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ELOVL2 overexpression enhances triacylglycerol synthesis in 3T3-L1 and F442A cells

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Abstract Elongation of very long-chain fatty acids (ELOVL) members were overexpressed in two preadipocyte cell lines, ELOVL2 and ELOVL3 in 3T3-L1 cells, and ELOVL1–3 in F442A cells. Cells overexpressing ELOVL2, whose preferred substrates are arachidonic acid (AA, C20:4*n*−6) and eicosapentaenoic acid (EPA, C20:5*n*−3), showed an enhanced triacylglycerol (TAG) synthesis and subsequent accumulation of lipid droplets. Incorporation of fatty acid (FA) but not of glucose into TAG was enhanced by ELOVL2-overexpression. Two lipogenic genes encoding diacylglycerol acyltransferase-2 (DGAT2) and fatty acid-binding protein-4 (FABP4, aP2) were induced in ELOVL2-overexpressing cells, whereas no such effect was seen on the fatty acid synthase (FAS) gene.
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Keywords: Elovl; Polyunsaturated fatty acid; Fatty acid; Triacylglycerol; Lipogenesis; Elongation

1. Introduction

Fatty acids (FAs), which are exogenously incorporated or endogenously synthesized/elongated and their CoA esters are essential molecules in cells, e.g. as a fuel for energy production, as membrane components, as signaling molecules, and as regulators of various transcriptional factors. In mammals, *de novo* FA synthesis up to palmitic acid (C16:0) is carried out in the cytosol by fatty acid synthase (FAS). Further FA-elongation activity making long chain- and very long chain-fatty acids (VLCFAs; ⩾C18) resides in the endoplasmic reticulum (ER), peroxisomes [1,2], and in mitochondria [3]. In the ER, a family of enzymes, elongation of very long-chain fatty acids (ELOVL; gene name *Elovl*), catalyze the first rate-limiting condensation step of the sequential VLCFA synthesis reaction [4,5].

Seven ELOVL members have been identified in mice and humans, each having a respective gene locus, a unique tissue specific expression pattern with overlapping substrate specificity [5–8]. Saturated and monounsaturated FAs are preferred substrates of ELOVL1, ELOVL3 and ELOVL6 [8–11], whereas ELOVL2, ELOVL4 and ELOVL5 use polyunsaturated fatty acids (PUFAs) primarily [10,12,13].

Saturated FA biosynthesis uses only FAS and ELOVL, whereas monounsaturated FA synthesis requires an additional desaturation reaction either by Δ9- or Δ6-desaturase [14,15]. Multiple reactions are included in PUFA synthesis pathways such as elongations (by ELOVL2, 4 and 5) and desaturations (by Δ5- and Δ6-desaturase), in that the existence of primer substrates such as linoleic acid (C18:2*n*−6) and α-linolenic acid (C18:3*n*−3) is a prerequisite (essential FAs). Furthermore, one round of peroxisomal β-oxidation is required in mammals to obtain docosahexaenoic acid (DHA, C22:6*n*−6) and C22:5*n*−6 [16]. Because vertebrates lack ω3(*n*−3) desaturase, *n*−3 and *n*−6 PUFA synthesis pathways are not interchangeable [15].

Both *n*−6 and *n*−3 PUFAs are a component of phospholipid thus influence the structure and function of the membrane [17]. The membrane of the adipose tissue, liver, muscle and the kidney is rich in *n*−6 PUFAs, whereas that of the brain, testis and of retina contain *n*−3 PUFAs predominantly [18].

The *n*−6 to *n*−3 ratio affects the production of an eicosanoid. An *n*−6 PUFA, arachidonic acid (AA, C20:4*n*−6) is the precursor of the highly inflammatory prostaglandin E2, whereas *n*−3 PUFAs such as eicosapentaenoic acid (EPA, C20:5*n*−3) become the low inflammatory prostaglandin E3 [19]. In the liver, *n*−3 PUFAs are known to catabolize FA by functioning as ligands for PPAR-α [20]. Despite the extensive knowledge on other enzymes, few reports are available as to the function of ELOVL members in PUFA synthesis pathways. The present study shows the first evidence that overexpression of a PUFA elongase ELOVL2 in preadipocytes enhances triacylglycerol (TAG) synthesis and accumulation of fat droplets.

2. Materials and methods

2.1. Buffer solutions

The following buffer solutions were used. Elongation assay buffer; 50 mM sodium phosphate, 50 μM fatty acyl-CoA (Sigma or Avanti),

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Abbreviations: AA, arachidonic acid (C20:4*n*−6); BSA, bovine serum albumin; DEX, dexamethasone; DHA, docosahexaenoic acid (Δ-4,7,10,13,16,19-C22:6*n*−3); DMEM, Dulbecco's modified Eagle's medium; DGAT, diacylglycerol acyltransferase; ELOVL, elongation of very long-chain fatty acids; EPA, eicosapentaenoic acid (Δ-5,8,11,14,17-C20:5*n*−3); FA, fatty acid; FABP, fatty acid-binding protein; FAS, fatty acid synthase; IBMX, 3-isobutyl-1-methyl-xanthine; Oil-Red-O, [1-(*p*-xyrylazoxyrylazo)-2-naphthol]; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; RT, reverse transcriptase; RXR, retinoid X receptor; SREBP, sterol regulatory element-binding protein; TAG, triacylglycerol; VLCFA, very long-chain fatty acid

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1 mM NADPH, 5 μM rotenone and 20 μM FA-free bovine serum albumin (BSA, Roche) (pH 6.8): RNA extraction solution; 38% (v/v) phenol, 0.8 M guanidium thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate and 5% (v/v) glycerol: Hybridization solution; 5× NaCl/Cit, 50 mM sodium phosphate (pH 7.0), 7% (v/v) sodium dodecyl sulfate, 50% (v/v) formamide and 2% (w/v) Blocking reagent (Roche): lipid extraction solution; 66% (v/v) chloroform, 34% (v/v) methanol and 0.5 mM (w/v) butylated hydroxy toluene: FA labeling medium; Dulbecco's modified Eagle's medium (DMEM) (5.5 mM glucose), 250 μM palmitic acid, 0.1 μCi/ml [U-^{14}C] palmitic acid (Amersham) and 25 μM FA-free bovine serum albumin.

2.2. Cell culture and in vitro differentiation

3T3-L1, F442A, and NIH3T3 cells were obtained from the European Collection of Cell Cultures consortium. Dulbecco's modified Eagle's medium (DMEM, 25 mM glucose) supplemented with 10% (v/v) bovine calf serum iron-fortified (SAFC Biosciences), streptomycin (100 μg/ml) and penicillin (100 U/ml) was used as a medium. For differentiation, two days after confluence, cells were incubated with a differentiation medium containing DMEM (25 mM glucose), 10% (v/v) fetal bovine serum, antibiotics, 1 μM insulin, 1 μM dexamethasone (DEX), and 500 μM 3-isobutyl-1-methyl-xanthine (IBMX). After 48 h, cells were treated with the differentiation medium without IBMX and DEX. This was renewed every second day and continued until the end of each experiment.

2.3. Construction and creation of ELOVLs-expressing cells

The XhoI-cloning site of a mammalian expression vector pCXN2 [21] was converted to a NotI-site and used for Elovl-expression units. For Elovl1 and Elovl3, an NheI-site in the reported constructs [5,22] was modified to the NotI-site, and each cDNA flanked by two NotI-sites isolated by NotI. For Elovl2, a cDNA in pEMR1023 [8] was excised by SalI and BglII, and both ends converted to NotI-sites. The Elovl1–3 cDNA fragments obtained were ligated to modified pCXN2 as above. These constructs were introduced into 3T3-L1 and F442A cells using a calcium phosphate coprecipitation method, then stably-transfected cells selected for three weeks with 1.2 μM G418 (Invitrogen). Established cells were maintained in the presence of 0.3 μM G418, without reaching confluence (until ≈70%).

2.4. RNA analysis

Total RNA was prepared from cells using RNA isolation solution, following a procedure for TRIzol (Invitrogen). Digoxygenin-labeled cRNA probes were prepared [23] using the following constructs as templates. For fatty acyl-CoA binding protein-4 (FABP4; aP2), a cDNA prepared by reverse transcriptase (RT)-PCR (see Supplementary Table 1 for primers) was ligated to pGemT (Promega). For Elovl1 and Elovl3, constructs in pCIneo [5,21] were used. For Elovl2, a cDNA in pEMR1023 [8] was subcloned into pGemT. For diacylglycerol acyl transferase (DGAT)2 and fatty acid synthase (FAS), two expressed sequence tag clones (IMAGE5123807 and IMAGE4195432) were used. Northern hybridization was carried out using hybridization solution and probes, and the hybrid detected [23]. Results were recorded with a CCD-camera; LAS1000 (Fuji).

For RT-PCR, cDNA was synthesized from total RNA (2 μg) using MMLV-RT (Invitrogen) and an oligo-dT primer. PCR was performed as follows; 92 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. At the completion of 30, 33, 36, and 39 cycles, a portion of the product (10 μl) was separated on a 1.2% agarose gel.

2.5. Preparation of microsomes and FA elongation assay

Microsomes were prepared from cells as described [24]. FA elongation assay was carried out as follows. Microsomes (40 μg/assay) were pre-incubated for 3 min at 37 °C in elongation assay buffer and assays (200 μl/assay) initiated by adding malonyl-CoA (50 μM, with 3 dpm/pmol of [2-^{14}C] malonyl-CoA; Hartman Analytic). Reactions were performed within the range of linearity (up to 60 min). At each time point, reactions were terminated with 2.5 M potassium hydroxide in 20% (v/v) methanol (200 μl), followed by saponification for 60 min at 65 °C, then the products acidified by adding 6 N hydrochloric acid in 50% (v/v) methanol (200 μl). The free-FA released was recovered by three extractions with hexane (each 1 ml), and upper-phases were mixed with liquid scintillation fluid (each 5 ml) (Perkin Elmer), followed by scintillation counting (LS-6500, Beckman).

2.6. Lipid analysis

Cells were fixed for 15 min with a 3.7% (w/v) paraformaldehyde solution in PBS, stained for 30 min with 2.5 mM Oil-Red-O [1-(*p*-xyrylazoxyrylazo)-2-naphthol] in 50% (v/v) 2-propanol. Following two 10 min washings with 50% (v/v) 2-propanol, photographs were taken with LAS1000.

To analyze the incorporation of FA into TAG, cells grown in 6-well plates were incubated for 16 h in DMEM (5.5 mM glucose) without serum. Following two washings with 30 μM BSA (FA-free) in PBS, cells were incubated with FA labeling medium (2 ml/well). For glucose incorporation, the medium containing [U-^{14}C] glucose was used instead of [U-^{14}C] palmitic acid.

Total lipid was extracted as follows; cells resuspended in PBS (1 ml/well) were subjected to sonication (twice each for 10 s), and a portion of the lysate (100 μl) was used for determination of protein concentration [23]. The remaining 900 μl was mixed with lipid extraction solution (3.6 ml, twice each for 15 s) followed by centrifugation at $1000 \times g$ for 5 min. The separated lower phase was dried in a vacuum oven and dissolved in lipid extraction solution. Lipid equivalent to 500 μg of protein was separated on a TLC plate (silica gel 60, Merck) [25], and ^{14}C-labeled lipid was detected using a phosphorimager (BAS3000, Fuji). All lipids were visualized by spraying of the plate with 500 mM copper sulphate in 50% (v/v) methanol followed by baking at 150 °C for 20 min, then the image was recorded with LAS1000. The location of individual lipid species was verified using lipid standards (Sigma).

2.7. Data analysis

Results of Northern blotting and TLC were analyzed using programs, NIH image (NIH, USA) or Image gauge (Fuji). For statistics, one-way repeated-measures ANOVA and Tukey's post hoc test for multiple comparisons were used (Prism, Graphpad software); **, $P < 0.001$; *, $P < 0.05$.

3. Results

3.1. Functional expression of ELOVLs

To evaluate how respective ELOVL members affect cellular lipid metabolism, we created cell lines stably expressing ELOVL2 and ELOVL3 in 3T3-L1 cells, and ELOVL1–3 in F442A cells (Fig. 1). These two preadipocytes retain a potency to differentiate into adipocytes and are thus used to study cellular lipid metabolism [26].

Endogenous Elovl1–3 mRNAs were not detectable by Northern blotting in all the cells examined, therefore semi-quantitative RT-PCR was applied. In all cells, although the amount was low, Elovl2 and Elovl3 mRNAs were detectable, whereas a certain amount of Elovl1 mRNA was observed (Fig. 1A). The order of endogenous Elovl1–3 mRNA levels estimated was Elovl 1> Elovl 2≅ Elovl 3. In stably-transfected cells, Elovl mRNAs were detected by Northern blotting with the predicted length (≈1.4 kbp) (Fig. 1B). Using microsomes prepared from ELOVL2- and ELOVL3-expressing cells, FA-elongation activity was analyzed (Fig. 1C). With palmitoyl-CoA (C16:0-CoA), a substantial elongation activity was seen in vector-transfected 3T3-L1 cells (≈170 pmol/min/mg). There was no increased activity in ELOVLs-expressing cells. Rather, ELOVL2-expressing cells showed a diminished activity (Fig. 1C). The vector-transfected F442A cells had a lower palmitoyl-CoA elongation activity than 3T3-L1 cells (Fig. 1C and D). Increased palmitoyl-CoA elongation activity was seen in F442A cells expressing ELOVL2 and ELOVL3, confirming the functional expression of ELOVLs (Fig. 1D).

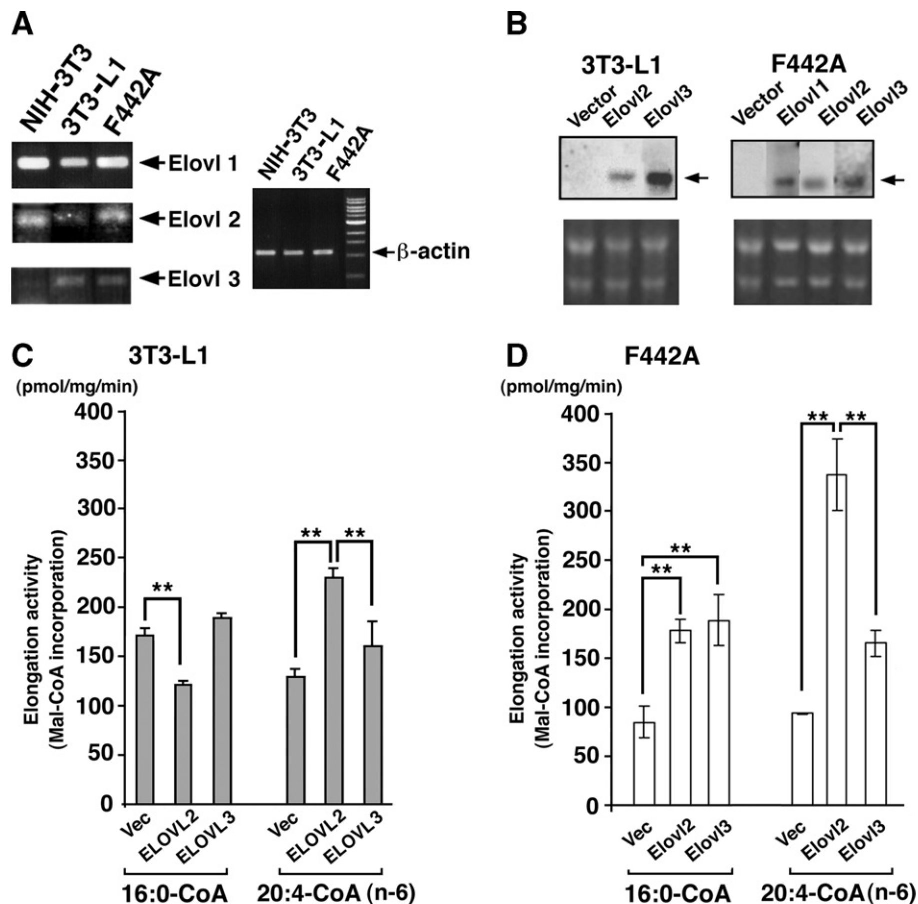

Fig. 1. Functional expression of ELOVLs in preadipocytes. (A) Endogenous Elovl1–3 mRNAs in 3T3-L1, F442A and parental NIH-3T3 cells detected by semi-quantitative RT-PCR. cDNAs were standardized for β-actin. (B) Elovl1–3 transcripts in stably transfected 3T3-L1 cells analyzed by Northern blotting. Arrows show positions of Elovl mRNAs (1.4 kbp). Mixed probes (Elovl1–3) were used. Data shown are combined photographs from different time exposures of the membrane. (C,D) FA elongation activity in microsomes from ELOVL2- and ELOVL3-expressing cells. (C) 3T3-L1-derived cells microsomes. (D) F442A-derived cells microsomes. Error bars represent the means ± S.D. of three different time points measured in duplicate; **, $P < 0.001$.

With arachidoyl-CoA (C20:4-CoA), both in 3T3-L1- and in F442A-derived cells, elongation activity was significantly increased by ELOVL2-overexpression (Fig. 1C).

3.2. ELOVL2 enhances accumulation of lipid droplets

Oil-Red-O staining showed a pronounced accumulation of lipid droplets in ELOVL2-expressing cells (Fig. 2A). In 3T3-L1 cells, differentiation was low in vector-transfected cells, however lipid accumulation was obvious in ELOVL2-expressing cells (Fig. 2A). In contrast, all F442A-derived cells differentiated moderately, in that ELOVL2-expressing cells nevertheless showed the highest accumulation of lipid droplets (Fig. 2A) and the TAG content (Fig. 2B and C). This enhanced TAG formation by ELOVL2 was continuous in the late stage of differentiation (on days 8 and 10) (Fig. 2D and E).

3.3. Utilization of FA and glucose for TAG synthesis

By adding [U-^{14}C] palmitic acid or [U-^{14}C] glucose into the media, their utilization for TAG synthesis was analyzed. ELOVL2-expressing cells incorporated [U-^{14}C] palmitic acid into TAG efficiently, both in the differentiated (Fig. 3A and C) and undifferentiated (Fig. 3B and D) states. With [U-^{14}C] glucose, although ^{14}C-labeled TAG was increased by time, the amount of ^{14}C-labeled TAG (at 10 h) was the same between the cell lines examined (Fig. 3E).

3.4. Analysis of mRNA levels for adipogenic genes

Whether the increased TAG synthesis by ELOVL2-overexpression coupled with lipogenic gene expression, the mRNA levels of three lipogenic genes, i.e. FABP4, FAS, and DGAT2, were examined by Northern blotting. A large amount of FABP4 mRNA was found in ELOVL2-expressing cells compared with other cells (Fig. 4). DGAT2 mRNA was also abundant in ELOVL2-expressing cells (Fig. 4). In contrast, there was no ELOVL2-dependent increase in the FAS (fatty acid synthase) mRNA level, but this was rather high in ELOVL3-expressing cells (Fig. 4).

4. Discussion

The present study demonstrated that excess ELOVL2 enhances TAG synthesis and loading of lipid in preadipocytes.

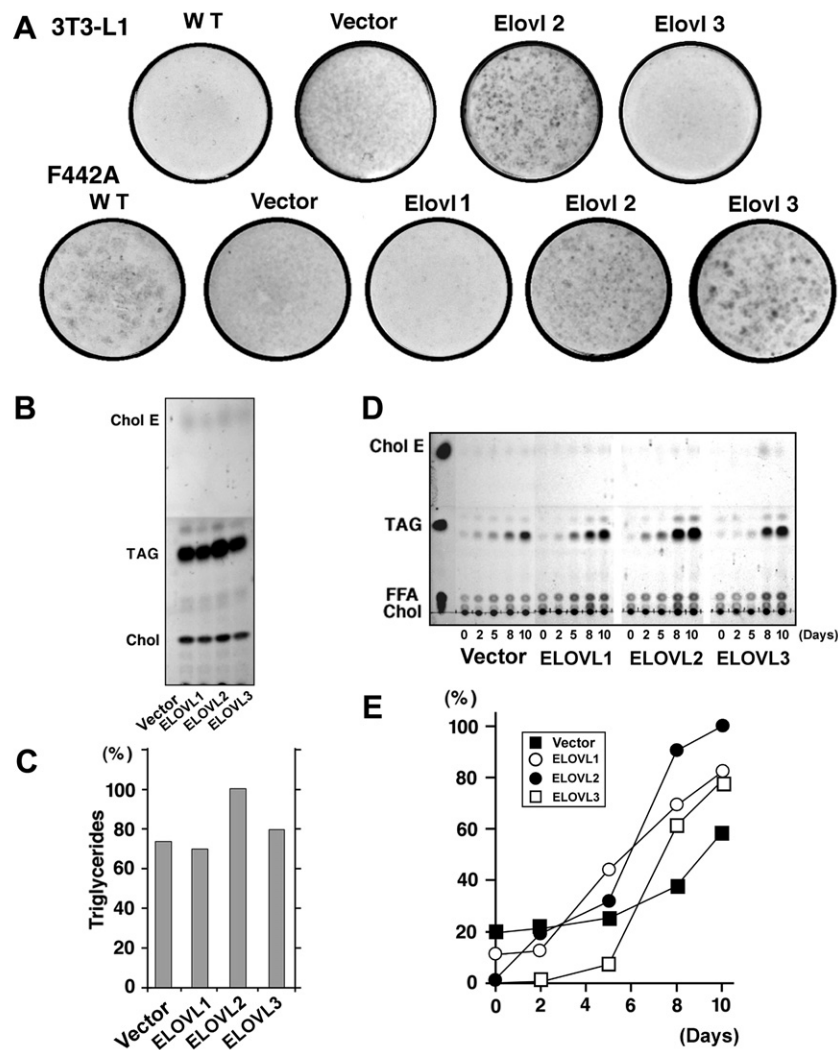

Fig. 2. ELOVL2-overexpression enhances accumulation of lipid droplets and the TAG content in 3T3-L1 and F442A cells. (A) ELOVLs-expressing cells and their wild-type (WT) cells were differentiated for 14 days, and stained with Oil-Red-O. (B–E) F442A cells transfected with ELOVL1–3 were differentiated for 10 days and lipid resolved by TLC. (B) Lipid samples were prepared at the late stage of differentiation (10 days) and separated by TLC. Each lane contains total lipid equivalent to 500 μg of protein. (C) The TAG contents in (B) are shown as a percentage of ELOVL2, after standardization for the cholesterol spot. (D) During differentiation, total lipid samples were sequentially taken (on days 0, 2, 5, 8, and 10) and separated by TLC. (E) The relative TAG content in (D) is expressed as a percentage of the maximum value. Chol, cholesterol; Chol E, cholesterol ester.

With our experimental condition, differentiation was less pronounced in 3T3-L1 cells, however this led us to identify the effect of ELOVL2-overexpression in preadipocytes by simple Oil-Red-O staining (Fig. 2).

The mouse Elovl2 cDNA was identified [8], by a homology search with ELOVL3, whose mRNA in brown adipose tissue greatly increases by cold exposure [22,27,28]. An abundant amount of Elovl2 mRNA in testis suggested that ELOVL2 elongates PUFAs [8]. This was subsequently demonstrated in mouse [10], human [29] and rat [30,31] orthologs, and in this study (Fig. 1C). The reduced palmitoyl-CoA elongation activity appeared in ELOVL2-expressing 3T3-L1 cells (Fig. 1C) might be the result of a compensational reduction of endogenous ELOVL(s) (e.g. ELOVL1).

Lipogenic pathways for TAG synthesis are transcriptionally regulated by sterol regulatory element-binding protein (SREBP)-1c and peroxisome proliferator-activated receptor (PPAR)-γ, where various PUFAs are known to regulate the function of these transcriptional factors [20,32,33]. We examined the expression levels of these target genes in established cell lines.

PUFA is known to inhibit the function of SREBP-1c [34,35]. However, ELOVL2-overexpression had no effect on an SREBP-1c target gene, FAS (Fig. 4). Another group also suggested that Elovl2 gene transcription is not regulated by SREBP-1c in rat hepatocytes [31]. Therefore the connection between ELOVL2 and SREBP-1c seems inconsiderable. The reason for the high FAS gene expression in ELOVL3-expressing cells remains to be elucidated.

An adipose tissue-specific differentiation marker FABP4 (aP2) was induced by ELOVL2 (Fig. 4). FABP4 gene expression is regulated by PPAR-γ [36]. Liver-type FABP acquires

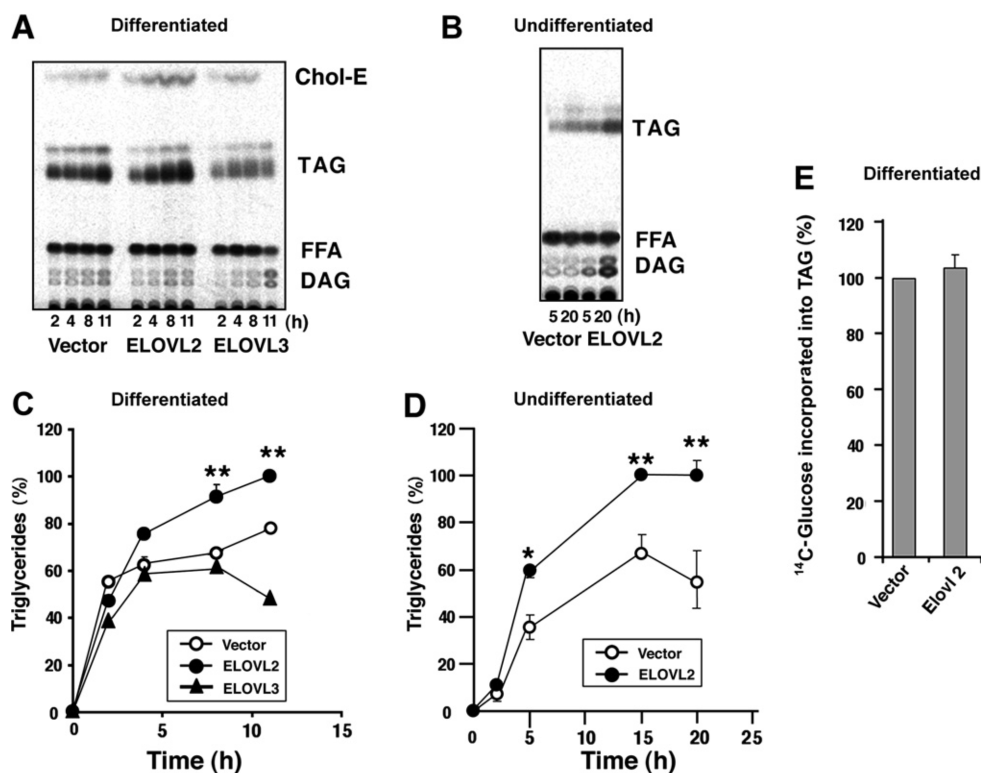

Fig. 3. Incorporation of extra-cellular FA and glucose into TAG in ELOVLs-overexpressing 3T3-L1 cells. (A–D) Incorporation of [U-^{14}C] palmitic acid into TAG in ELOVLs-expressing cells was analyzed with differentiated (A,C, 10 days) and with undifferentiated (B,D) conditions. (A,B) Representative TLC results visualized on the phosphorimager. (C,D) The relative amount of ^{14}C-labeled TAG is expressed as a percentage of the maximum value (from two differentiation experiments measured in duplicate). DAG, diacylglycerol; FFA, free fatty acid; Chol E, cholesterol ester. (E) [U-^{14}C] glucose incorporation into TAG in differentiated cells. Radiolabeled TAG synthesized from glucose (glycerol-3-phosphtate) was measured at 10 h after the addition of [U-^{14}C] glucose. Statistical significances were shown versus control (vector) values of corresponding time points; **, $P < 0.001$; *, $P < 0.05$.

FA in the cytosol, followed by translocation of the complex into the nucleus, and transfer of ligands (FA) to PPAR-γ, then trans-activates target genes [37]. Moreover, a heterodimer partner of PPAR-γ, retinoid X receptor (RXR)-α [37], is activated by DHA (an $n-6$ PUFA) [38].

DGAT2, a last-step enzyme in the TAG synthesis pathway, was also induced by ELOVL2-overexpression (Fig. 4). DGAT2 mRNA increases along with differentiation of 3T3-L1 cells ([39] and Fig. 4). Currently, there is no evidence that DGAT2 is under the control of SREBP-1c or PPAR-γ. However at the protein level, EPA-CoA (PUFA) has been shown to inhibit DGAT activity [40].

As to catabolic effects, $n-3$PUFAs are reported to induce FA-oxidation through the action of PPAR-α [20]. Overexpression of a PUFA elongase ELOVL2 in cells was therefore expected to accelerate cellular catabolism. However, the present study showed enhanced TAG synthesis, suggesting no major correlation between catabolism and enhanced TAG synthesis by ELOVL2.

With the time scale we analyzed (10 h), extracellular FA but not glucose was shown as the major source of TAG, especially in ELOVL2-expressing cells (Fig. 3A–E). Transport of FA is one of the factors to be considered for this effect. A compositional change in various types of FA species such as incorporated FA and its elongated intracellular VLCFA modifies the membrane fluidity and thus the localization of caveolae [15]. This could affect the functions of fatty acid transporter FATP1 [41] and other related transporters.

5. Conclusion

How one particular ELOVL member, i.e. ELOVL2, augments TAG synthesis is not revealed, yet the present study showed evidence supporting the finding. One hypothesis is as follows; in ELOVL2-expressing cells, an increased amount of elongated-PUFAs (products) or consumption of PUFAs (as substrates) change the composition of a cellular FA (-CoA) pool, induce PPAR-γ-controlled lipogenic genes, enhance TAG synthesis followed by accumulation of lipid droplets in cells. Another possibility is the regulation of various enzyme activities by PUFAs, e.g. DGAT2. It should be noted herein that our observation was found in preadipocytes whereas numerous catabolic effects of $n-3$ PUFAs are mainly reported in vivo. Further mechanistic as well as more broad investigations are needed to delineate and generalize the finding presented, yet quests for specific inhibitors, inducers and connected pathways for ELOVL2 will provide medically useful information to control lipid metabolism.

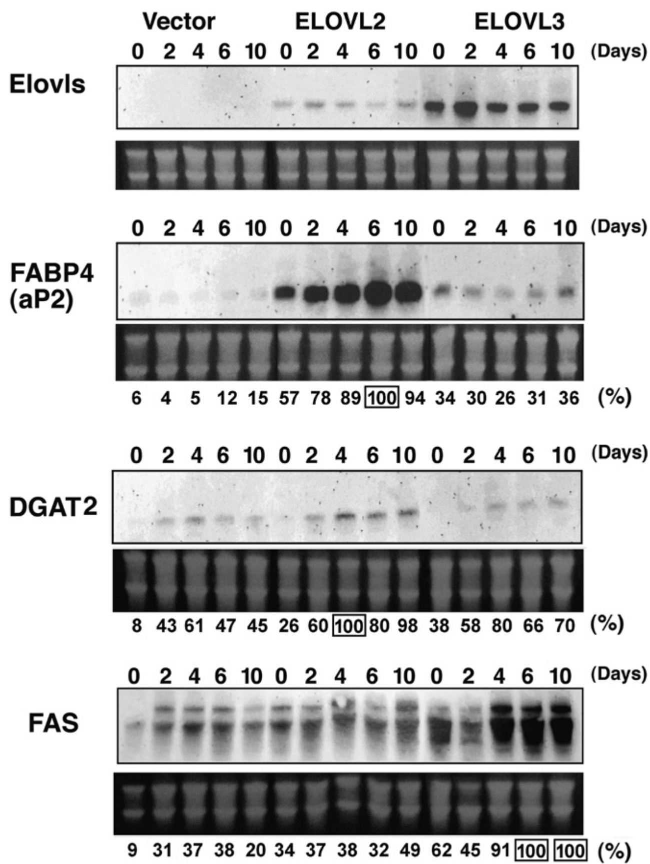

Fig. 4. Differentiation-dependent changes in the mRNA levels of lipogenic genes in 3T3-L1 cells expressing ELOVL2 and ELOVL3 Total RNA isolated during differentiation of 3T3-L1 cells (days 0, 2, 4, 6 and 10) was subjected to Northern blotting using FABP4, DGAT2 and FAS as probes. Each lane contains 5 μg of total RNA. RNA photographs are shown as a reference of loading. The mRNA level was represented as a percentage of the maximum value, after standardization with 18S RNA.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.05.081.

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