycerol-3-phosphate dehydrogenase 1 *(Gpd1)* were downregulated by EPA ($P \leq 0.05$, fold change ≥ 2, RPKM ≥0.3) [29].

We identified 15 miRNAs which were differentially expressed between HF and HF-EPA diets (Table 2). Candidates with high differential expression were further validated; these include miR-455–3p, miR-129–5p, miR-129–2-3p, miR-129–1-3p, miR-181c-3p, miR-199a-3p, and miR-31023p. Of these, miR-455 was previously reported to play a critical role in BAT adipogenesis [19]. Also, miR-129–5p which was identified in this study, was also shown previously to regulate thermogenesis and energy expenditure [30]. However, to our knowledge, no studies have identified regulation of these miRNAs in response to EPA. The rest of the miRNAs we identified are novel and not previously known to be involved in BAT regulation, specifically in response to EPA.

3.4. Validation of gene and miRNA profiling results

qPCR was performed for validation of target genes and miRNAs using BAT from HF vs. HF-EPA groups. As previously reported, we observed significantly higher expression of key thermogenesis genes, peroxisome proliferator-activated receptor gamma coactivator 1 alpha *(Pgc1α),* PR domain containing 16 (*Prdm16)*, Ppar-γ, and *Ucp1* in HF-EPA Vs. HF diet [11]. Additionally, we observed significantly lower expression of genes known to have a negative correlation with thermogenesis, such as Tgfbr3, Smad3, and Ncor2 ($P \leq 0.05$, Figure 2.A). We further tested 5 miRNAs (miR-455–3p, miR-129–1-3p, miR-1292–3p, miR-129–5p, and miR-181c-3p), which were significantly upregulated by EPA and 2 miRNAs (miR-3102–3p and miR-199a-3p), significantly downregulated by EPA in our miRNA profiling. qPCR validation showed that the expression levels of miR-455–3p and miR-129–5p were significantly higher ($P \leq 0.05$) in HF-EPA compared to HF groups. Other miRNAs validated showed trends towards significance ($P<0.1$) (Figure 2.B). The validation of gene and miRNA (HF vs. HF-EPA) compared to low LF diet is included in supplementary figure 5.

We conducted further analysis of the DE miRNAs in canonical pathways to create a proposed mRNA/ miRNA network model in IPA (Figure 3). **We previously reported** that mRNAs such as *Ppar-γ*, *Ppar-α, Prdm16, Pgc1α, and Ucp1* mainly involved in energy expenditure and fatty acid oxidation were upregulated by EPA [11], while some genes

negatively associated with thermogenesis such as *Hif1α.n* [19], *Tgfβr3* [24], *Notch*1 [18], *Smad3* [24], *Ncor2 [21], Igf2 [23], and Egr1 [22],* were all downregulated by EPA. Also, in our main network analysis, miR-455–3p and miR-129–5p, were upregulated by EPA, consistent with their role in BAT thermogenic function, and were therefore incorporated from our miRNA profiling analysis [19, 30].

3.5. IPA analysis including canonical pathways and networks

Using IPA, we identified that EPA upregulated pathways such as peroxisome proliferator-activated receptor (PPAR), which is involved in lipid metabolism and fatty acid oxidation and pathways involved in energy balance such as PPAR and retinoid X receptors (PPAR/RXR) [31]. Additionally, energy expenditure pathways such as phosphatase and tensin homolog (PTEN) [32], Rho GDP-dissociation inhibitor (Rho-GDI) signaling pathway which is involved in multi-molecular functions such as shuttling capability for the particular membrane microenvironment were also upregulated by EPA (Table 3) [33]. As expected, inflammatory pathways were downregulated by EPA. They include transforming growth factor beta 1 (TGFβ1) [34], signal transducer and activator of transcription 3 (STAT3) [35], nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) [36], high mobility group box 1 (HMGB1) [37], integrin linked kinase (ILK) [38], interleukin 6 (IL6) [39], tumor necrosis factor receptor 1 (TNFR1) [40], and toll like receptor signaling (TLR) [41] (Table 3).

4. Discussion

Our study is the first report of transcriptomic and miRNA profiling in BAT from mice fed a HF compared to HF diet supplemented with EPA. The depth of transcriptomic and miRNA profiling performed in this study uncovered many genes and miRNAs involved in pathways for thermogenesis, energy expenditure, brown fat development, which may link to lipid homeostasis, and fatty acid oxidation. Our findings suggest that EPA increased markers of thermogenesis, similar to cold exposure or diet induced thermogenesis.

In the previous report [11], we investigated the effects of both EPA and DHA on cultured brown adipocytes. We observed more mitochondrial content with EPA than DHA. Therefore, for this study we only used EPA to detect its effect on BAT metabolism related to thermogenesis. While, DHA is a key component of membranes specifically in the central nervous system and contribute to brain development [42], studies have shown that EPA suppresses inflammation and major coronary events better than DHA [43].

Out of four canonical pathways activated by EPA, we focused on PTEN and PPAR pathways, which are related to thermogenesis. PTEN is a tumor suppressor protein which inhibits the activity of phosphatidylinositol 3-kinase (*PI3K*). Moreover, transgenic mice with higher copies of *Pten* displayed increased energy expenditure and higher levels of *Ucp1* in BAT [32]. Corroborating with this, deficiency of *Pten* in mice is linked to obesity and insulin resistance suggesting *Pten* activation is beneficial [44]. These reports and our transcriptomic profiling together suggests that EPA could activate PTEN in BAT, and this in turn may be involved in increasing thermogenesis and energy expenditure.

Another pathway that is modulated by EPA in BAT includes PPAR. This is consistent with previous studies which show that omega-3 fatty acids are ligands for PPARs, namely *Ppar-α* and *Ppar-γ*, leading to activation of adipogenesis, thermogenesis, fat catabolism, mitochondrial fatty acid oxidation rates and energy expenditure in BAT and WAT [45]. *Ppar-α* is highly expressed in BAT and increases levels of thermogenic marker including *Pgc1α* and *Ucp1* [46], all of which are suitable targets for BAT activation and metabolic disorders. Moreover, some studies have already reported that n-3 PUFAs induce thermogenesis in BAT by increasing the level of *Ppar-α* and *Pparγ* [47–49]. Beside the activation of BAT, omega 3 fatty acids act as natural ligands and activators of *Ppar-α*, which blocks NF-KB activity in WAT to prevent inflammation [50]. Furthermore, the free fatty acid receptor 4 (Ffar4), also known as G-protein-coupled receptor 120 (Gpr120), was also significantly upregulated by EPA in our study. However, it was not listed in the differentially expressed genes in the tables above, due to the higher cut off used in our data analyses. These findings are consistent with a recent report demonstrating that Ffar4 was a functional receptor for omega 3 fatty acids in mouse BAT [51].

Our results show that EPA significantly increased miR-455 expression (3). This miRNA targets key brown adipogenic signaling molecules including *Hif1αn*, *Ppar-γ*, and *Tgfβr3* [19]. Mir-455 inhibits *Hif1an*, which in turn induces *Ucp1*. Indeed, higher BAT *Ucp1* levels were reported in *Hif1αn* whole-body knockout mice [52]. Furthermore, *Hif1αn* is upstream of the AMPK-PGC1α regulatory pathway [19], and miR-455 also activates protein kinase, AMP-activated, alpha1 (AMPKa1), an enzyme which catalytic subunit of AMP-activated protein kinase (AMPK) by targeting *Hif1an* [19]. AMPK1 activation in turn induces *Pgc1α* and *Ucp1* expression to induce mitochondrial biogenesis and thermogenesis [53]. Thus, our findings are consistent with these reports and validate the importance of this pathway in BAT regulation.

Moreover, the main network pathway in figure 3 shows that *Hif1αn* serves as an intermediate target between miR-455 and another gene, *Notch1*. Mir-455 reduces the expression of *Notch1,* which in turn promotes browning of white adipose tissue to prevent obesity [54, 55]. Additionally, mRNA levels of beige cell markers such as *CD137* and T-Box1 (*Tbx1*), and mitochondria markers such as carnitine palmitoyltransferase 1A (Cpt1a) and Cpt2, were also higher, suggesting beneficial effects of *Notch* depletion [54]. This has been also confirmed in mouse studies, where *Notch* specific deletion in inguinal WAT induced *Ucp1*, cell death-inducing DFFA-like effector A (*Cidea)*, *Pgc1α* and *Prdm16* expression [56]. Consistent with these reports, our data predict an inhibitory effect of EPA on *Hif1αn* and *Notch1* genes, which may increase thermogenesis and energy expenditure by increasing levels of *Prdm16*, *Pgc1α*, and *Ucp1* as the key regulators of thermogenesis.

Our results further demonstrated that miR-455 also downregulated *Tgfβr3* level and its downstream member, *Smad2/3*. TGF-β signals through dual serine/threonine kinase receptors and its transcription factors named Smads, with Smad3 serving as the major facilitator of TGF-β signal [57]. Blockade of TGF-β/Smad3 signaling leads to enhanced metabolic profile and energy expenditure [24]. Interestingly, *Smad3* global knockout mouse is resistant to diet induced obesity and has elevated thermogenic markers and increased basal rate of oxygen consumption in the WAT compared to controls [24]. These results confirm

that loss of *Tgfβ/Smad3* triggers browning of WAT. In agreement with these studies, our study validates these findings and identified a link between miR-455 and its *Tgfβ/Smad3 targets*.

PPAR-γ is a nuclear receptor highly expressed in BAT and WAT, which serves as a master transcriptional regulator of brown adipocyte differentiation and it is crucial for tissue development, function, and survival [58]. In our study, EPA induced miR-455 and *PPAR-γ* expression, inhibiting *Ncor* expression, known for its role in skeletal muscle and BAT energy homeostasis [59]. *Ncor1* is a direct corepressor of *PPAR-γ*. Hence, clearance of *Ncor* from the *PPAR-γ* complex is necessary for recruitment of the brown cofactor *Prdm16* [21]. Furthermore, inhibition of *Ncor* leads to higher levels of *PPAR-α,* which also stimulates mitochondrial activity, fatty acid β-oxidation via *Ucp1* [60]. Thus, our results demonstrate that EPA increased thermogenic markers, in part via regulation of miR-455, a novel EPA target, upstream of *PPAR-γ* and *PPAR-α*, which inhibit *Ncor* to induce key thermogenic biomarkers. However, other studies have also reported potential roles of miR-455 in cancer, specifically as a tumor suppressor [61–63]. Taken together these studies suggest potential dual benefits for miR-455 in both cancer and energy balance.

Another miRNA, miR-129–5p was also significantly upregulated by EPA. Its reported targets are *Igf2* and *Egr1* [30, 64]. Interestingly, mouse mutant for *Igf2* gene demonstrated massive BAT hypertrophy, less lean mass and higher levels of *Ucp1* and *Prdm16* compared to wildtype mice [23]. Additionally, inactivation of *Igf2* in preadipocytes increased *Ucp1* and *Prdm16* expression consistent with *in vivo* studies. The other target for miR-129–5p, Egr1 when knocked down in the WAT induced expression of BAT *Ucp1* and *Pgc1α* [22]. These data indicate that miR-129–5p regulates important genes involved in thermogenesis. Although the fold differences in the expression of miRNAs were small in the current study, previous studies have shown that even a small (2-fold) change will have a significant impact on target protein levels [65].

In the present study, we used a diet containing 36 g/kg EPA, which is equivalent to 6.75% of energy intake in diet. In comparison, the intakes of EPA and DHA in the United States are low for omega 3s ~0.1–0.2 g/d [66] and the current recommendations vary from 1 g/d to 4 g/d [67] with upper levels necessary to reduce hypertriglyceridemia. Higher doses have been used in human studies from 4.2 g/d [68] to doses up to 15 g/d described in a recent meta-analysis [69, 70]. However, in human studies, the effect of fish oil in improving fat oxidation and whole-body metabolism is inconsistent. Some factors responsible for theses inconclusive results include usage of lower doses in human studies compared to animal studies, genetic variabilities in humans compared to selected animals for research, and different ratio of EPA/DHA which were used for clinical studies [71, 72]

5. Conclusion

We report several previously identified as well as new genes, miRNAs, and pathways involved in thermogenesis, energy expenditure, and brown fat development. Some of the identified targets of EPA in BAT include miRNAs 455, 129 and PPAR/NCOR, and SMAD3/TGF pathways. These all may lead to improved metabolic homeostasis, suggesting

that omega-3 fatty acids, and potentially other nutritional interventions are viable therapies for preventing and/or treating obesity and related metabolic disorders, in part via regulation of BAT. While our study has many strengths and novel findings, it has some limitations. We did not measure the energy expenditure directly in our mice, thus we did not validate whether increased expression of thermogenic biomarkers by EPA translated into increased energy expenditure in the EPA fed mice. Moreover, additional molecular and physiological studies are necessary to further validate the mRNA-miRNA regulatory pairs that we identified in our unbiased screens, their direct role in thermogenesis and mechanism of their regulation by EPA both in vitro and in vivo. Such studies include using miRNA mimics and inhibitors in cells and animals, to validate target genes which are beyond the scope of the current report. Lastly, it will be worthwhile in future to determine if lower doses of EPA compared to DHA or whole fish oil recapitulate the responses reported here for EPA in animal models; and whether these findings can be translated in clinical studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank members of the Dr. Moustaid-Moussa's lab for their assistance with this project, especially Shane Scoggin for his assistance with various technical aspects of this research. We also would like to thank the center of Biotechnology and Genomics at Texas Tech University, the department of Biology and Biochemistry, University of Houston and Baylor College of Medicine, for assistance from their facilities used in this study. The authors' responsibilities were as follows N.M.M. and N.S.K. designed the study; M. P.; N. N. W; N. S. K conducted research; M. P. drafted the first version of this paper; M. P; N.M.M. L. R; P. G., C. C; and K. R. were involved in the data analyses; P. K; P. H. G; C.C. and N. M. M. provided facilities for research; N. M. M; provided facilities and materials for this research, and is the primary responsibility for this research and final content of the manuscript. Funding for this research was supported in part by NIH/NCCIH grant #R15AT008879-01A1 (NMM), USDA NIFA Exploratory award (2015-67030-23452), startup funds from Texas Tech University, and The Obesity Research Cluster.

Abbreviations used:

Adra1	adenosine A1 receptor
AMPK	AMP-activated protein kinase
Arhgap35	Rho GTPase activating protein 35
Arhgef17	Rho guanine nucleotide exchange factor 17
Atg1	autophagy related 1
BAT	brown adipose tissue
BMPR	bone morphogenetic protein receptor
Casp7	caspase 7
Cbl	Cbl proto-oncogene
Cidea	cell death-inducing DFFA-like effector A

Cish	cytokine inducible SH2 containing protein
Cpt-1	carnitine palmitoyl transferase I
Crebbp	CREB binding protein
DE	differentially expressed
DHA	docosahexaenoic acid
DMEM	dulbecco's modified eagle medium
Egr1	early growth response 1
EPA	eicosapentaenoic Acid
Esr1	estrogen receptor 1
FDR	false discovery rate
Ffar4	Free fatty acid receptor 4
Fnbp1	formin binding protein 1
Fos	fos proto-oncogene
Foxo1	forkhead box O1
Gapdh	glyceraldehyde 3phosphate dehydrogenase
GEO	gene expression omnibus
Gnao1	G protein subunit alpha O1
Gpd1	glycerol-3-phosphate dehydrogenase 1
(Gpr120)	G-protein-coupled receptor 120
HC	hierarchical clustering
HF	high-fat diet
HF-EPA	HF diet supplemented with EPA
Hif1an	hypoxia inducible factor 1 alpha subunit inhibitor
Hi-Seq	high sequencing
HMGB	high mobility group box
HS	hematopoietic lineage cell-specific protein
Igf2	insulin like growth factor 2
IL	interleukin
ILK	integrin linked kinase

Inhbb	inhibin beta B
Insr	insulin receptor
IPA	ingenuity pathway analysis
Jun	Jun proto-oncogene
Kat6a	lysine acetyltransferase 6A
Klhl6	kelch-like family member 6
LF	low fat
miRNA and miR	microRNA
mRNA	messenger RNA
Myh9	myosin heavy chain 9
Ncor	nuclear receptor corepressor
NF-κB	nuclear factor kappa-light-chainenhancer of activated B cells
Notch1	Notch homolog 1, translocation-associated (Drosophila)
Pdpk1	3-phosphoinositide dependent protein kinase 1
Pgc1α	peroxisome proliferator-activated receptor gamma coactivator 1 alpha
Pik3c2b	phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 beta
Pmepa1	prostate transmembrane protein, androgen induced 1
Pon	Paraoxonase
Ppar	peroxisome proliferator-activated receptor
Prdm16	PR domain containing 16
Prl41	catalyze protein synthesis, ribosomal protein L41
PTEN	phosphatase and tensin homolog
Ptpn11	protein tyrosine phosphatase, Non-Receptor Type 11
PUFA	polyunsaturated fatty acid
Rhob	ras homolog family member B
RHO-GDI	Rho GDP-dissociation inhibitor
PI3K	phosphatidylinositol 3-kinase

RIN	RNA integrity numbers
RNA-Seq	RNA sequencing
RPKM	reads per kilo base per million mapped reads
RT-qPCR	quantitative reverse transcription
RXR	retinoid X receptor
SAT	subcutaneous adipose tissue
SEM	standard error of mean
Sgk	serine/threonine-protein kinase
Smad	SMAD family member
SNS	sympathetic nervous system
Sp1	Sp1 transcription factor
SRA	sequence read archive
STAT	signal transcription and activator of transcription
Tbx	T-box transcription factor
TG	triglycerides
TGFβ	transforming growth factor –β
TGFβR	transforming growth factor beta receptor
TLR	toll-like receptor
Tnfaip3	TNF Alpha Induced Protein 3
TNFR	tumor necrosis factor receptor
UCP	uncoupling protein
VAT	visceral adipose tissue
Wasf2	WAS protein family member 2
WAT	white adipose tissue

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Highlights:

- EPA upregulated thermogenic markers similar to cold exposure or exercise.
- MiR-455 and −129 are novel miRNAs involved in regulation of thermogenesis by EPA.
- Smad3, Tgfbr3, and Notch are novel RNAs down-regulated by EPA for thermogenesis.
- Modulation of BAT function using EPA is a potential novel target for obesity

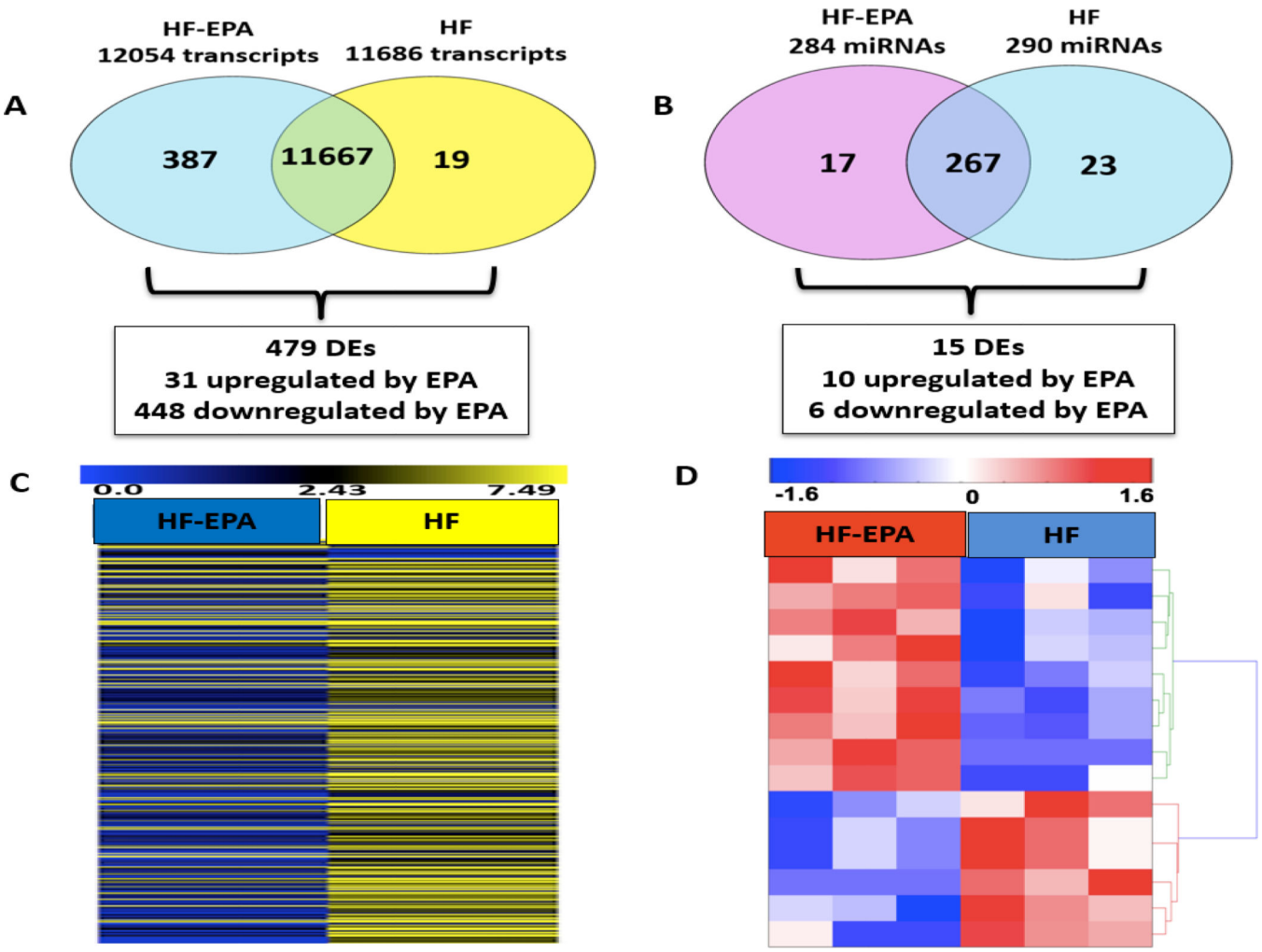

Figure 1. Gene and miRNA profiling and Hierarchical clustering process in B6 mice.

(A) Gene filtering process using venn diagram following RNA sequencing for HF vs. HF-EPA in BAT. Four hundred and seventy nine DE genes were identified using QSeq software of which 31 and 448 genes were upregulated and downregulated by EPA respectively ($P \leq 0.05$, fold change ≥2, RPKM ≥0.3). (B) MiRNA filtering process using venn diagram following miRNA profiling for HF vs. HF-EPA in BAT. Fifteen DE miRNAs were identified of which 9 and 6 miRNAs were upregulated and downregulated by EPA respectively ($P \leq 0.05$, log 2 fold change > 1.25, RPKM ≥ 3.76). (C) Four hundred and seventy nine DE genes in BAT, which were differentially regulated by EPA. The vertical line represents the different diets (HF vs. HF-EPA) and the horizontal line represents the list of genes. Lower values are represented with blue tones while higher values have more intense yellow tones ($P \leq 0.05$, fold change ≥ 2, RPKM ≥ 0.3). (D) Fifteen DE miRNAs in BAT, which were differentially regulated by EPA. The vertical line represents the different diets (HF vs. HF-EPA) and the horizontal line represents the list of miRNAs. Lower values are represented with blue tones while higher values have hotter red tones ($P \leq 0.05$, log 2 fold change > 1.25, RPKM ≥ 3.76). HF, high fat; HF-EPA, HF diet supplemented with EPA; DE, differentially expressed.

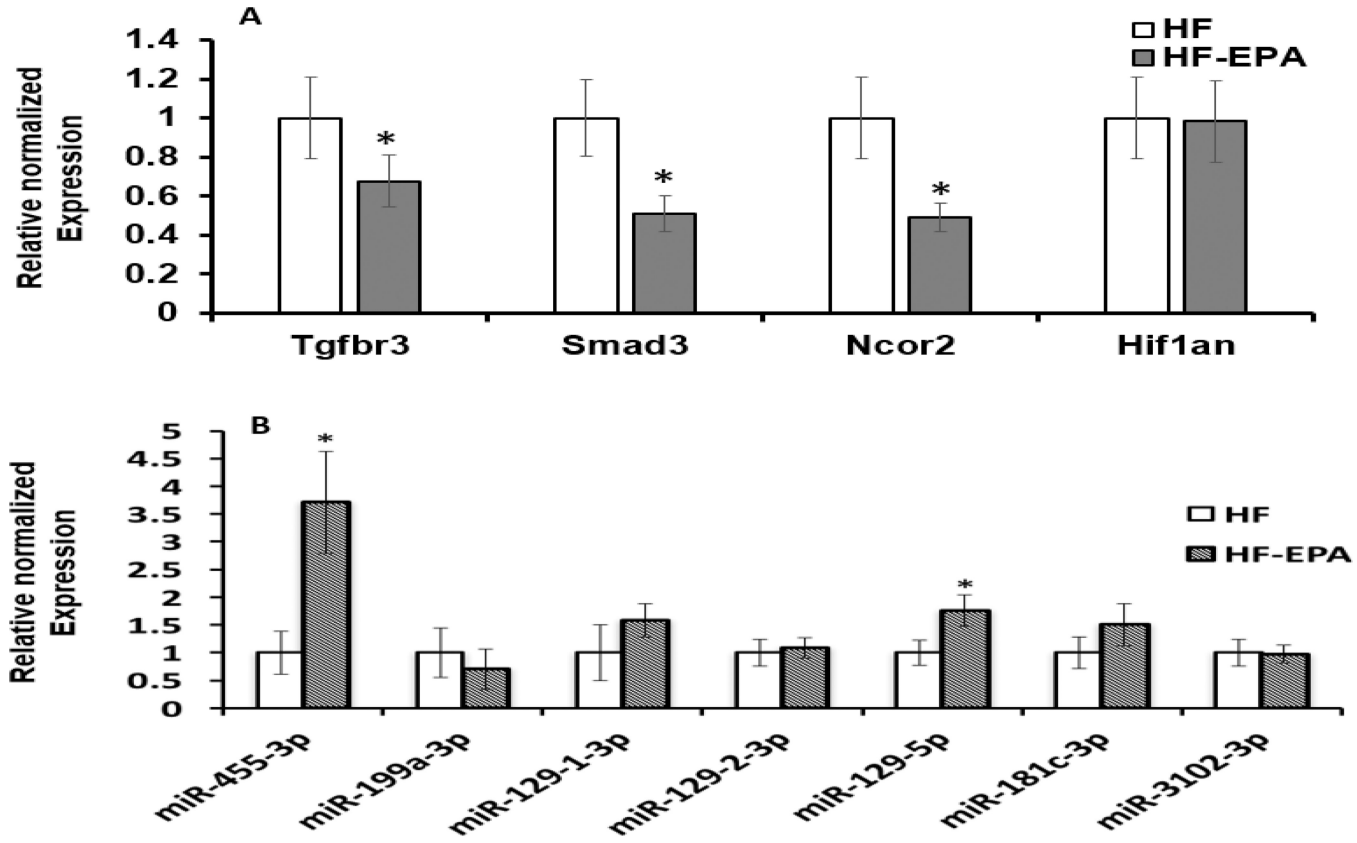

Figure 2. Validation of genes and miRNAs in B6 mice:

Genes and miRNAs were validated using RT-qPCR (A) Gene expression of thermogenesis markers Tgfbr3, Smad3, Ncor2, and Hif1an. Data are expressed as mean ± SEM, P ≤ 0.05, n=5. (B) MiR-455–3p, miR-199a3p, miR-129–1-3p, miR-129–2-3p, miR-129–5p, miR-181c-3p, and miR-3102–3p were validated. Data are expressed as mean ± SEM, P ≤ 0.05, n=5. HF, high fat; HF-EPA, HF diet supplemented with EPA.

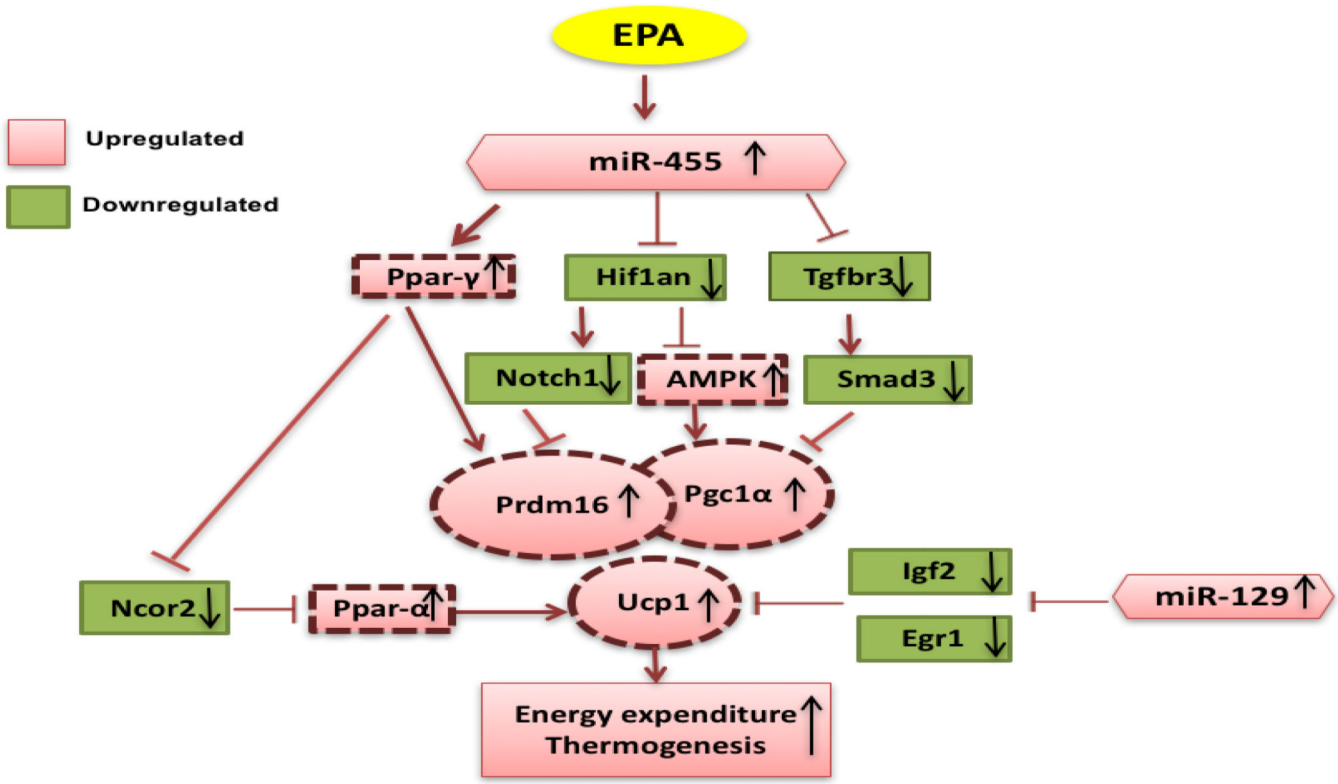

Fig 3. mRNAs and miRNA networks leading to activation of BAT in B6 mice.

Solid lines around boxes represent the genes, which were identified by our study (mean ± SEM, $P \leq 0.05$, n=5), while dashed lines represent genes from our previously published work or published literature.

Table 1.

Top upregulated and downregulated mRNA transcripts in BAT from B6 mice (HF vs. HF-EPA), which were differentially regulated by EPA using QSeq software ($P \leq 0.05$, fold change ≥2, RPKM ≥0.3). HF, high fat; HF-EPA, HF diet supplemented with EPA; RPKM, reads per kilo base per million mapped reads.

	Gene Name	RPKM HF	RPKM HF-EPA	Fold Change	P value
Top upregulated genes					
Klhl6	Kelch-Like Family Member 6	0.65	1.39	2.14	0.01
Sgk2	Serine/threonine-protein kinase	0.5	1.05	2.11	0
Pon1	Paraoxonase 1	8.43	17.8	2.11	0
Rrl41	Catalyze Protein Synthesis, Ribosomal Protein L41	5.48	11.49	2.1	0.01
Top downregulated genes					
Notch1	translocation-associated Notch protein TAN-1	9.43	4.66	0.49	0.01
Casp7	Caspase 7	3.8	1.79	0.47	0.05
Hif1an	Hypoxia Inducible Factor 1 Alpha Subunit Inhibitor	2.44	1.13	0.46	0
Gpd1	Glycerol-3-Phosphate Dehydrogenase 1	3.53	1.63	0.46	0.00
Crebbp	CREB Binding Protein	4.57	2.04	0.45	0
Adora1	Adenosine A1 Receptor	19	8.25	0.43	0
Foxo1	Forkhead Box O1	4.65	1.95	0.42	0
Smad3	Smad Family Member 3	2.51	0.9	0.36	0
Igf2	Insulin Like Growth Factor 2	2.67	0.97	0.36	0.02
JUN	Jun Proto-Oncogene	28.37	9.56	0.34	0
Inhbb	Inhibin Beta B	1.57	0.52	0.33	0.01
Sp1	Sp1 Transcription Factor	2.96	0.95	0.32	0.01
Egr1	Early Growth Response 1	5.41	1.75	0.32	0
Tgfbr3	Transforming Growth Factor Receptor 3	5.52	1.64	0.3	0
Ncor2	Nuclear Receptor Corepressor 2	14.14	5.58	0.2	0.01

Table 2.

List of miRNAs regulated by EPA.

Fifteen miRNAs were differentially expressed between HF and HF-EPA diets in B6 mice. Nine and 6 miRNAs were upregulated and downregulated by EPA respectively. HF, high fat; HF-EPA, HF diet supplemented with EPA; RPKM, reads per kilo base per million mapped reads.

miRNA Name	RPKM HF	RPKM HF-EPA	Fold Change	P value
Upregulated miRNA				
miRNA-17–3p	3.74	5.33	1.59	0.03
miRNA-129–2-3p	9.75	11.30	1.55	0.01
miRNA-129–5p	8.70	10.27	1.57	0.01
miRNA-129–1-3p	3.32	4.94	1.61	0.01
miRNA-148a-5p	6.56	7.22	0.67	0.02
miRNA-150–5p	14.52	15.26	0.74	0.02
miRNA-181c-3p	3.85	5.33	1.7	0.04
miRNA-2137	12.48	13.27	0.79	0.03
miRNA-455–3p	12.15	13.52	1.38	0.05
Downregulated miRNA				
miRNA-125b-1–3p	7.66	6.44	−1.22	0.03
miRNA-199a-3p	10.3	9	−1.03	0.03
miRNA-199b-3p	10.3	9	−1.03	0.03
miRNA-3102–3p	7.50	4.26	−3.24	0.03
miRNA-503–3p	4.89	3.32	−1.57	0.00
miRNA-674–5p	7.22	5.52	−1.7	0.02

Table 3.

Top upregulated and downregulated ingenuity canonical pathways, which were differentially regulated by EPA in BAT from B6 (HF vs. HF-EPA) using IPA. HF, high fat; HFEPA, HF diet supplemented with EPA.

Ingenuity Canonical Pathway	Gene Name	-log (p-value)	Ratio
Top upregulated			
PPAR Signaling	Crebbp, Fos, Insr, Jun, Ncor2, Ppard, Rxra	2.47E	7.53E-02
PTEN Signaling	Bmpr2, Cbl, Foxo1, Foxo3, Foxo4, Igf1r, Insr, Pdpk1, Tgfbr3	3.07E	7.63E-02
PPAR/RXR Activation	Bmpr2, Crebbp, Gpd1, Insr, Jun, Map2k6, Ncor2, Rxra, Smad3, Tgfbr3	2.88E	6.21E-02
RHO-GDI Signaling	Arhgap35, Arhgef17, Crebbp, Esr1, Fnbp1, Gnao1, Pip4k2b, Rhob, Wasf2	1.96E	5.20E-02
Top downregulated			
TGFB1 Signaling	Bmpr2, Crebbp, Fos, Inhbb, \Jun, Map2k6, Pmepa1, Smad3	3.32E	9.20E-02
STAT3 Pathway	Bmpr2, Cish, Igf1r, Insr, Tgfbr3	1.73E	6.85E-02
NF-κB Signaling	Bmpr2, Crebbp, Igf1r, Insr, Map2k6, Map3k1, Pik3c2b, Tgfbr3, Tnfaip3	1.98E	5.23E-02
HMGB1 Signaling	Fnbp1, Fos, Jun, Kat6a, Map2k6, Pik3c2b, Rhob, Sp1	2.42E	6.67E-02
IL-k Signaling	Crebbp, Fnbp1, Fos, Irs1, Irs2, Jun, Map2k6, Myh9, Pdpk1, Pik3c2b, Rhob	2.73E	5.95E-02
IL-6 Signaling	Fos, IL-6r, Jun, Map2k6, Pik3c2b, Ptpn11	1.45E	5.17E-02
CD 40 Signaling	Fos, Jon, Map2k6, Pik3c2b, Tnfaip3	1.93E	7.69E-02
TNFR1 Signaling	Fos, Jun, Map3k1, Tnfaip3, Casp7	2.45E	1.02E-01
Toll-like Receptor Signaling	Map2k6, Fos, Jun, Map3k1, Tnfaip3	1.71E	6.76E-02