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Docosahexaenoic Acid Modulates the Enterocyte Caco-2 Cell Expression of MicroRNAs Involved in Lipid Metabolism[1–3]

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Abstract

Consumption of the long-chain ω-3 (n–3) polyunsaturated fatty acid docosahexaenoic acid (DHA) is associated with a reduced risk of cardiovascular disease and greater chemoprevention. However, the mechanisms underlying the biologic effects of DHA remain unknown. It is well known that microRNAs (miRNAs) are versatile regulators of gene expression. Therefore, we aimed to determine if the beneficial effects of DHA may be modulated in part through miRNAs. Loss of dicer 1 ribonuclease type III (*DICER*) in enterocyte Caco-2 cells supplemented with DHA suggested that several lipid metabolism genes are modulated by miRNAs. Analysis of miRNAs predicted to target these genes revealed several miRNA candidates that are differentially modulated by fatty acids. Among the miRNAs modulated by DHA were miR-192 and miR-30c. Overexpression of either miR-192 or miR-30c in enterocyte and hepatocyte cells suggested an effect on the expression of genes related to lipid metabolism, some of which were confirmed by endogenous inhibition of these miRNAs. Our results show in enterocytes that DHA exerts its biologic effect in part by regulating genes involved in lipid metabolism and cancer. Moreover, this response is mediated through miRNA activity. We validate novel targets of miR-30c and miR-192 related to lipid metabolism and cancer including nuclear receptor corepressor 2, isocitrate dehydrogenase 1, *DICER*, caveolin 1, ATP-binding cassette subfamily G (white) member 4, retinoic acid receptor β, and others. We also present evidence that in enterocytes DHA modulates the expression of regulatory factor X6 through these miRNAs. Alteration of miRNA levels by dietary components in support of their pharmacologic modulation might be valuable in adjunct therapy for dyslipidemia and other related diseases. J. Nutr. 144: 575–585, 2014.

Introduction

The ω-3 long-chain FAs DHA (22:6n–3) and EPA (20:5n–3) are the FAs most frequently associated with diverse health-promoting activities. Although their cardioprotective effects of lowering TGs are well known, they have a role in other biologic mechanisms such as anti-inflammation mechanisms, neuroprotection, and chemoprevention (1).

Several molecular mechanisms have been proposed to explain the beneficial effects of DHA. Cardioprotective effects are thought to occur because of increased degradation of apo B in hepatocytes (which reduces VLDL concentrations), lower blood pressure via activation of vascular large-conductance Ca^{2+}- and voltage-activated K^+ channels (2), and anti–atrial fibrillation. DHA's actions on inflammation are mediated through a variety of molecules, including NAD(P)H oxidase and the G protein–coupled receptor 120 (GPR120),[11] in addition to the formation of protectins, resolvins, and maresins, among others (3).

To date, the effect of noncoding RNAs has been poorly characterized with regard to their cellular activity in response to

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[3] Supplemental Figures 1–7 and Supplemental Tables 1–5 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

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[11] Abbreviations used: ABCG4, ATP-binding cassette subfamily G (white) member 4; *ACC1*, acetyl-CoA carboxylase alpha; *ACOX1*, acyl-CoA oxidase 1; *ACSL1*, acyl-CoA synthetase long-chain family member 1; *ACVR2B*, activin A receptor type IIB; Ago, Argonaute; *ALCAM*, activated leukocyte cell adhesion molecule; *APOL1*, apolipoprotein L1; *ATF1*, activating transcription factor 1; *CAV1*, caveolin 1; *CAV2*, caveolin 2; *CLOCK*, clock circadian regulator; *CRTC2*, CREB regulated transcription

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DHA. MicroRNAs (miRNAs) are ~21-nt regulatory noncoding RNAs that bind Argonaute (Ago) proteins to affect gene expression through base-pairing with target mRNAs. miRNAs generally repress target gene expression either by translation inhibition or by mRNA degradation (4). The biogenesis of miRNAs is a multistage process and comprises the action of 2 endoribonuclease enzymes before they are loaded onto an Ago protein [reviewed in (5)]. Drosha ribonuclease type III (*DROSHA*) is responsible for the first endonucleolytic reaction in the nucleus, and dicer 1 ribonuclease type III (*DICER*) catalyzes the second reaction essential for miRNA maturation. The loss of *DICER* results in accumulation of miRNA precursors, and knockout of *Dicer* in mice is lethal (6). Although the primary role of miRNAs appears to be the "fine tuning" of gene expression, it is in response to stress that their functions become pronounced in the maintenance of health and disease progression (7).

There is increasing evidence that dietary fats may modulate the expression of miRNAs (8). Therefore, it is not surprising that the effects of DHA on gene expression could be partially mediated through regulation of miRNAs (3). Indeed, evidence suggests that ω-3 PUFAs, including DHA, can modify the expression of miRNAs in gliomas (9) or breast (10) and colon (11) cancers. In this study, we searched for miRNAs that might regulate enterocyte lipid metabolism in response to DHA stimulation. Our results suggest a role for the miRNA-mediated control of gene expression in enterocytes in response to DHA.

Materials and Methods

Cell culture. Caco-2 and HepG2 cells were obtained from the American Type Culture Collection, and 1321N1 cells were purchased from the European Collection of Cell Culture. Cells were maintained in DMEM containing 10% FBS and antibiotics. For experiments on differentiated cells, Caco-2 cells were split and seeded at a density of 0.5×10^6 cells/well on 24 mm diameter polycarbonate Transwell filter inserts with a 0.4 μm pore size (Corning). The inserts were placed onto 6-well culture plates, and monolayers were cultured for 21 d. For transfection experiments, Caco-2, HepG2, or 1321N1 cells were split and, when they reached ~60–70% confluence, were transfected as described below.

Dicer knockdown. A small interfering RNA (siRNA) pool targeting the human Dicer mRNA was obtained from Dharmacon. Caco-2 cells were transfected with Dicer siRNA (75 nmol/L) by using Oligofectamine (Invitrogen) in Opti-MEM medium (Invitrogen) following the manufacturer's instruction. An equal concentration of a nontargeting siRNA control was used in each control condition (nonsilencing).

Lipid micelles preparation. Lipid micelles were prepared as previously described (12). Briefly, stock solutions of FAs and phosphatidylcholine (Sigma) were prepared in chloroform. A stock solution of sodium taurocholate was prepared in 95% ethanol. Lipids and bile salts were mixed and evaporated under a stream of nitrogen. The dried lipids were dissolved in DMEM, and the resulting solution was mixed vigorously on a vortex until clear before use.

RNA isolation and qRT-PCR. Total RNA was isolated by using the miRNeasy Mini Kit (Qiagen). For mRNA quantification, cDNA was synthesized by using Taqman Reverse Transcription Reagents (Applied Biosystems). qRT-PCR was performed by using the miScript SYBR Green PCR kit (Qiagen) on a 7900 HT Fast Real-Time PCR System (Applied Biosytems). mRNA levels were normalized to those of the housekeeping gene *18S*. Relative expressions were calculated by the comparative threshold cycle method and presented as a relative expression ratio (2-$\Delta\Delta$ threshold cycle). For miRNA quantification, total RNA was reverse-transcribed by using the miScript II Reverse Transcription Kit (Qiagen). Specific primers for each miRNA (miScript Primer Assay) were also obtained from Qiagen. Relative expression was calculated as described above by using U6 for normalization.

Transfection of miRNA mimics and inhibitors. Caco-2 and HepG2 cells were transfected with 40-nmol/L miRIDIAN miRNA mimics (miR-30c or miR-192) or 40-nmol/L miRIDIAN miRNA inhibitors (anti-miR-30c or anti-miR-192) (Dharmacon) as previously described (13). All control samples were treated with an equal concentration of a nontargeting control mimic sequence (Con-miR) or an inhibitor negative control sequence (Con-Inh; Dharmacon) Verification of miRNA overexpression or endogenous inhibition was determined by qRT-PCR, as described above.

3′ Untranslated region luciferase reporter assays. cDNA fragments corresponding to the entire 3′ untranslated region (UTR) of human isocitrate dehydrogenase 1 (IDH1), retinoic acid receptor (RAR) β (RARB), caveolin 1 (CAV1), nuclear receptor corepressor 2 (NCOR2), and regulatory factor X6 (RFX6) were amplified by reverse-transcriptase PCR from total cDNA from Caco-2 cells and subcloned into the psiCHECK-2 vector (Promega) as previously described (13). For experiments, HEK293cells were cotransfected for 48 h with the indicated 3′UTR luciferase reporter vectors and the miR-30c, miR-192 mimic, or negative control mimic (Con-miR) luciferase activity was assayed by using the Dual-Glo Luciferase Assay System (Promega). Luciferase activity was plotted as a percentage of the control (cells cotransfected with the corresponding Con-miR). For Caco-2 cells, after transfection with the indicated 3′UTR luciferase reporter vectors, they were exposed the last 24 h with lipid micelles and prepared as described above before luciferase activity measurement. At least 3 independent experiments were performed for each determination.

Pathway analysis and prediction. For functional analysis, we only included gene ontology terms that were found to be over-represented (adjusted P value < 0.05) in the gene input list after false discovery rate correction following the hypergeometric statistical test.

Statistical analysis. Normality of variables was tested by using the Shapiro-Wilk test. In light of the results, all data were reported as means ± SEMs (n is noted in the figure legends), and parametric methods were used for analytical statistics. Equality of variances was tested by using the Levene test. An unpaired Student's t test was then used to analyze the effects of DHA treatment and the overexpression and inhibition of

coactivator 2; *CTGF*, connective tissue growth factor; *DBT*, dihydrolipoamide branched chain transacylase E2; *DDIT4*, DNA-damage-inducible transcript 4; *DHFR*, dihydrofolate reductase; *DICER*, dicer 1 ribonuclease type III; *DLL4*, delta-like 4; DROSHA, drosha ribonuclease type III; *ELOVL1*, ELOVL fatty acid elongase 1; *ELOVL5*, ELOVL fatty acid elongase 5; *FABP2*, fatty acid binding protein 2; *FABP3*, fatty acid binding protein 3; *FABP7*, fatty acid binding protein 7; *FASN*, fatty acid synthase; *FOXO3*, forkhead box O3; *GALNT2*, UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2; GPR120, G protein–coupled receptor 120; *HMGCS1*, 3-hydroxy-3-methylglutaryl-CoA synthase 1; *IDH1*, isocitrate dehydrogenase 1; *IGF1*, insulin-like growth factor 1; *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *LCLAT1*, lysocardiolipin acyltransferase 1; *LDLR*, low density lipoprotein receptor; LIN28B, lin-28 homolog B (*Caenorhabditis elegans*); *LIPC*, lipase hepatic; *LPGAT1*, lysophosphatidylglycerol acyltransferase 1; miRNA, microRNA; *MTA1*, metastasis associated 1; *MTTP*, microsomal TG transfer protein; *MVD*, mevalonate (diphospho) decarboxylase; *NCOR2*, nuclear receptor corepressor 2; *NEDD4*, neural precursor cell expressed, developmentally down-regulated 4; *NR1H4*, nuclear receptor subfamily 1 group H member 4; *PCK1*, phosphoenolpyruvate carboxykinase 1; *PCSK9*, proprotein convertase subtilisin/kexin type 9; *PGC1A*, peroxisome proliferator-activated receptor gamma coactivator 1 α; *PGC1B*, peroxisome proliferator-activated receptor gamma coactivator 1 β; *PPARA*, peroxisome proliferator-activated receptor alpha; *PPARD*, peroxisome proliferator-activated receptor delta; RAR, retinoic acid receptor; *RARB*, retinoic acid receptor β; *RARG*, retinoic acid receptor γ; *RB1*, retinoblastoma 1; RFX6, regulatory factor X6; *RUNX2*, runt-related transcription factor 2; *SERPINE1*, serpin peptidase inhibitor clade E member 1; siRNA, small interfering RNA; *SORT1*, sortilin 1; *SREBF1*, sterol regulatory element binding transcription factor 1; *TGFB1*, transforming growth factor β 1; *TRPS1*, trichorhinophalangeal syndrome I; *TWF1*, twinfilin actin-binding protein 1; UTR, untranslated region; *VLDLR*, very low density lipoprotein receptor; *WNK1*, WNK lysine deficient protein kinase 1; *ZEB1*, zinc finger E-box binding homeobox 1; *ZEB2*, zinc finger E-box binding homeobox 2.

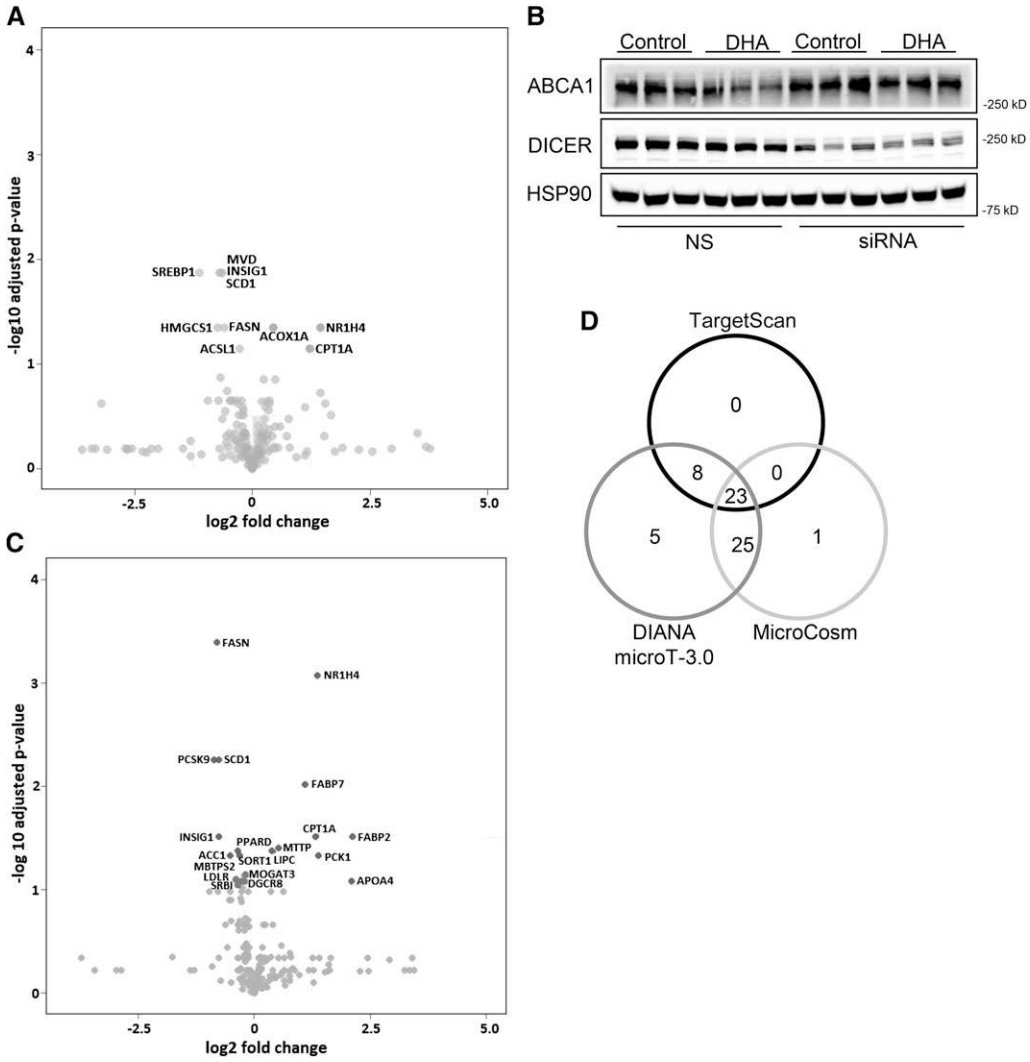

FIGURE 1 Loss of *DICER* in DHA-treated enterocytes reveals the regulation of lipid metabolism genes through miRNAs. (*A*) Volcano plot of DHA-modulated genes in Caco-2 cells. (*B*) Representative Western blot of *DICER* knockdown in Caco-2 cells and treated with or without DHA. HSP90 was used as the loading control. (*C*) Volcano plot of DHA-modulated genes in *DICER*-reduced cells. *DICER* was knocked down in Caco-2 cells and treated with 200 μmol/L DHA (in lipid micelles) or vehicle for 24 h. Gene expression was analyzed by qRT-PCR. Highlighted genes correspond to significant adjusted *P* values. (*D*) Venn diagram of predicted miRNAs that target the various lipid metabolism genes that were regulated in a manner that might be dependent on both *DICER* and DHA by 3 different prediction algorithms. ABCA1, ATP-binding cassette, sub-family A, member 1; *ACC1*, acetyl-CoA carboxylase alpha; ACOX1A, acyl-CoA oxidase 1A; ACSL1, acyl-CoA synthetase long-chain family member 1; *APOA4*, apolipoprotein A-IV; *CPT1A*, carnitine palmitoyltransferase 1A; *DICER*, dicer 1 ribonuclease type III; *DGCR8*, DGCR8 microprocessor complex subunit; *FABP2*, fatty acid binding protein 2; *FABP7*, fatty acid binding protein 7; *FASN*, fatty acid synthase; HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1; HSP90, heat shock protein 90; *INSIG1*, insulin induced gene 1; *LDLR*, low density lipoprotein receptor; *LIPC*, lipase hepatic; *MBTPS2*, membrane-bound transcription factor peptidase site 2; miRNA, microRNA; *MOGAT3*, monoacylglycerol O-acyltransferase 3; *MTTP*, microsomal TG transfer protein; MVD, mevalonate (diphospho) decarboxylase; *NR1H4*, nuclear receptor subfamily 1 group H member 4; NS, nonsilencing; *PCK1*, phosphoenolpyruvate carboxykinase 1; *PCSK9*, proprotein convertase subtilisin/kexin type 9; *PPARD*, peroxisome proliferator-activated receptor delta; *SCD1*, stearoyl-CoA desaturase; siRNA, small interfering RNA; *SORT1*, sortilin 1; *SRBI*, scavenger receptor class B member 1; SREBP1, sterol regulatory element binding protein 1.

specific miRNAs (miR-30c and miR-192). To determine the effect of various FAs [DHA, oleic (18:1n–9) acid, and palmitic (16:0) acid], 1-factor ANOVA was used. When differences were noted, a Dunnett's post hoc test was used to identify specific differences to the control. To identify specific differences between FAs, a Bonferroni post hoc test was used. When equality of variances was not verified, a Games-Howell post hoc test was used. The level of significance was set at $P < 0.05$ for all analyses. Statistical analysis was performed by using IBM SPSS (version 19.0) for Windows.

Results

Loss of DICER reveals novel miRNAs that may regulate lipid metabolism in enterocytes. To assess the effect of DHA on gene expression in enterocytes, we first analyzed the expression of >150 genes (**Supplemental Table 1**) related to cholesterol, lipoproteins, FA metabolism, and other related processes in Caco-2 cells exposed to DHA lipid micelles for 24 h. A variety of genes analyzed were either up- or downregulated in response to DHA treatment (**Fig. 1***A*).

To determine if the modulation of these and other lipid metabolism–related genes observed above are mediated through miRNAs, we used siRNA knock-down of *DICER*. siRNA treatment reduced DICER protein amounts by ~70% (Fig. 1*B*). qRT-PCR analysis in *DICER*-reduced enterocytes exposed to DHA lipid micelles for 24 h showed the modulation of distinct genes and some that overlapped (Fig. 1C) with the Caco-2 cells expressing *DICER*. These data suggest that ω-3 PUFAs may regulate gene expression through miRNA-mediated

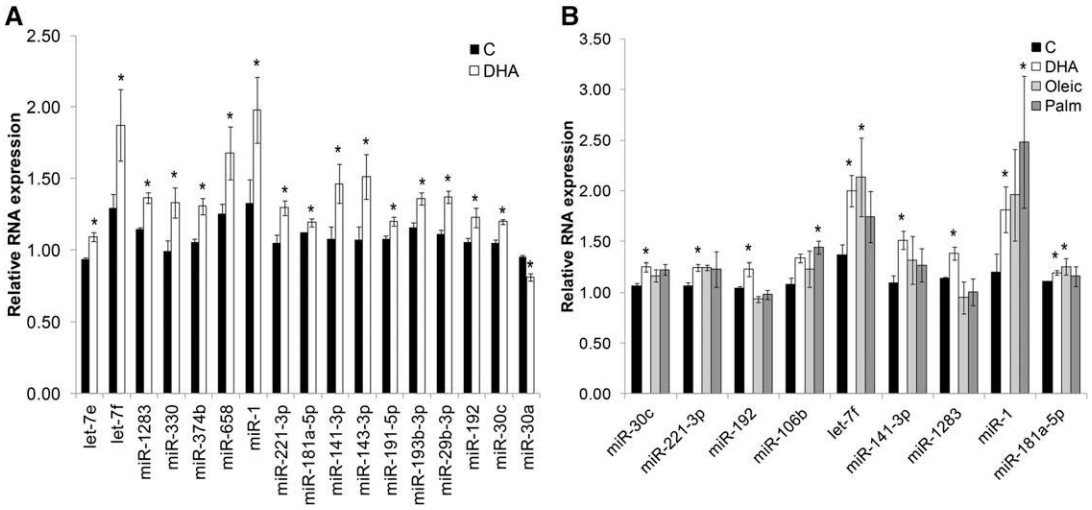

FIGURE 2 DHA modulates the expression of miRNAs in enterocytes. (*A*) The effect of DHA on the expression of miRNAs. Differentiated Caco-2 cells were treated with DHA or with vehicle for 24 h, and miRNAs were assessed by qRT-PCR. (*B*) Comparative analysis of the effect of other FAs on miRNA expression. Differentiated Caco-2 cells were treated with vehicle, DHA, oleic acid, or palmitic acid for 24 h, and miRNAs were measured by qRT-PCR. Values are means ± SEMs; $n \geq 3$ independent experiments. *Different from control, $P < 0.05$. C, control; miRNA, microRNA; Palm, palmitic acid.

pathways. However, we cannot exclude the possibility that some of the observed changes in gene expression are nonspecific or indirect.

To examine the regulation of DHA-responsive genes by miRNAs in enterocytes we used the bioinformatic tool miR2gene (14). We analyzed those genes that were differentially expressed between the 2 cell types: the enterocytes with *DICER* knocked down and the enterocytes expressing *DICER*. These genes were as follows: acyl-CoA oxidase 1 (*ACOX1A*), 3-hydroxy-3-methylglutaryl-CoA synthase 1 (*HMGCS1*), mevalonate (diphospho) decarboxylase (*MVD*), sterol regulatory element binding transcription factor 1 (*SREBF1*), fatty acid synthase (*FASN*), nuclear receptor subfamily 1 group H member 4 (*NR1H4*), acetyl-CoA carboxylase alpha (*ACC1*), acyl-CoA synthetase long-chain family member 1 (*ACSL1*), fatty acid binding protein 2 (*FABP2*), fatty acid binding protein 7 (*FABP7*), lipase hepatic (*LIPC*), microsomal triglyceride transfer protein (*MTTP*), phosphoenolpyruvate carboxykinase 1 (*PCK1*), proprotein convertase subtilisin/kexin type 9 (*PCSK9*), peroxisome proliferator-activated receptor delta (*PPARD*), and sortilin 1 (*SORT1*). As shown in Figure 1*D*, 23 miRNAs are predicted to target these genes.

We next assessed which of the miRNAs predicted to target the genes were differentially regulated by DHA. To do this, Caco-2 cells were differentiated and treated with DHA micelles for 24 h. By using qRT-PCR we evaluated the expression of 55 miRNAs, including the 23 common miRNAs identified above. Our results showed that only a subset were modulated by DHA (**Fig. 2***A*, **Supplemental Fig. 1***A*).

DHA modulates the expression of specific enterocyte miRNAs. To determine if the modulation of enterocyte miRNAs is specific to DHA or a general response to a wider range of FAs, we differentiated Caco-2 cells and treated them with equal concentrations (200 μmol/L) of oleic and palmitic acid for 24 h. As shown in Figure 2*B* and Supplemental Figure 1*B*, different FA treatments resulted in different expression patterns of miRNAs. However, some miRNAs showed no difference in expression between the different FA treatments. It is important to note that we only analyzed those miRNAs that were differentially regulated by DHA lipid micelles (Fig. 2*A*), and thus we cannot rule out the possibility that either oleic or palmitic acid may produce different effects on other miRNAs.

We found that miR-141-3p, miR-221-3p, miR-192, miR-30c, and miR-1283 were induced only by DHA. We also found that DHA, but not oleic or palmitic acids, repressed the expression of miR-30a. We observed that let-7f and miR-181a-5p were induced by DHA and oleic acid but not palmitic acid, miR-1 was induced by DHA and palmitic but not oleic acid, and miR-106b was induced only by palmitic acid. Even though the changes in expression of many miRNAs were subtle, these changes were consistent and reproducible across all experiments.

miR-30c targets predicted genes regulating lipid metabolism. We decided to further validate miR-30c and miR-192 because there is evidence that the former can be modulated by PUFAs in glioma cells (9) and the latter plays a role in obesity (15) and colon adenocarcinomas (11). To better understand the role of miR-30c in lipid metabolism, we first verified its expression in different mouse tissues. Mature miR-30c (**Supplemental Fig. 2**) is detectable in the small intestine and in the colon. However, its expression is low compared with that in other tissues. To determine the genes that might be modulated by miR-30c in enterocytes, we analyzed the predicted target genes by using the miRWalk database (16). We identified >6000 mRNAs potentially regulated by miR-30c (**Supplemental Table 2**). Pathway analysis by the GenCodis3 application (17) indicated that ~600 target genes are involved in primary metabolic processes (**Supplemental Fig. 3***A*). This analysis suggests that miR-30c may regulate a variety of genes modulating lipid metabolism (Supplemental Fig. 3*B*). In addition, we performed a literature-based analysis of these predicted targets and from this selected a list of target genes for further validation (**Supplemental Table 3**). We next investigated protein-protein interactions between the genes in this list by using the String-db database (18). Our results suggest that many of these gene products interact in vivo (**Fig. 3***A*).

To determine the contribution of miR-30c to lipid metabolism in enterocytes, we evaluated TG accumulation in Caco-2 cells exposed to 24-h treatment of oleic acid lipid micelles (Fig. 3*B*). The modulation of miR-30c expression in Caco-2 cells

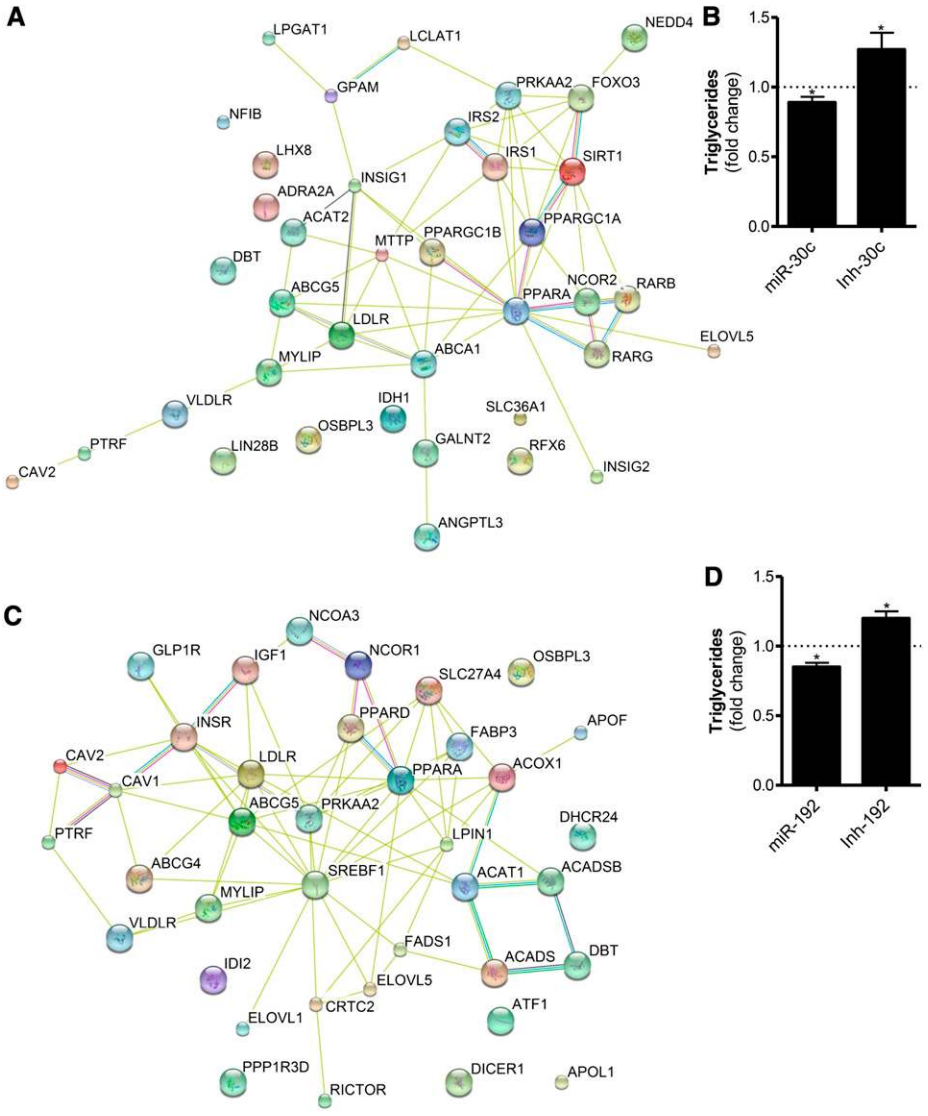

FIGURE 3 miR-30c and miR-192 have multiple predicted target genes related to lipid metabolism. (*A*) Protein-protein interaction analysis of the miR-30c selected predicted target genes related to lipid metabolism using String software (18). (*B*) Fold change of cellular TG concentrations of Caco-2 cells exposed to lipid micelles. Caco-2 cells were transfected by using miR-30c mimics or inhibitors or their controls and treated with 200 μmol/L DHA (in lipid micelles) for 24 h. Data are represented as fold change from that of the respective controls. (*C*) Protein-protein interaction analysis of the miR-192 selected predicted target genes related to lipid metabolism using String software. (*D*) Cellular TG concentrations of Caco-2 cells exposed to lipid micelles. Caco-2 cells were transfected by using miR-192 mimics or inhibitors or their controls and treated with 200 μmol/L DHA (in lipid micelles) for 24 h. Values are means ± SEMs; $n \geq 3$ independent experiments. Data are represented as fold change from that of the respective controls. *Different from control, $P < 0.05$. *ABCG5*, ATP-binding cassette subfamily G (white) member 5; *ABCA1*, ATP-binding cassette, subfamily A, member 1; *ACOX1*, acyl-CoA oxidase 1; *ADRA2A*, adrenoceptor alpha 2A; *ANGPTL3*, angiopoietin-like 3; *APOF*, apolipoprotein F; *APOL1*, apolipoprotein L1; *ATF1*, activating transcription factor 1; *CAV1*, caveolin 1; *CAV2*, caveolin 2; *CRTC2*, CREB regulated transcription coactivator 2; *DBT*, dihydrolipoamide branched chain transacylase E2; *DHCR24*, 24-dehydrocholesterol reductase; *DICER1*, dicer 1 ribonuclease type III; *ELOVL1*, ELOVL fatty acid elongase 1; *ELOVL5*, ELOVL fatty acid elongase 5; *FABP3*, fatty acid binding protein 3; *FADS1*, fatty acid desaturase 1; *FOXO3*, forkhead box O3; *GALNT2*, UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2; *GLP1R*, glucagon-like peptide 1 receptor; *GPAM*, glycerol-3-phosphate acyltransferase; *IDH1*, isocitrate dehydrogenase 1; *IGF1*, insulin-like growth factor 1; Inh-30c, inhibitor of miR-30c; Inh-192, inhibitor of miR-192; *INSIG1*, insulin induced gene 1; *INSIG2*, insulin induced gene 2; *INSR*, insulin receptor; *IRS1*, insulin receptor substrate 1; *IRS2*, insulin receptor substrate 2; *LCLAT1*, lysocardiolipin acyltransferase 1; *LDLR*, low density lipoprotein receptor; *LHX8*, LIM homeobox 8; *LIN28B*, lin-28 homolog B (Caenorhabditis elegans); *LPGAT1*, lysophosphatidylglycerol acyltransferase 1; *LPIN1*, lipin 1; *MTTP*, microsomal triglyceride transfer protein; *MYLIP*, myosin regulatory light chain interacting protein; *NCOA3*, nuclear receptor coactivator 3; *NCOR1*, nuclear receptor corepressor 1; *NCOR2*, nuclear receptor corepressor 2; *NEDD4*, neural precursor cell expressed, developmentally down-regulated 4; *NFIB*, nuclear factor I/B; *OSBPL3*, oxysterol binding protein-like 3; *PPARA*, peroxisome proliferator-activated receptor α; *PPARD*, peroxisome proliferator-activated receptor delta; *PPARGC1A*, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; *PPARGC1B*, peroxisome proliferator-activated receptor gamma coactivator 1 beta; *PPP1R3D*, protein phosphatase 1 regulatory subunit 3D; *PRKAA2*, protein kinase AMP-activated alpha 2 catalytic subunit; *PTRF*, polymerase I and transcript release factor; RARB, retinoic acid receptor β; RARG, retinoic acid receptor γ; *RFX6*, regulatory factor X6; *RICTOR*, RPTOR independent companion of MTOR complex 2; *SIRT1*, sirtuin 1; *SLC27A4*, solute carrier family 27 member 4; *SLC36A1*, solute carrier family 36 member 1; *SREBF1*, sterol regulatory element binding transcription factor 1; *VLDLR*, very low density lipoprotein receptor.

resulted in decreased or increased accumulation of TGs, supporting a role for it in enterocyte lipid metabolism.

miR-192 targets predicted genes regulating lipid metabolism. In contrast to miR-30c, miR-192 expression was highly expressed in the intestine and liver (**Supplemental Fig. 4**). The high expression of miR-192 also occurred in tissues related to nutrient absorption (e.g., stomach, intestines, and liver). Target prediction analysis of miR-192 yielded >3000 mRNAs (**Supplemental Table 4**). Pathway analysis of predicted targets indicated that miR-192 may regulate processes related to cancer, focal adhesion, endocytosis, insulin signaling, and lipid metabolism (data not shown). Interestingly, this analysis showed that ~300 predicted targets are involved in primary metabolic processes (**Supplemental Fig. 5***A*). Further bioinformatic analysis of these predicted targets suggested that miR-192 is also a strong candidate to regulate lipid metabolism (Supplemental Fig. *5B*).

In addition, we performed a literature-based analysis on these predicted targets, and from this we selected a list of target genes for further validation (**Supplemental Table 5**). A subsequent protein-protein interaction analysis confirmed that many of these genes interact with one another, warranting their further

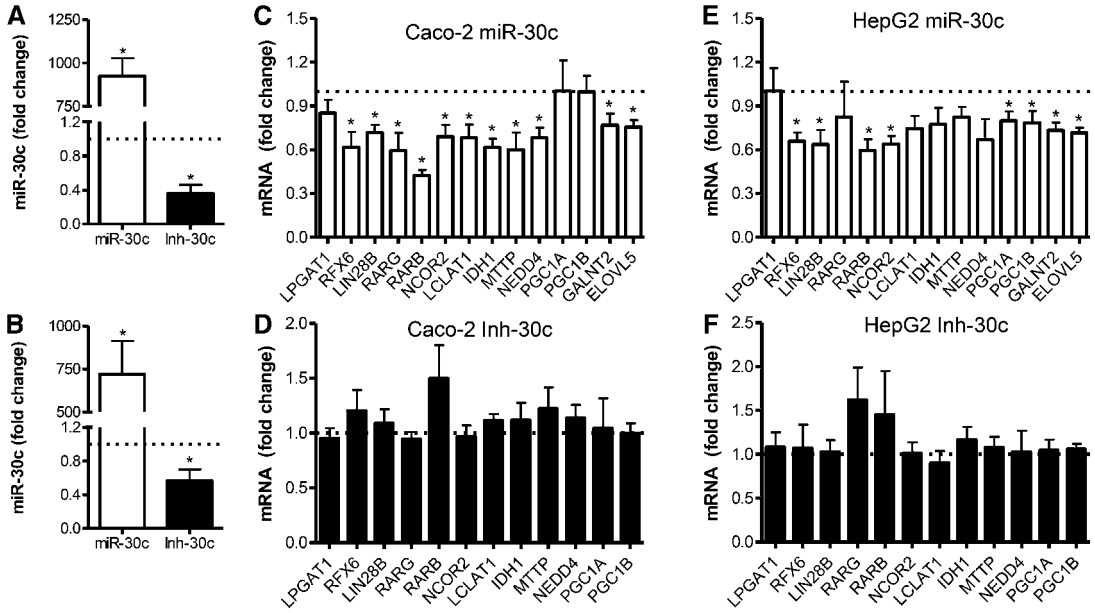

FIGURE 4 miR-30c regulates the mRNA expression of several genes related to lipid metabolism in enterocytes and in hepatocytes. Relative miRNA levels of miR-30c expression in Caco-2 cells transfected for 48 h with the miRNA mimic miR-30c or the miRNA inhibitor oligonucleotide miR-30c (*A*) or HepG2 cells (*B*). qRT-PCR analysis of predicted targets of miR-30c in Caco-2 cells and HepG2 cells treated with the miRNA mimic of miR-30c (*C, E*) or the miRNA inhibitor of miR-30c (*D, F*). Values are means ± SEMs; $n \geq 3$ independent experiments. *Different from control, $P < 0.05$. *ELOVL5,* ELOVL fatty acid elongase 5; *GALNT2,* UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2; *IDH1,* isocitrate dehydrogenase 1; Inh-30c, inhibitor of miR-30c; *LCLAT1,* lysocardiolipin acyltransferase 1; *LIN28B,* lin-28 homolog B; LPGAT1, lysophosphatidylglycerol acyltransferase 1; miRNA, microRNA; *MTTP,* microsomal triglyceride transfer protein; *NCOR2,* nuclear receptor corepressor 2; *NEDD4,* neural precursor cell expressed, developmentally down-regulated 4; *PGC1A,* peroxisome proliferator-activated receptor gamma coactivator 1 alpha; *PGC1B,* peroxisome proliferator-activated receptor gamma coactivator 1 beta; *RARB,* retinoic acid receptor β; *RARG,* retinoic acid receptor gamma; *RFX6,* regulatory factor X6.

validation (Fig. 3C). Moreover, overexpressing or endogenously inhibiting miR-192 resulted in changes in total TG accumulation in Caco-2 cells exposed to oleic lipid micelles (Fig. 3*D*), which also suggests its role in enterocyte lipid metabolism.

miR-30c and miR-192 target genes related to lipid metabolism: validation of mRNA expression of selected miRNA target genes. To identify the true targets of these miRNAs, we first evaluated by qRT-PCR the effects of overexpression of either miR-30c (**Fig. 4***A*, *B*) or miR-192 (**Fig. 5***A*, *B*) on mRNA levels of their predicted target genes. Transfection of Caco-2 cells with miR-30c significantly inhibited the mRNA expression of regulatory factor X6 (*RFX6*), lin-28 homolog B (*LIN28B*), retinoic acid receptor gamma (*RARG*), retinoic acid receptor beta (*RARB*), nuclear receptor corepressor 2 (*NCOR2*), lysocardiolipin acyltransferase 1 (*LCLAT1*), isocitrate dehydrogenase 1 (*IDH1*), *MTTP*, neural precursor cell expressed developmentally down-regulated 4 (*NEDD4*), UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 2 (*GALNT2*), ELOVL fatty acid elongase 5 (*ELOVL5*) and adrenoceptor alpha 2A (*ADRA2A*) (Fig. 4C, **Supplemental Fig. 6***A*). Other predicted target genes did not change significantly (Fig. 4C, Supplemental Fig. 6*A*). The endogenous inhibition of miR-30c using anti-miR-30c oligonucleotides did not exhibit significant changes on selected genes (Fig. 4*D*). To confirm these data, we also evaluated the expression of these genes in HepG2 cells. Transfection of HepG2 cells with miR-30c also inhibited the mRNA expression of *RFX6, LIN28B, RARB, NCOR2, GALNT2,* and *ELOVL5* (Fig. 4*E*). In contrast to what we observed in Caco-2 cells, miR-30c repressed the expression of peroxisome proliferator-activated receptor gamma coactivator 1 α (*PGC1α*) and peroxisome proliferator-activated receptor gamma coactivator 1 β (*PGC1β*) in HepG2 cells, without modifying the mRNA expression of *LCLAT1*. Similar to Caco-2 cells, other predicted target genes were not repressed in HepG2 cells (Supplemental Fig. 6*B*). The endogenous inhibition of miR-30c in this cell line also showed no changes on selected genes (Fig. 4*F*).

The overexpression of miR-192 in Caco-2 cells resulted in an inhibition of the mRNA expression of caveolin 1 (*CAV1*), *DICER*, ELOVL fatty acid elongase 1 (*ELOVL1*), *ELOVL5*, very low density lipoprotein receptor (*VLDLR*), fatty acid binding protein 3 (*FABP3*), caveolin 2 (*CAV2*), dihydrolipoamide branched chain transacylase E2 (*DBT*), and insulin-like growth factor 1 (*IGF1*) (Fig. 5*C*). Other predicted target genes were not significantly modified (Fig. 5*C*, **Supplemental Fig. 7***A*). The inhibition of miR-192 increased the mRNA expression of *CAV1*, *DICER*, and *FABP3*, and there was a trend to increase the expression of ATP-binding cassette subfamily G (white) member 4 (*ABCG4*) ($P = 0.062$) (Fig. 5*D*). To further validate these data, we also transfected HepG2 cells with an miR-192 mimic and an anti-miR-192. As with Caco-2 cells, overexpression of miR-192 in this cell type repressed the basal expression of *CAV1, DICER1, ELOVL1, ELOVL5, VLDLR, CAV2,* and *DBT.* Other predicted target genes that were repressed in this cell type, although not in Caco-2, were peroxisome proliferator-activated receptor alpha (*PPARA*), activating transcription factor 1 (*ATF1*), CREB regulated transcription coactivator 2 (*CRTC2*), and apolipoprotein L1 (*APOL1*) (Fig. 5*E*, Supplemental Fig. 7*B*). As with Caco-2 cells, we also inhibited the endogenous miR-192 in HepG2 cells (Fig. 5*F*). We note that, in contrast to what we observed for miR-30c, the inhibition of endogenous miR-192 was ~25% lower after using anti-miR oligonucleotides, probably because of the high expression of this miRNA in both cell lines (Fig. 5*A*, *B*). However, this inhibition of miR-192 in HepG2 resulted in increased expression of *CAV1* (Fig. 5*F*). Our findings suggest

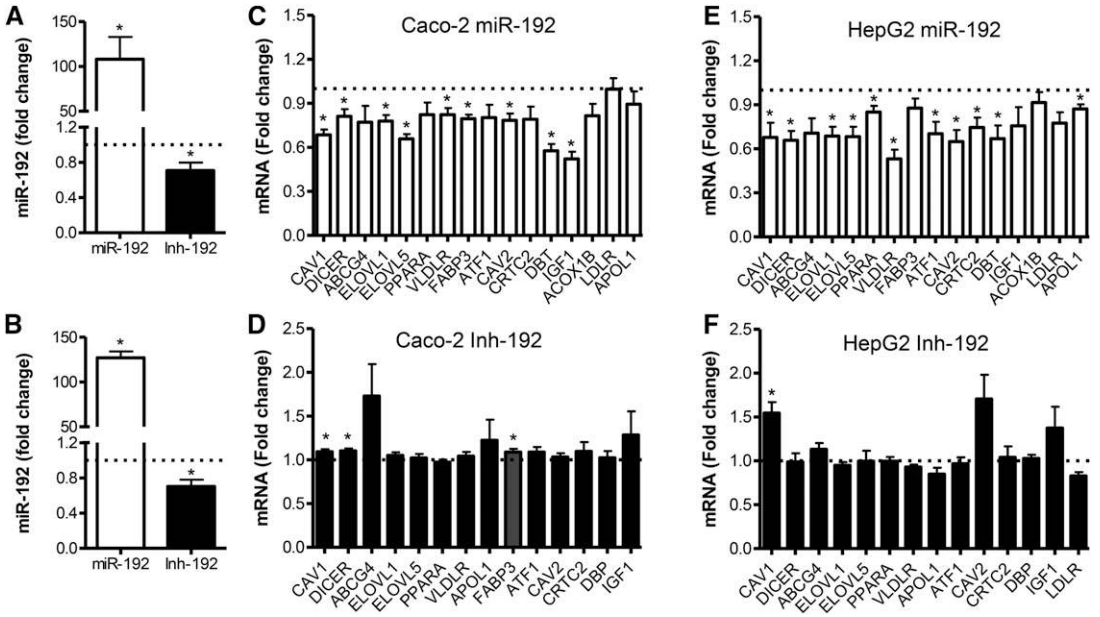

FIGURE 5 miR-192 regulates the mRNA expression of several genes related to lipid metabolism in enterocytes and in hepatocytes. Relative miRNA levels of miR-192 expression in Caco-2 cells transfected for 48 h with the miRNA mimic miR-192 or the miRNA inhibitor oligonucleotide miR-192 in Caco-2 cells (*A*) or HepG2 cells (*B*). qRT-PCR analysis of predicted targets of miR-192 in Caco-2 cells and HepG2 cells treated with the miRNA mimic miR-192 (*C*, *E*) or the miRNA inhibitor of miR-192 (*D*, *F*). Values are means ± SEMs; $n \geq 3$ independent experiments. *Different from control, $P < 0.05$. ABCG4, ATP-binding cassette subfamily G (white) member 4; *ACOX1B*, acyl-CoA oxidase 1; *APOL1*, apolipoprotein L1; *ATF1*, activating transcription factor 1; CAV1, caveolin 1; CAV2, caveolin 2; *CRTC2*, CREB regulated transcription coactivator 2; *DBT*, dihydrolipoamide branched chain transacylase E2; DICER, dicer 1 ribonuclease type III; *ELOVL1*, ELOVL fatty acid elongase 1; *ELOVL5*, ELOVL fatty acid elongase 5; *FABP3*, fatty acid binding protein 3; *IGF1*, insulin-like growth factor 1; Inh-192, inhibitor of miR-192; *LDLR*, low density lipoprotein receptor; miRNA, microRNA; *PPARA*, peroxisome proliferator-activated receptor alpha; *VLDLR*, very low density lipoprotein receptor.

that miR-192 might also play important roles in hepatocyte lipid metabolism.

miR-30c and miR-192 mediate some of the metabolic actions of DHA. Not only does miR-30c repress the enterocytic mRNA expression of NCOR2 (Fig. 4*C*, *E*), but it also represses its protein expression (**Fig. 6***A*). Moreover, endogenous inhibition of miR-30c resulted in an increased protein expression of NCOR2 (Fig. 6*C*). Similar results were obtained in HepG2 cells (Fig. 6*B*, *D*). Overexpression of miR-30c in both Caco-2 and HepG2 cells resulted in a clear decrease in IDH1 protein amounts (Fig. 6*A*, *B*), whereas the endogenous inhibition of this miRNA increased its protein expression (Fig. 6C, *D*). In agreement with previous studies (19), we confirm that LIN28B is a target of miR-30c, because both its overexpression and inhibition resulted in decreased and increased protein expression, respectively, in both Caco-2 and HepG2 cells (Fig. 6*A*–*D*).

Overexpression of miR-192 in Caco-2 cells resulted in a reduced expression of CAV1 mRNA and protein (Fig. 6*E*). Conversely, the endogenous inhibition of miR-192 resulted in an increased expression of CAV1 (Fig. 6*G*). The low protein amounts of CAV1 in HepG2 cells does not allow us to replicate these data in this cell line. DICER is also a target of miR-192: overexpression or inhibition of miR-192 in Caco-2 and HepG2 cells resulted in a decrease or an increase in its protein amounts, respectively (Fig. 6*E*–*G*).

Because ABCG4 plays a major role in lipid homeostasis (20), we evaluated ABCG4 protein expression in the astrocytoma cell line 13121N1. Overexpression of miR-192 in this cell line resulted in a clearly reduced expression of the ABCG4 protein (Fig. 6*F*, lower panel). However, the effects of miR-192 inhibition in the 1321N1 cell line were less pronounced (Fig. 6*H*).

To investigate the direct regulation of 1 of these miRNA-mediated targets of DHA, we first evaluated the expression of the majority of qRT-PCR–validated targets of either miR-30c or miR-192. As shown in Fig. 6*I*, differentiated Caco-2 cells treated with DHA micelles clearly repressed the expression of most, yet not all, possible mRNAs targets of miR-30c and miR-192, indicating that their effect may either be mediated by other miRNAs (Fig. 2*A*) or be a consequence of other regulatory processes. To further confirm that these genes are a DHA target (via either miR-30c or miR-192), we treated Caco-2 cells with DHA micelles in the presence or absence of the endogenous inhibitor oligonucleotide of either miR-30c or -192. As shown in Fig. 6*J*, incubation of Caco-2 cells with the inhibitor of miR-30c resulted in a de-repression of *RFX6* mRNA expression induced by DHA treatment. Also, *RARB* repression by DHA was partially abrogated by the inhibition of miR-30c (Fig. 6*K*), suggesting that both *RFX6* and *RARB* may be miR-30c–mediated effectors of DHA.

To further confirm that the DHA-dependent changes in *RFX6* and *RARB* expression are mediated directly by miRNAs, we examined the DHA-dependent regulation of reporter gene constructs that contain these 3′UTRs. For this, we generated a luciferase reporter construct of the human 3′UTR of *IDH1*, *RARB*, *CAV1*, *NCOR2*, and *RFX6*. miR-30c markedly repressed the activity of *IDH1*, *RARB*, *NCOR2*, and *RFX6* but not that of *CAV1* 3′UTR luciferase constructs (**Fig. 7***A*). miR-192 repressed the activity of the *CAV1* 3′UTR luciferase construct (Fig. 7*B*). Unexpectedly, we observed that miR-192 also repressed *RFX6* 3′UTR luciferase construct activity (Supplemental Table 4). Finally, we transfected Caco-2 cells with the *RFX6* and *RARB* 3′UTR luciferase constructs and exposed them to either control, oleic, or DHA micelles for 24 h. DHA

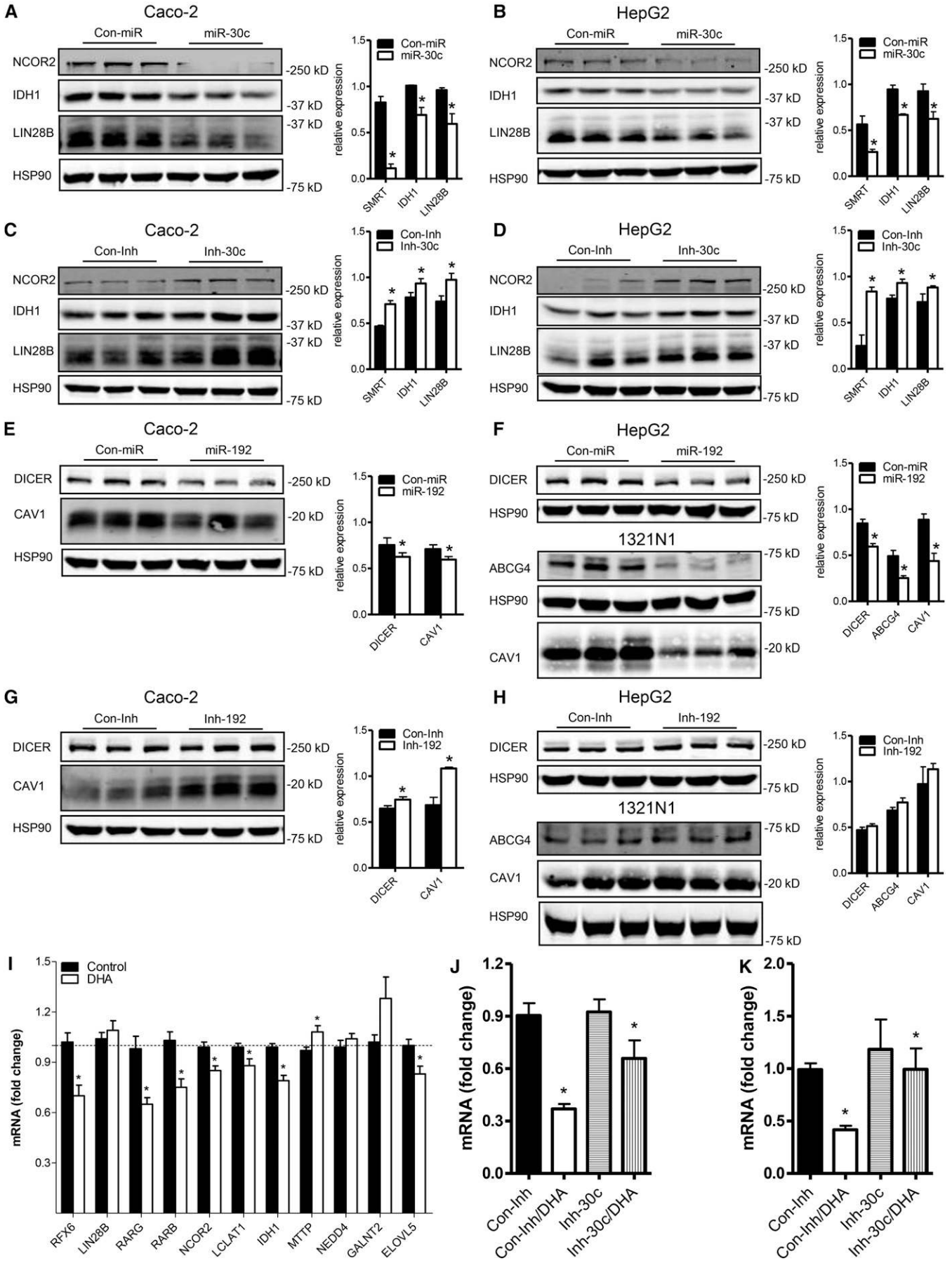

FIGURE 6 Novel molecular DHA targets related to lipid metabolism and cancer. (*A–H*) Western blots of protein targets of miR-30c or miR-192. Caco-2 or HepG2 cells were transfected with a control mimic or the miRNA mimic miR-30c (*A, B*) or miR-192 (*E, F*) for 48 h before protein analysis by Western blot. Cells were transfected with a control inhibitor or the oligonucleotide inhibitor of miR-30c (*C, D*) or the inhibitor of miR-192 (*G, H*) for 48 h before protein analysis. Blots are representative. (*I*) Relative expression of novel targets of DHA. Caco-2 cells were differentiated and treated for 24 h with vehicle or DHA, and gene expression was assayed by qRT-PCR. (*J, K*) DHA modulates gene expression through miRNAs. Caco-2 cells were transfected with either a control inhibitor or the inhibitor of miR-30c in the presence or absence of DHA. *RFX6* (*J*) or *RARB* (*K*) was assayed by qRT-PCR. Values are means ± SEMs; $n \geq 3$ independent experiments. (*A–I*) *Different from control, $P < 0.05$. (*J, K*) *Different from control in the absence or presence of DHA, $P < 0.05$. ABCG4, ATP-binding cassette subfamily G (white) member 4; CAV1, caveolin 1; Con-Inh, control inhibitor; Con-miR, control mimic; DICER, dicer 1 ribonuclease type III; *ELOVL5*, ELOVL fatty acid elongase 5; *GALNT2*, UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2; HSP90, heat shock protein 90; IDH1, isocitrate dehydrogenase 1; Inh-30c, inhibitor of miR-30c; Inh-192, inhibitor of miR-192; LIN28B, lin-28 homolog B; *LCLAT1*, lysocardiolipin acyltransferase 1; miR-30c, miR-30c mimic; miR-192, miR-192 mimic; miRNA, microRNA; *MTTP*, microsomal triglyceride transfer protein; NCOR2, nuclear receptor corepressor 2; *NEDD4*, neural precursor cell expressed, developmentally down-regulated 4; *RARB*, retinoic acid receptor β; *RARG*, retinoic acid receptor gamma; *RFX6*, regulatory factor X6.

micelles, but not oleic acid, repressed *RFX6* 3′UTR luciferase construct activity but not that of *RARB* (Fig. 7*C*), thus clearly indicating that DHA-dependent change in *RFX6* expression could be mediated by either miR-30c, miR-192, or both.

Discussion

Elucidating the molecular signaling pathways and effectors of ω-3 PUFAs is important to reinforce the advice of consuming adequate amounts of fish and/or the use of dietary supplements containing fish oil. Here, we report that DHA modulates the expression of enterocyte miRNAs that target not only several key genes related to lipid metabolism but also genes important to different aspects of cancer biology.

The miRNA 30 family, including miR-30c, is required for vertebrate hepatobiliary development and has been implicated in the pathology of breast tumors (21), endothelial cell function (22), vascular calcification, and myocardial matrix remodeling. Notably, in vitro and in vivo research indicates that ω-3 PUFAs affect the expression of different miRNAs in adipose tissue (23), gliomas (9), breast cancer cell lines (10), or inflammatory cells [reviewed in (3)] with potential physiologic consequences. Genes controlled by miR-30c include twinfilin actin-binding protein 1 (*TWF1*), runt-related transcription factor 2 (*RUNX2*), DNA-damage-inducible transcript 4 (*DDIT4*), delta-like 4 (*DLL4*), serpin peptidase inhibitor clade E member 1 (*SERPINE1*), Kirsten rat sarcoma viral oncogene homolog (*KRAS*), trichorhinophalangeal syndrome I (*TRPS1*), metastasis associated 1 (*MTA1*), and connective tissue growth factor (*CTGF*). There is also evidence that miR-30c promotes adipocyte differentiation by targeting *SERPINE1* and *ACVR1* (24), pointing to their role in lipid metabolism. In this study, we show that, in enterocytes, miR-30c is an important regulator of lipid metabolism by targeting several other genes, including *LPGAT1, RFX6, LIN28B, RARG, RARB, NCOR2, LCLAT1, IDH1, MTTP, NEDD4, GALNT2, ELOVL5*, forkhead box O3 (*FOXO3*), and *ADRA2A*, many of which were also regulated by miR-30c in HepG2 cells. *NCOR2* is a key player in the repression of inflammatory gene transcription (25) and metabolic disease associated with diet-induced obesity (26). *IDH1* interconverts isocitrate to α-ketoglutarate (2-oxoglutarate), which is a metabolic step in the citric acid cycle, a central component of glucose metabolism. Mutations in this enzyme are often found in human glioblastoma (27) and acute myeloid leukemia (28). The RNA binding protein LIN28B is overexpressed in a wide variety of tumors (29); by blocking the let-7 tumor suppressor miRNA, LIN28B not only promotes malignancy but may also regulate glucose metabolism (30). RFX6 is a novel transcription factor with a major role in the regulation of islet formation and insulin production (31). Overexpression of RFX6 has been shown to increase gastric inhibitory polypeptide expression, which may ultimately lead to high-fat-diet–induced obesity (32). RARs mediate multiple biologic processes, including morphogenesis, development, and cell differentiation. Apart from their documented function in cancer biology, it has been shown that RARB expression may be modulated by dietary patterns and may also be involved in lipid metabolism (33).

The miRNA miR-192 was originally found to play key roles in renal physiopathology, by repressing targets such as zinc finger E-box binding homeobox 1 (*ZEB1*), zinc finger E-box binding homeobox 2 (*ZEB2*), and WNK lysine deficient protein kinase 1 (*WNK1*) (34). Its expression is regulated in part by transforming growth factor β 1 (*TGFB1*) and p53 (34). In addition, miR-192 was also found to be deregulated in certain carcinomas, where its target genes include retinoblastoma 1 (*RB1*), activated leukocyte cell adhesion molecule (*ALCAM*), dihydrofolate

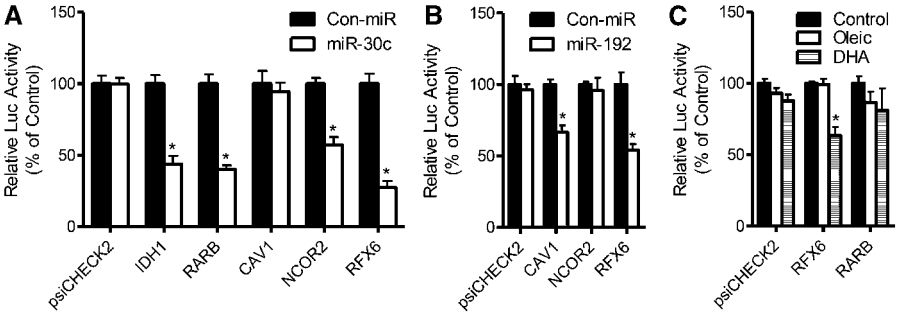

FIGURE 7 DHA-dependent change in *RFX6* expression is mediated by miRNAs. (*A, B*) Luciferase (Luc) activity of HEK293 cells after cotransfection with miR-30c, miR-192, or control miR and different human 3′UTR constructs of selected target genes. (*C*) Luciferase activity of Caco-2 cells after transfection with the human 3′UTR constructs of *RFX6*, *RARB*, or empty construct (psiCHECK2) and incubated in the presence of oleic acid or DHA lipid micelles. Results are expressed as percentages of the 3′UTR activity of the control. Values are means ± SEMs; $n \geq 3$ independent experiments. *Different from control, $P < 0.005$. CAV1, caveolin 1; Con-miR, control mimic; IDH1, isocitrate dehydrogenase 1; miR-30c, miR-30c mimic; miR-192, miR-192 mimic; miRNA, microRNA; NCOR2, nuclear receptor corepressor 2; RARB, retinoic acid receptor β; RFX6, regulatory factor X6; UTR, untranslated region.

reductase (*DHFR*), and activin A receptor type IIB (*ACVR2B*) (35). Moreover, miR-192 also regulates the *Period* gene family and the circadian gene clock circadian regulator (*CLOCK*) and may also be implicated in β-cell function [e.g., in diabetes (36)] or in adipose tissue physiology [e.g., in obesity (15)]. We now reveal novel targets related to lipid metabolism that can be modulated by miR-192 (i.e., *CAV1, ABCG4, ELOVL1, ELOVL5, PPARA, VLDLR*, *FABP3, ATF1, CAV2, CRTC2, DBT,* and *IGF1*). CAV1 is the major membrane protein of caveolae and plays a major role in lipid metabolism (37). ABCG4 regulates cellular cholesterol homeostasis (38). Even though ABCG4 is mainly expressed in the adult central nervous system, it is highly yet transiently expressed both in hematopoietic cells and in enterocytes of the developing intestine (39).

The exact molecular mechanism by which DHA modulates the expression of these miRNAs is not known. However, its effect on these miRNAs seems to be specific, because the other FA tested did not exert the same effects. In combination with previous work on FAs and miRNAs (9,11,23), the data here provide a framework for exploring which miRNAs could be modulated specifically by FAs.

The research on ω-3 DHA largely focuses on the molecular mechanisms underlying its hypotriglyceridemic effects due to increases in apo B degradation or by inducing autophagy (3). In intestinal epithelial cells, DHA reduces apo B secretion and the expression of NPC1-like 1 (NPC1L1) (40), in turn producing lipid-lowering effects. In this study, we demonstrate that, in enterocytes, DHA is able to differentially modulate the expression of several miRNAs, including miR-30c and miR-192, which subsequently modulate the expression of genes involved in lipid metabolism. However, we cannot rule out the possibility that other mechanisms governed by noncoding RNAs could be involved in this process, because competing endogenous RNAs, long-noncoding RNAs, and circular RNAs are involved in the interplay regulation of gene expression of our entire genome (41).

An important finding of this study is the identification of novel miR-30c and miR-192 targets in relation to lipid metabolism. Some of these gene targets are also implicated in cancer biology, corroborating the hypothesis that adequate ω-3 PUFA is chemopreventive (42).

In agreement with our results, Soh et al. (43) recently confirmed that miR-30c targets MTTP, reducing apo B secretion in the liver. In addition, miR-30c also reduces lipid synthesis, probably by targeting other genes related to lipid metabolism, including lysophosphatidylglycerol acyltransferase 1 (*LPGAT1*). The authors suggested that miR-30c mimics might have therapeutic potential as antihyperlipidemic and antiatherosclerosis agents (43). Our data suggest that DHA appears to be a promising alternative to increase endogenous miR-30c amounts and thus reduce lipid concentrations. Indeed, overexpression of miR-30c in the liver of individuals consuming DHA could exert its well-characterized hypotriglyceridemic effect by 2 different mechanisms: *1*) by the well-recognized effects of DHA on apo B degradation and *2*) through miR-30c. Indeed, in terms of therapy, miRNAs have recently attracted considerable attention because of their ability to target different genes putatively involved in numerous diseases. Anti-miRNA pharmacology is rapidly evolving as a tool to regulate miRNA levels in vivo, and clinical trials are being performed (44). However, the use of adeno-associated viruses as a promising medium to deliver miRNA mimics is still in its infancy, and therefore the use of a safe molecule such as DHA might theoretically represent a novel approach to modulate the expression of certain miRNAs.

A limitation of this study is that we only explored the effects of DHA on 2 specific cell types: epithelial enterocytes and hepatocytes. miRNAs are tightly regulated to allow the shaping of gene expression in a temporally restrained and tissue-specific manner; therefore, it remains to be determined whether the supplementation of DHA to other different cell types (e.g., adipocytes) could differentially modulate the expression of the same miRNAs.

In summary, our data add novel evidence to the premise that essential FAs "fine tune" gene expression (3) in a temporally restrained and tissue-specific manner, through the modulation of miRNAs. In addition, our data suggest that the modulation of the expression of miRNAs through a pharma-nutrition approach might be a viable alternative or adjunct to current pharmacologic therapy targeting endogenous miRNAs. The precise mechanisms underlying the beneficial effects on lipid metabolism and cancer incidence observed in populations with high DHA and ω-3 PUFA consumption deserve further investigation.

Acknowledgments

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