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Role of Elovl4 Protein in the Biosynthesis of Docosahexaenoic Acid

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Abstract

The disk membranes of retinal photoreceptor outer segments and other neuronal and reproductive tissues are enriched in docosahexaenoic acid (DHA, 22:6n3), which is essential for their normal function and development. The fatty acid condensing enzyme Elongation of Very Long chain fatty acids-4 (ELOVL4) is highly expressed in retina photoreceptors as well as other tissues with high 22:6n3 content. Mutations in the *ELOVL4* gene are associated with autosomal dominant Stargardt-like macular dystrophy (STGD3) and results in synthesis of a truncated protein that cannot be targeted to the endoplasmic reticulum (ER), the site of fatty acid biosynthesis. Considering the abundance and essential roles of 22:6n3 in ELOVL4-expressing tissues (except the skin), it was proposed that the ELOVL4 protein may be involved in 22:6n3 biosynthesis. We tested the hypothesis that the ELOVL4 protein is involved in 22:6n3 biosynthesis by selectively silencing expression of the protein in the cone photoreceptors derived cell line 661W and showed that the ELOVL4 protein is not involved in DHA biosynthesis from the short chain fatty acid precursors 18:3n3 and 22:5n3.

27.1 Introduction

Mammalian fatty acid elongases are a group of condensing enzymes that mediate elongation of fatty acids by the addition of two carbon units of malonyl-CoA. Currently seven members of these condensing enzymes named ELOngation of Very Long chain fatty acids (ELOVL) have been reported. Their roles in fatty acid chain elongation, their fatty acid specificity, as well as the steps within the carbon chain they act on, have been studied and reviewed (Leonard et al., 2004; Meyer et al., 2004; Tvrdik et al., 2000; Westerberg et al., 2004). The fourth member of this group, ELOVL4, was first discovered and reported in 2001 as a truncated protein associated with autosomal dominant Stargardt-like macular dystrophy (STGD3), an inherited juvenile form of macular degeneration (Bernstein et al., 2001; Edwards et al., 2001; Zhang et al., 2001). On the basis of features of the ELOVL4 protein, which are characteristic of fatty acid elongases, and its tissue distribution, the ELOVL4 protein was proposed by Zhang et al. (2001) to be involved in biosynthesis of

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docosahexaenoic acid (DHA, 22:6n3), the most abundant fatty acid in the retina, other neural tissues, and the testis.

We used an immortalized cell line derived from mouse cones (661W) (Al-Ubaidi et al., 1992) that expresses ELOVL4 and possesses the machinery for fatty acid elongation and desaturation. These cells were able to elongate short chain polyun-saturated fatty acids (PUFA) of the n3 and n6 family to longer chain fatty acids up to C24 when they were incubated with 18:3n3, 20:5n3 or 22:5n3. We tested the hypothesis that the ELOVL4 protein is involved in the elongation of PUFA to 22:6n3 by selectively silencing the expression of endogenous ELOVL4 in 661W cells and determined that the ELOVL4 protein is not involved in the biosynthesis of 22:6n3 from short chain PUFA precursors.

27.2 Materials and Methods

27.2.1 RNA Interference

A pool of siRNA designed for the silencing of the mouse *Elovl4* gene (genome smartpool reagent M-054863-00-0005) was purchased from Dharmacon, Inc. (Lafayette, CO). 661W cells (2×10^5 cells in 2 ml of medium/well using a 24-well plate or 2×10^4 cells in 200 μl of medium/well using a 96-well plate) were transfected with the *Elovl4* siRNA or the control siRNA using I-fect transfection reagent (Neuromics, Edina, MN), according to the manufacturer's instructions. All transfections were performed in a mixture of Opti-MEM (Invitrogen, Carlsbad, CA) and complete medium without antibiotics. Transfections were optimized by varying the doses of the transfection reagents and the siRNA. Cells were incubated with the transfection siRNA/I-fect complexes for 12 h in Opti-MEM and then switched to complete DMEM. Cell lysis and protein isolation were done 72 h post transfection. The efficacy of gene silencing was assessed by RT-PCR and Western blotting using a rabbit polyclonal antibody against mouse ELOVL4 described previously (Agbaga et al., 2008).

27.2.2 Construction of Mouse Anti Elovl4 Gene shRNA

From the four siRNA pools, two individual siRNAs that efficiently silenced the *Elovl4* expression in 661W cells were selected and converted to short-hairpin oligonucleotides that were synthesized by Integrated DNA Technologies (IDTDNA, Coralville, IA). The short hairpin oligonucleotides were annealed and cloned into the *Hin*dIII/*Sfi*I site under the U6 promoter of pSilencer U6 AR1 CMV IRES-EGFP vector (Ambion Inc, Austin, TX) or into Genscript pRNAT-U6.2/Lenti (Piscataway, NJ) vector to generate shRNA vectors. A scrambled sequence, which does not target mouse *Elovl4* expression, was used as a control. The shRNAs were individually transfected into 661W cells and green fluorescent protein (GFP) positive cells were sorted by flow cytometry, cultured, and used for fatty acid supplementation experiments.

27.2.3 Tissue Culture

661W cells were plated at a density of 2×10^6 cells/ml in DMEM supplemented with 10% calf serum and antibiotics, and treated for 72 h with 5–30 μg/ml of the sodium salts of either 18:3n3 or 22:5n3. After 72 h, the cells were collected and washed once in 0.1 M phosphate

buffer containing 50 μM of fraction V fatty acid-free BSA (Sigma, St Louis MO) and then in 0.1 M phosphate buffer. Finally, cells were pelleted and stored at −80°C until used. For radioactive tracer studies, the cells were plated as above and then incubated with 2–4 μCi/ml of fraction V fatty acid-free BSA conjugated [1-^{14}C]-18:3n3 for 48–72 h. The cells were collected and stored as before.

27.2.4 Fatty Acid Analysis

Total lipids were extracted from fatty acid supplemented and control cells by the method of Bligh and Dyer (Bligh and Dyer 1959) and converted to fatty acid methyl esters (FAMEs) by the procedure of Morrison and Smith (1964). FAMEs were analyzed by gas-liquid chromatography as described (Tanito et al., 2008). Fatty acid phenacyl esters (FAPES) were prepared from total lipid extracts for high performance liquid chromatography (HPLC) analysis as previously described (DeMar and Anderson 1997; DeMar et al., 1996).

27.3 Results

27.3.1 661W Cells Express Elovl4 and Can Elongate 18:3n3 and 22:5n3 to Longer Chain Fatty Acids

To determine the specific step(s) in fatty acid elongation that the ELOVL4 protein catalyses, we determined that an immortalized retinal cell line (661W) expresses *Elovl4* mRNA and protein (Fig. 27.1). Addition of 18:3n3 and 22:5n3 to the culture media showed that 661W cells possess the metabolic machinery necessary for elongation of these PUFA to C-24 PUFA. 18:3n3 was efficiently elongated to C20 and C24 PUFA (Fig. 27.2a) and 22:5n3 was elongated to 24:5n3 with some retro-conversion to 20:5n3 (Fig. 27.2b). Since formation of 22:6n3 involves desaturation of 24:5n3 to 24:6n3, which is then metabolized in the peroxisome by β-oxidation to 22:6n3 (Voss et al., 1991), we conclude that 661W cells have the biochemical machinery necessary for elongation of n3 PUFA.

27.3.2 Knock-Down of Endogenous Elovl4 Does Not Affect C18–C24 PUFA Synthesis

siRNA and shRNA mediated silencing of the *Elovl4* message in 661W cells resulted in knock-down of the ELOVL4 protein as determined by Western blot analysis (Fig. 27.3). As shRNA-D2 was efficient at silencing ELOVL4 protein expression (Fig. 27.3d), we chose cells transfected with this construct and sorted for fatty acid biosynthesis studies. As shown in Fig. 27.4, ELOVL4 knock-down did not affect the ability of the 661W cells to elongate 18:3n3 to 20:5n3, 22:5n3, and 24:5n3 as determined by HPLC (Fig. 27.4a) and GC-FID (Fig. 27.4b). If ELOVL4 catalyzed any particular one of these elongation steps, there would have been an increase in the radioactivity (or mass) in the precursor fatty acid and a decrease in radioactivity (or mass) of the product of the accumulated precursor in the silenced condition.

27.4 Discussion

The highest expression of ELOVL4 is found in the retina, followed by testis, skin and brain. Except for the skin, all of these tissues have high contents of 22:6n3, which is required for their normal development and function (Benolken et al., 1973; Salem et al., 2001). This fatty

acid must either be obtained from diet or be synthesized through sequential desaturation and elongation of dietary n3 PUFA such as 18:3n3 or 22:5n3 to 24:6n3 (Bazan et al., 1982; Delton-Vandenbroucke et al., 1997; Sprecher 2000; Voss et al., 1991). The 24:6n3 is then converted to 22:6n3 through peroxisomal β-oxidation (Sprecher 1999; Sprecher et al., 1999; Voss et al., 1991). The high content of 22:6n3 in all ELOVL4 expressing tissues except the skin suggested a probable role of ELOVL4 protein in the biosynthetic pathway of 22:6n3 from its short chain precursors (Zhang et al., 2001). However, mutations in the *ELOVL4* gene results in a truncated protein that misroutes both mutant and wild-type protein from the endoplasmic reticulum, the site of fatty acid biosynthesis (Grayson and Molday 2005; Karan et al., 2005; Vasireddy et al., 2005)

We tested whether ELOVL4 protein is involved in the pathway of 22:6n3 synthesis by silencing its expression in cells that have endogenous expression of this protein, and determining the effect on C-24 PUFA formation from shorter chain PUFA precursors. Using non-radioactive and [^{14}C]-18:3n3, we showed that cone photoreceptor-derived 661W cells express the ELOVL4 protein (Fig. 27.1) and have the metabolic machinery necessary for the elongation of either 18:3n3 or 22:5n3 to longer chain n3 fatty acids up to C24 (Fig. 27.2a, b). However, when we silenced the ELOVL4 expression in 661W cells, no detectable changes were observed in the elongation of either 18:3n3 or 22:5n3 to longer chain fatty acids. Elongation and desaturation of 18:3n3 and 22:5n3 fatty acids in these cells proceeded unabated in the same way as in the wild-type cells. These elongation steps could be mediated by the Δ5–7 elongase activity of the mouse ELOVL2 protein (Meyer et al., 2004). Elongation of 20:5n3 to 22:5n3 was also probably catalyzed by Δ5 elongase activity of the same enzyme (Meyer et al., 2004). This suggests ELOVL4 may not be involved in elongation of these steps in the pathway of 22:6n3 biosynthesis. We then evaluated Δ7 elongase activity in the pathway of 22:6n3 biosynthesis by incubating the cells with 22:5n3 while knocking down the expression of *Elovl4*. ELOVL4 knock-down did not have any effect on the elongation of 22:5n3 to 24:5n3 (data not shown). We, therefore, concluded that the ELOVL4 protein could either be playing a redundant role in biosynthesis of 22:6n3 or it may not be involved in the biosynthesis of 22:6n3 in the retina or in any tissues where it is expressed. These conclusions are also supported by recent studies on ELOVL4 using different mouse models (Cameron et al., 2007; Li et al., 2007; McMahon et al., 2007a, b; Vasireddy et al., 2007). Further, we designed experiments to test the hypothesis that the ELOVL4 protein is involved in synthesizing C26–C38 Very Long Chain Polyunsaturated Fatty Acids (VLC-PUFA), which are also found in ELOVL4 expressing tissues in minor quantities (Aveldano 1987). We presented an unequivocal evidence that indeed the ELOVL4 protein is involved in the biosynthesis of VLC-PUFA (Agbaga et al., 2008). The findings that ELOVL4 is involved in the biosynthesis of VLC-PUFA are in agreement with previous studies that showed that the skin of mice homozygous for *Elovl4* 5-bp deletion knock-in and *Elovl4* knock-out animals have greater total saturated FA (esp. 24:0 and 26:0) than that of their wild-type kin (Cameron et al., 2007; Li et al., 2007; McMahon et al., 2007a; Vasireddy et al., 2007). It also supported the studies by McMahon et al. (2007b) which showed that the retinas of *Stgd3*-knockin mice that carry one copy of the human pathogenic 5-bp deletion in the mouse *Elovl4* gene and one normal copy of the mouse *Elovl4* gene have deficiency in C32-C36 acyl phosphatidylcholine. Moreover, the 22:6n3

composition of those retinas was not different from the composition of the retinas from wild-type mice (McMahon et al., 2007b). The conclusion drawn from the current study is that ELOVL4 is not involved in the biosynthesis of 22:6n3 from shorter chain PUFA precursors.

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References

Agbaga MP, Brush RS, Mandal MN, et al. Role of Stargardt-3 macular dystrophy protein (ELOVL4) in the biosynthesis of very long chain fatty acids. Proc Natl Acad Sci USA. 2008; 105:12843–12848. [PubMed: 18728184]

Al-Ubaidi MR, Font RL, Quiambao AB, et al. Bilateral retinal and brain tumors in transgenic mice expressing simian virus 40 large T antigen under control of the human interphotoreceptor retinoid-binding protein promoter. J Cell Biol. 1992; 119:1681–1687. [PubMed: 1334963]

Aveldano MI. A novel group of very long chain polyenoic fatty acids in dipolyunsaturated phosphatidylcholines from vertebrate retina. J Biol Chem. 1987; 262:1172–1179. [PubMed: 3805015]

Bazan HE, Careaga MM, Sprecher H, et al. Chain elongation and desaturation of eicos-apentaenoate to docosahexaenoate and phospholipid labeling in the rat retina in vivo. Biochim Biophys Acta. 1982; 712:123–128. [PubMed: 6288109]

Benolken RM, Anderson RE, Wheeler TG. Membrane fatty acids associated with the electrical response in visual excitation. Science. 1973; 182:1253–1254. [PubMed: 4752217]

Bernstein PS, Tammur J, Singh N, et al. Diverse macular dystrophy phenotype caused by a novel complex mutation in the ELOVL4 gene. Invest Ophthalmol Vis Sci. 2001; 42:3331–3336. [PubMed: 11726641]

Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol. 1959; 37:911–917. [PubMed: 13671378]

Cameron DJ, Tong Z, Yang Z, et al. Essential role of Elovl4 in very long chain fatty acid synthesis, skin permeability barrier function, and neonatal survival. Int J Biol Sci. 2007; 3:111–119. [PubMed: 17304340]

DeMar JC Jr, Anderson RE. Identification and quantitation of the fatty acids composing the CoA ester pool of bovine retina, heart, and liver. J Biol Chem. 1997; 272:31362–31368. [PubMed: 9395466]

DeMar JC Jr, Wensel TG, Anderson RE. Biosynthesis of the unsaturated 14-carbon fatty acids found on the N termini of photoreceptor-specific proteins. J Biol Chem. 1996; 271:5007–5016. [PubMed: 8617777]

Delton-Vandenbroucke I, Grammas P, Anderson RE. Polyunsaturated fatty acid metabolism in retinal and cerebral microvascular endothelial cells. J Lipid Res. 1997; 38:147–159. [PubMed: 9034209]

Edwards AO, Donoso LA, Ritter R 3rd. A novel gene for autosomal dominant Stargardt-like macular dystrophy with homology to the SUR4 protein family. Invest Ophthalmol Vis Sci. 2001; 42:2652–2663. [PubMed: 11581213]

Grayson C, Molday RS. Dominant negative mechanism underlies autosomal dominant Stargardt-like macular dystrophy linked to mutations in ELOVL4. J Biol Chem. 2005; 280:32521–32530. [PubMed: 16036915]

Karan G, Yang Z, Howes K, et al. Loss of ER retention and sequestration of the wild-type ELOVL4 by Stargardt disease dominant negative mutants. Mol Vis. 2005; 11:657–664. [PubMed: 16145543]

Leonard AE, Pereira SL, Sprecher H, et al. Elongation of long-chain fatty acids. Prog Lipid Res. 2004; 43:36–54. [PubMed: 14636670]

Li W, Sandhoff R, Kono M, et al. Depletion of ceramides with very long chain fatty acids causes defective skin permeability barrier function, and neonatal lethality in ELOVL4 deficient mice. Int J Biol Sci. 2007; 3:120–128. [PubMed: 17311087]

McMahon A, Butovich IA, Mata NL, et al. Retinal pathology and skin barrier defect in mice carrying a Stargardt disease-3 mutation in elongase of very long chain fatty acids-4. Mol Vis. 2007a; 13:258–272. [PubMed: 17356513]

McMahon A, Jackson SN, Woods AS, et al. A Stargardt disease-3 mutation in the mouse Elovl4 gene causes retinal deficiency of C32-C36 acyl phosphatidylcholines. FEBS Lett. 2007b; 581:5459–5463. [PubMed: 17983602]

Meyer A, Kirsch H, Domergue F, et al. Novel fatty acid elongases and their use for the reconstitution of docosahexaenoic acid biosynthesis. J Lipid Res. 2004; 45:1899–1909. [PubMed: 15292371]

Morrison WR, Smith LM. Preparation of Fatty Acid Methyl Esters and Dimethylacetals from Lipids with Boron Fluoride–Methanol. J Lipid Res. 1964; 5:600–608. [PubMed: 14221106]

Salem N Jr, Litman B, Kim HY, et al. Mechanisms of action of docosahexaenoic acid in the nervous system. Lipids. 2001; 36:945–959. [PubMed: 11724467]

Sprecher H. An update on the pathways of polyunsaturated fatty acid metabolism. Curr Opin Clin Nutr Metab Care. 1999; 2:135–138. [PubMed: 10453344]

Sprecher H. Metabolism of highly unsaturated n-3 and n-6 fatty acids. Biochim Biophys Acta. 2000; 1486:219–231. [PubMed: 10903473]

Sprecher H, Chen Q, Yin FQ. Regulation of the biosynthesis of 22:5n-6 and 22:6n-3: a complex intracellular process. Lipids. 1999; 34(Suppl):S153–S156. [PubMed: 10419131]

Tanito M, Brush RS, Elliott MH, et al. High levels of retinal membrane docosahexaenoic acid increase susceptibility to stress-induced degeneration. J Lipid Res. 2008; 50(5):807–819. [PubMed: 19023138]

Tvrdik P, Westerberg R, Silve S, et al. Role of a new mammalian gene family in the biosynthesis of very long chain fatty acids and sphingolipids. J Cell Biol. 2000; 149:707–718. [PubMed: 10791983]

Vasireddy V, Uchida Y, Salem N Jr, et al. Loss of functional ELOVL4 depletes very long-chain fatty acids (> or =C28) and the unique omega-O-acylceramides in skin leading to neonatal death. Hum Mol Genet. 2007; 16:471–482. [PubMed: 17208947]

Vasireddy V, Vijayasarathy C, Huang J, et al. Stargardt-like macular dystrophy protein ELOVL4 exerts a dominant negative effect by recruiting wild-type protein into aggresomes. Mol Vis. 2005; 11:665–676. [PubMed: 16163264]

Voss A, Reinhart M, Sankarappa S, et al. The metabolism of 7,10,13,16,19-docosapentaenoic acid to 4,7,10,13,16,19-docosahexaenoic acid in rat liver is independent of a 4-desaturase. J Biol Chem. 1991; 266:19995–20000. [PubMed: 1834642]

Westerberg R, Tvrdik P, Unden AB, et al. Role for ELOVL3 and fatty acid chain length in development of hair and skin function. J Biol Chem. 2004; 279:5621–5629. [PubMed: 14581464]

Zhang K, Kniazeva M, Han M, et al. A 5-bp deletion in ELOVL4 is associated with two related forms of autosomal dominant macular dystrophy. Nat Genet. 2001; 27:89–93. [PubMed: 11138005]

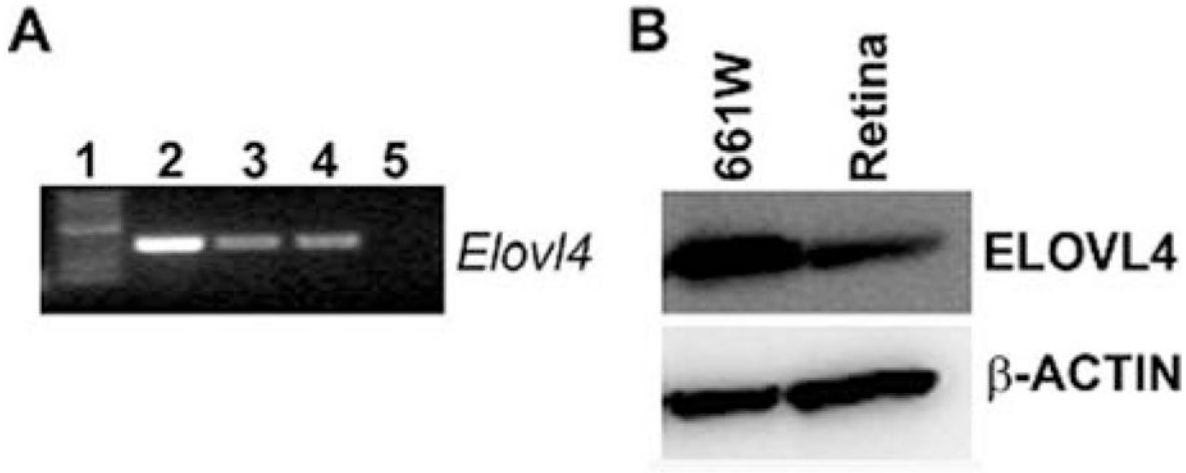

Fig. 27.1.

Endogenous expression of Elovl4 in 661W cells; (**a**) RT-PCR of *Elovl4* cDNA from 661W cells. Complementary DNA from the 661W cells was used as a template to amplify the *Elovl4* transcript with actin used as a control (data not shown). Representative results are presented. Lanes: 1, 100-bp markers; 2, mouse retina cDNA; 3–4, 661W cDNA; 5, no reverse transcriptase control. (**b**) A representative Western blot analysis of 30 μg protein from 661W cells and mouse retina confirmed the expression of ELOVL4 in 661W cells. Bottom panel is β-actin loading control

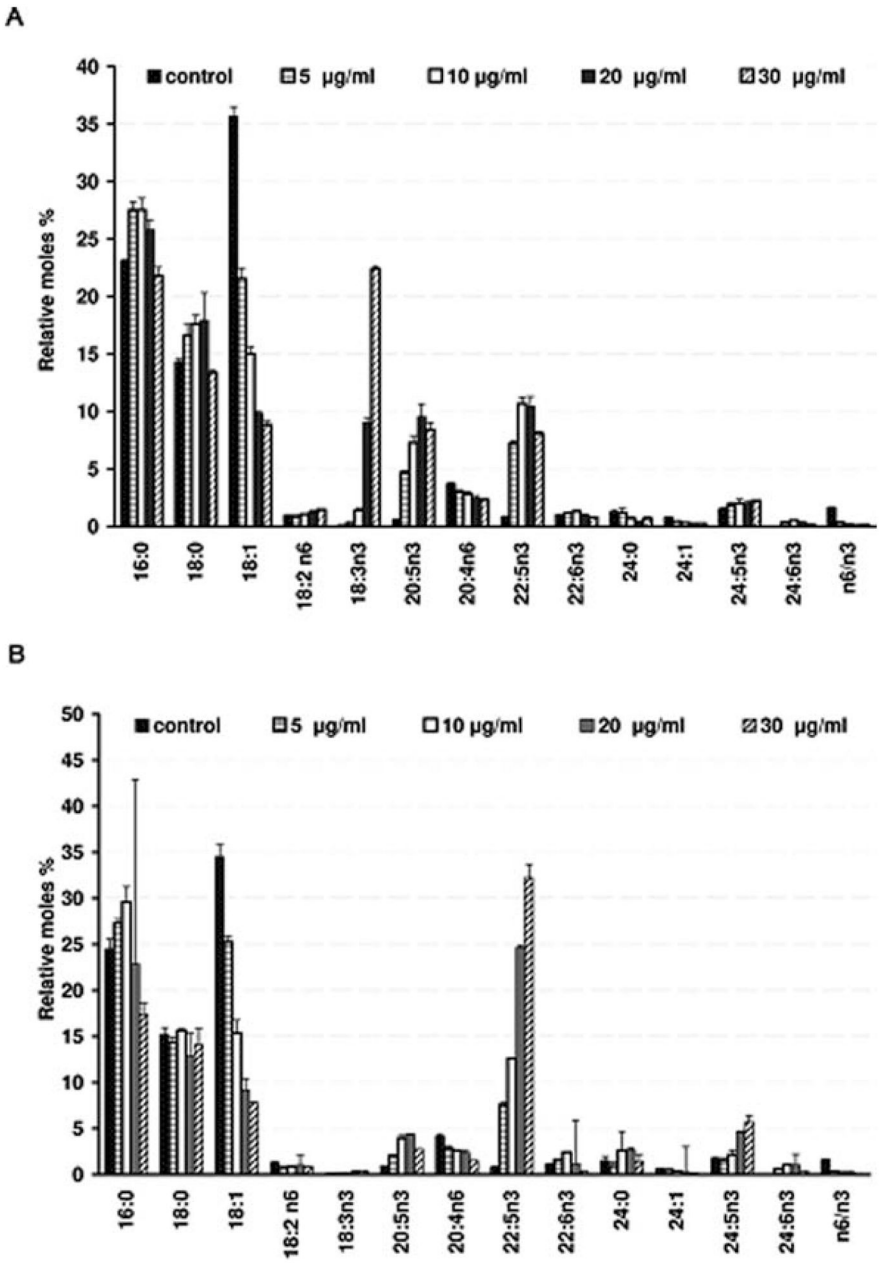

Fig. 27.2.

Elongation of 18:3n3 and 22:5n3 in 661W cells; (**a**) 661W cells were cultured in medium supplemented with increasing concentrations of 18:3n3 or (**b**) 22:5n3 and grown for 72 h. Total cellular lipids were extracted, converted to fatty acid methyl esters (FAMEs), and analyzed by gas-liquid chromatography. 18:3n3 was elongated and desaturated to 20:5n3, 22:5n3, and 24:5n3. However, there was negligible conversion to 24:6n3 and 22:6n-3. Similarly, 22:5n3 (**b**) was incorporated into cellular lipids and some was elongated to 24:5n3

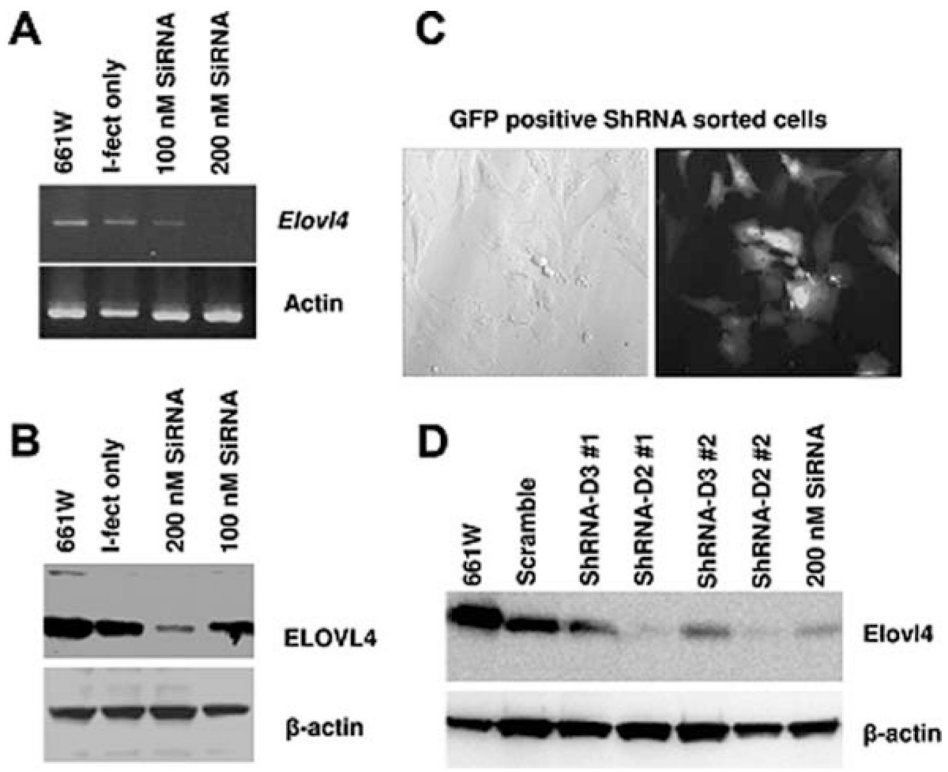

Fig. 27.3.

siRNA and shRNA knock-down of mouse *Elovl4* in 661W cells; (**a**) *Elovl4* knock-down in 661W cells transfected with 100 or 200 nM anti-*Elovl4* siRNA smart-pools. *Elovl4* knock-down by the siRNA duplexes was assayed by RT-PCR. (**b**) Western blot analysis of ELOVL4 knockdown in 661W cells transfected with the pool of 4 siRNA duplexes 72 h after transfection. The *bottom panel* is β-actin loading control. (**c**) 661W cells transfected and sorted for GFP-positive cells expressing the pSilencer-anti-*Elovl4*-shRNA under the human U6 promoter and GFP under the CMV promoter. After sorting, the GFP positive cells were expanded and used for subsequent experiments. (**d**) Western blot analysis of GFP-positive 661W cells stably expressing anti-*Elovl4*-shRNA and controls post-sorting

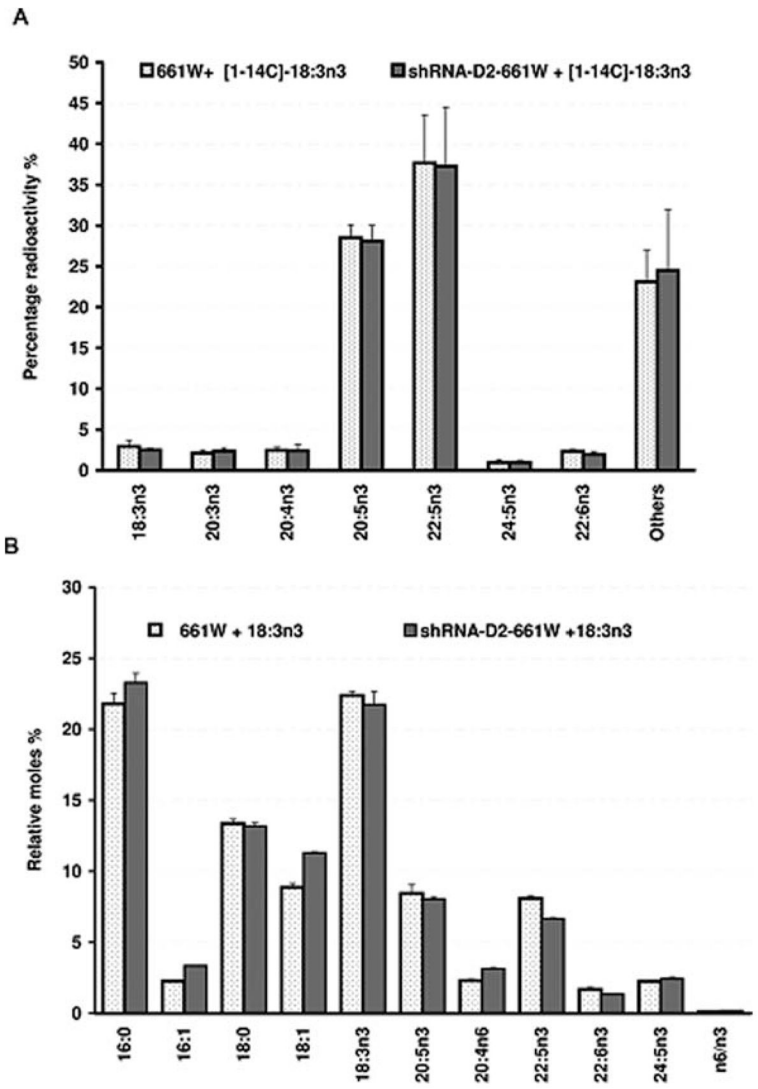

Fig. 27.4.

Knock-down of ELOVL4 did not affect the elongation of [1-^{14}C]-18:3n3 to [^{14}C]-20:5n3, [^{14}C]-22:5n3 and [^{14}C]-24:5n3.; (**a**) Relative percentage radioactivity in ELOVL4 knock-down and wild type 661W cells supplemented with [1-^{14}C]-18:3n3. No differences in the formation of [^{14}C]-20:5n3, [^{14}C]-22:5n3, and [^{14}C]-24:5n3 were found between control (non-shRNA expressing cells) and the ELOVL4-knock-down cells. This suggests that ELOVL4 is not involved in the elongation of 18:3n3 to 20:5n3, of 20:5n3 to 22:5n3, and of 22:5n3 to 24:5n3 in these cells. (**b**) GC-FID results of elongation of 18:3n3 in wild type 661W cells and shRNA-D2-GFP positive cells. There were no differences in the elongation products