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Novel Lipogenic Enzyme ELOVL7 Is Involved in Prostate Cancer Growth through Saturated Long-Chain Fatty Acid Metabolism

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Abstract

A number of epidemiologic studies have indicated a strong association between dietary fat intake and prostate cancer development, suggesting that lipid metabolism plays some important roles in prostate carcinogenesis and its progression. In this study, through our genome-wide gene expression analysis of clinical prostate cancer cells, we identified a novel lipogenic gene, *ELOVL7*, coding a possible long-chain fatty acid elongase, as overexpressed in prostate cancer cells. ELOVL7 expression is regulated by the androgen pathway through SREBP1, as well as other lipogenic enzymes. Knockdown of ELOVL7 resulted in drastic attenuation of prostate cancer cell growth, and it is notable that high-fat diet promoted the growth of *in vivo* tumors of ELOVL7-expressed prostate cancer. *In vitro* fatty acid elongation assay and fatty acid composition analysis indicated that ELOVL7 was preferentially involved in fatty acid elongation of saturated very-long-chain fatty acids (SVLFA, C20:0 ~). Lipid profiles showed that knockdown of ELOVL7 in prostate cancer cells affected SVLFAs in the phospholipids and the neutral lipids, such as cholesterol ester. Focusing on cholesterol ester as a source of *de novo* steroid synthesis, we show that ELOVL7 affected *de novo* androgen synthesis in prostate cancer cells. These findings suggest that EVOLV7 could be involved in prostate cancer growth and survival through the metabolism of SVLFAs and their derivatives, could be a key molecule to elucidate the association between fat dietary intake and prostate carcinogenesis, and could also be a promising molecular target for development of new therapeutic or preventive strategies for prostate cancers. [Cancer Res 2009;69(20):8133–40]

Introduction

Prostate cancer is the most common malignancy in males and the is the second leading cause of cancer-related death in the United States and Europe (1). Prostate cancer incidence has been increasing significantly in most developed countries probably due to prevalence of Western lifestyle including Western diet and the explosion of the aging population (1, 2). Epidemiologic evidence (3, 4) and migrant studies (5, 6) indicate that the prostate cancer incidence is much lower in parts of the world where people eat a predominantly low-fat diet, and dietary fat or lipid metabolism is likely to be critical in prostate carcinogenesis. Furthermore, high-fat dietary intake predicts unfavorable prognosis and relapse after treatment for localized prostate cancer (7), and experimental evidence also suggests that high-fat or high-calorie diet promotes prostate cancer cell proliferation (8, 9). Hence, there are some proposals that lifestyle intervention from high-fat diet to low-fat diet (10, 11) or drug intervention for hyperlipidemia or hypercholesterolemia (12, 13) could improve prostate cancer prognosis as well as prostate cancer incidence, and several clinical studies of such interventions for prostate cancer are now on-going. However, how high-fat diet and lipid metabolism can affect prostate carcinogenesis and prostate cancer progression at the molecular level has been a puzzle.

In this study, through our gene expression profiles of prostate cancer cells, we identified and characterized a novel gene, *ELOVL7*, which was overexpressed in prostate cancer cells and could play critical roles in lipid metabolism in prostate cancer cells. Elongation of very-long-chain fatty acids (ELOVL) family members are human homologues of yeast ELOs and catalyze the elongation reaction of very-long-chain fatty acids (14, 15). The elongation system, which is responsible for the addition of two carbon units to the carboxyl end of a fatty acid chain, is composed of four enzymes: a condensing enzyme (elongase, β-ketoacyl CoA synthase), β-ketoacyl CoA reductase, β-hydroxyacyl CoA dehydrase, and *trans*-2, 3-enoyl-CoA reductase (14). The rate of this fatty acid elongation is determined by the activity of the elongase (15). Six distinct fatty acid elongase subtypes (ELOVL1–6) are reported in mammals thus far, and each of these multiple elongation enzymes is thought to work specifically for different chain length saturated or unsaturated fatty acids (14–16). The metabolic pathways of long-chain fatty acids play an important role in the maintenance of membrane lipid composition as well as in the generation of precursors for cell signaling molecules, such as eicosanoids and sphingosine-1 phosphate (14, 15). Thus, these metabolic pathways are expected to be involved in some essential processes of cell physiology and cancer pathology.

We here validated overexpression of ELOVL7 in clinical prostate cancers and showed its involvement in prostate cancer cell viability as well as its preferential fatty acid elongation activity toward saturated very-long-chain fatty acids (SVLFA). We also showed that

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

The data reported in this article have been deposited in the Genebank (accession no. AB181393) and the Gene Expression Omnibus database (accession no. GSE14852).

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ELOVL7 could affect fatty acid composition in phospholipids and neutral lipids including cholesterol ester in prostate cancer cells, possibly contributing to *de novo* steroidogenesis in prostate cancer. Our data provide new insights in prostate carcinogenesis related to lipid metabolism and some clues for developing new therapeutic or preventive strategies against prostate cancers.

Materials and Methods

Cell lines and tissues. Prostate cancer cell lines LNCaP, DU-145, 22Rv1, and PC-3 were obtained from American Type Culture Collection, and the LNCaP-derived cell line C4-2B was purchased from ViroMed Laboratories. Both were cultured in the recommended medium with 10% fetal bovine serum (FBS). Frozen or paraffin-embedded prostate cancer tissues were obtained from prostate cancer patients who underwent radical prostatectomy at Kochi University, Kyoto Prefectural Medical School, and Iwate Medical University with appropriate informed consent (17).

Semiquantitative reverse transcription-PCR and Northern blot analysis. Total RNA extraction, reverse transcription-PCR (RT-PCR), and T7-based RNA amplification for minimal RNAs from microdissected prostate cancer cells were described previously (17). Primer sequences were the following: 5′-TTGGCTTGACTCAGGATTTA-3′and 5′-ATGCTATCACCTCCCCTGTG-3′ for *ACTB*, and 5′-TCTATGAATCCTTGAGGGCCTA-3′, and 5′- TGACAACATCCACAGAATGTTCC-3′ for *ELOVL7*. Human multiple tissues Northern blots (BD Bioscience) were hybridized for 20 h with ^{32}P-labeled *ELOVL7* cDNA, which was labeled using Megaprime DNA labeling system (GE Healthcare Bioscience). Probe cDNA of *ELOVL7* was prepared as a 785-bp PCR product. Prehybridization, hybridization, and washing were performed according to the manufacture's instruction. The blots were autoradiographed at −80°C for 7 d.

Generating antibody to EVOVL7 and immunohistochemical analysis. The NH_2-terminal peptide (SDLTSRTVHLYDNWIKDA) and COOH-terminal peptide (CHFWYRAYTKGQRLPKTVK) of human ELOVL7 protein were synthesized and used to immunize rabbits. The immune sera were purified on affinity columns packed with Affi-Gel 10 activated affinity media (Bio-Rad Laboratories) conjugating each of the peptide antigens. Immunohistochemical study was carried out using the Ventana automated immunohistochemical systems (Discovery, Ventana Medical Systems, Inc.). Sections were incubated with a 1:100 diluted solution of purified anti-ELOVL7 antibody for 16 min. The automated protocol was based on an indirect biotin-avidin system using a biotinylated universal secondary antibody and diaminobenzidine substrate with hematoxylin counterstaining. The specificity of the binding was confirmed by negative staining using rabbit nonimmune serum as a primary antibody.

Androgen stimulation. After incubation for 2 d in phenol red–free RPMI 1640 with 10% charcoal-stripped FBS, LNCaP cells were treated with 10 nmol/L R1881 (Sigma-Aldrich). Cells were harvested for RNA isolation at 0-, 3-, 6-, 12-, 24-, and 48-h time points. Primer sequences were 5′-GATAGGATGGGGTGTCTGTGTT-3′ and 5′-AGTCCCTCTCCTTACTTCATCC-3′ for *PSA* (*KLK3*), and 5′-CCGCACTGAGATCAACAAGA-3′ and 5′-AATCTGTTCCTGGCATTAAGCTC-3′ for *PRL3* as a loading control. The primer sequences of *ELOVL7* were described above.

Short hairpin RNA–expressing vectors and cell viability assay. Plasmids designed to express short hairpin RNA (shRNA) were prepared by cloning of double-stranded oligonucleotides into psiU6BX vector (18). The target sense-strand sequences of *ELOVL7* are 5′-CAAGCAACAACAACAACAA-CAA-3′ (si1): 5′-GCCTTCAGTGATCTTACAT-3′ (si2) and 5′-GAAGCAGCAC-GACTTCTTC-3′(siEGFP) as a negative control. The sense-strand sequence of *S1PR3* shRNA is 5′-TGAATGTTCCTGGGGCGCT-3′. LNCaP or 22Rv1 cells (2×10^6) were grown on 10-cm dishes, transfected with each of si1, si2, and siEGFP vectors using FuGene6 reagent (Roche), and cultured in appropriate medium containing 800 μg/mL of geneticin (Sigma-Aldrich) for 2 wk. The cells were fixed with 100% methanol, stained with 0.1% of crystal violet-H_2O for colony formation assay. In MTT assay, cell viability was measured using Cell-counting kit-8 (DOJINDO) at 10 d after transfection. Absorbance was measured at 490 and 630 nm with Microplate Reader 550 (Bio-Rad). We also used synthesized siRNA duplexes to *ELOVL7* (siELOVL7) and *SREBP1* (siSREBP1), purchased from Dharmacon, which showed significant knockdown effect on ELOVL7 or *SREBP1* expression. ON-TARGETplus siCONTROL-oligo (Dharmacon) was used as a negative control RNA duplex.

Fatty acid analysis by gas chromatography mass spectrometry. LNCaP cells were transfected with shRNA-expression vector (si1 or siEGFP) and incubated with geneticin for 7 d. Lipids were extracted from the cells by Folch liquid (methanol/chloroform 1:2, vol:vol) and evaporated under nitrogen gas. After hydration by 0.5 mol/L HCl, free fatty acid was extracted by chloroform and methyl esterized by 0.4 K methoxide/methanol and 14% boron trifluoride methanol. Each fatty acid level in the cells was subjected to gas chromatography mass spectrometry (GC/MS) analysis (GC-17A, Shimadzu).

Recombinant ELOVL7 protein and *in vitro* fatty acid elongation assay. Full-length *ELOVL7* cDNA was amplified by PCR using primers that were designed to contain 6×His-tag sequences at the NH_2 terminus, and was cloned into the pBacPAK9 vector (Clontech). Sf21 cells were cultured in Grace's insect medium (Life Technologies) supplemented with 10% FBS and 50 μg/mL gentamicin at 27°C and were infected with the indicated recombinant baculovirus. The cells were collected 72 h after infection, and their microsomes were isolated by differential centrifugation with the modified procedure described by Moon and colleagues (19). For Western blot analysis, the isolated microsomal proteins were denatured in SDS sample buffer at 4°C overnight, and 30 μg of proteins were loaded onto 15% SDS-PAGE gel and blotted onto nitrocellulose membranes. Membranes were incubated with HA-tag antibody (Roche) or anti-6×His antibody (B&D), and protein bands were visualized by chemiluminescent detection system (ECL, GE Healthcare Biosciences). The microsomes (10–200 μg) from the infected Sf21 cells or noninfected Sf21 cells as a control were incubated in a total reaction volume of 0.45 mL containing 0.1 mol/L Tris-Cl (pH 7.4), 3 mmol/L arachidoyl-CoA, 7.5 mmol/L malonyl-CoA, 20 mmol/L NADPH, and 0.6 mmol/L fatty acid–free bovine serum albumin (Sigma-Aldrich). Reaction was performed in a glass tube where the vapor phase was substituted by nitrogen gas and kept on ice. The reaction was incubated at 37°C for 5 min and stopped with Folch liquid (methanol/chloroform, 1:2, vol:vol). Each of fatty acid composition in the reaction samples was analyzed by GC/MS as described above.

Establishment of LNCaP-ELOVL7 cells and *in vivo* study. Full-length *ELOVL7* cDNA tagged with HA was cloned into the pCAGGS vector. LNCaP cells were seeded into 100-mm dish (5×10^5 cells per a dish) and transfected with 6 μg of pCAGGS empty vector alone or pCAGGS-HA-ELOVL7 vector using FuGENE6 reagent (Roche). Cells were selected with 400 μg/mL of geneticin for 2 wk and discrete colonies were isolated. Each clone was assayed for ELOVL7 protein expression by Western blot analysis using anti-HA antibody (Roche). For *in vivo* growth assay, 5×10^6 cells of LNCaP-ELOVL7 cells or LNCaP-Mock cells were inoculated to the flank of male 7-wk nude mice with Matrigel (B&D), and these mice were bred with normal diet or high-fat diet containing 10% beef fat (CLEA). Norman diet composition was as follows: 6% fat, 25% protein, and 343 kcal/100 grams. High-fat diet composition was as follows: 14% fat, 25% protein, and 507 kcal/100 grams. The tumor volumes were calculated by L×S×S×0.52 mm^3. The mice were bred under the Ethnical committee of The University of Tokyo.

Lipid subfractionation and fatty acid analysis by GC/MS. C4-2B cells were transfected with siRNA duplex (siELOVL7 or siCONTROL) to knock down ELOVL7. After 3 d of incubation, total lipids were extracted from the cells by Folch liquid. After nitrogen evaporation, the total lipid extract was dissolved in chloroform and loaded on silica column (Iatro beads). The neutral lipid fraction and the phospholipid fraction were eluted by chloroform and chloroform/methanol (1:1, v/v), respectively. Neutral lipid was separated by TLC (HP-TLC) with the solvent system (Hexane/diethylether/acetic acid, 80:20:2). After primuline spray, the spots corresponding to triglycerides and cholesterol ester were extracted with chloroform/methanol/water (2:2:1.8, v/v/v) and analyzed for fatty acid composition. Phospholipid was loaded onto a DEAE-sephadex A25 column and the neutral and acidic lipid fractions were successively eluted (20). Fatty acid composition was determined by GC/MS after transmethylation using

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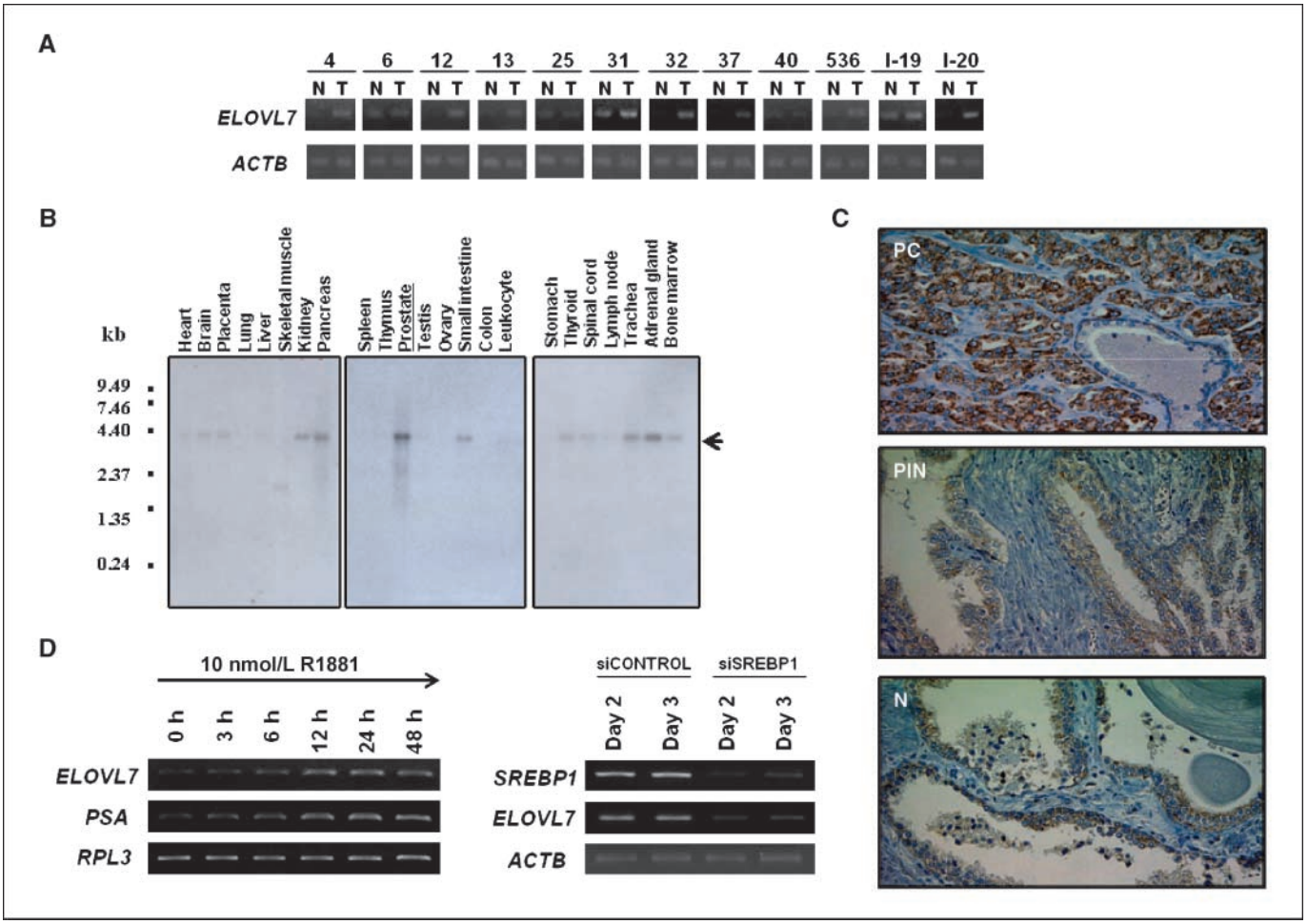

Figure 1. Overexpression of ELOVL7 in prostate cancer cells and regulation of ELOVL7 expression. *A,* semiquantitative RT-PCR analysis of *ELOVL7* in prostate cancer cells (*T*) and normal prostatic epithelial cells (*N*) that were microdissected from 10 prostate cancer tissues. *Horizontal lines,* clinical N/T pair cases. *ACTB* (β-actin) was used to quantify cDNA contents. *B,* multiple tissue Northern blot analysis showed that *ELOVL7* was strongly expressed in prostate, kidney, pancreas, and adrenal gland. *C,* immunoreactivity with anti-ELOVL7 antibody was observed in prostate cancer tissues, exhibiting strong positive immunostaining in the cytoplasm of prostate cancer cells (*top*), whereas a weak immunopositivity was observed in PIN precursors (*middle*) and noncancerous prostate epithelium (*bottom*). *D,* semiquantitative RT-PCR showed that androgens stimulated *ELOVL7* expression as well as prostate-specific antigen (PSA) in LNCaP cells (*left*). *RPL3* was used to normalize expression. Semiquantitative RT-PCR showed that knockdown of *SREBP1* (*siSREBP1*) decreased *ELOVL7* expression in LNCaP cells (*right*). *ACTB* was used to normalize expression.

boronfluoride-methanol reagent under nitrogen (100°C, 90 min) as previously described (21).

Quantitative analysis of testosterone and dihydrotestosterone by liquid chromatography tandem mass spectrometry. The amount of testosterone and dihydrotestosterone (DHT) in cells or tissues was measured by highly sensitive liquid chromatography tandem mass spectrometry analysis in Teikoku Hormone Medical Co., Ltd, as described previously (22). Briefly, 1 ng of T-d3 [T-19-C^2H_3] and 1 ng of DHT-d3 [17,16,16-$^2H_3$]-DHT were added as internal standards to the individual homogenized cells or tissues. After the purification by reverse-phase chromatography using a 3-mL Bond Elut C18 cartridge column (Varian), the dried steroidal fraction was reacted with 100 μL of reagent mixture (20 mg 2-methyl-6-nitrobenzoic anhydride, 10 mg 4-dimethyl-aminopyridine, and 20 mg picolinic acid in 1 mL of tetrahydrofuran) and 15 μL of triethylamine for 60 min at room temperature. The collected steroidal fraction was applied to liquid chromatography tandem mass spectrometry instrument 4000QTRAP (Applied Biosystems) equipped with ESI ion source and a Shimadzu HPLC system (Shimadzu).

Genome-wide cDNA microarray analysis and S1PR3 expression. Synthesized siRNA duplexes to *ELOVL7* (siELOVL7) or siCONTROL-oligo (Dharmacon) were transfected to C4-2B cells as described above, and total RNAs were extracted 48 h after the transfection. Total RNAs were also extracted from the ELOVL7-overexpressing tumors of the mice bred with high-fat diet or normal diet. After RNA amplification and labeling, these RNAs (siELOVL7 versus siCONTROL or high-fat diet versus normal diet) were competitively hybridized on the gnome-wide cDNA microarrays and analyzed as described previously (18). The hybridization was performed triplicated for each experiment. *S1PR3* expression was evaluated by RT-PCR using the primers 5′-TCTAATTGAAAAGAGACCTAATGC-3′ and 5′-TAACAAGACTTTTGAGCAAT-3′.

Results

Identification of a novel gene, *ELOVL7*, and its expression pattern. Through our genome-wide expression profiles of microdissected prostate cancer cells (17), we identified a dozen of transactivated genes in prostate cancer cells. Among them, we here focused on a novel gene, *ELOVL7*. Semiquantitative RT-PCR confirmed up-regulation of *ELOVL7* in prostate cancer cells from 8 of 10 prostate cancer tissues (Fig. 1*A*). As for the tissue distribution of *ELOVL7*, multiple tissue Northern blot analysis

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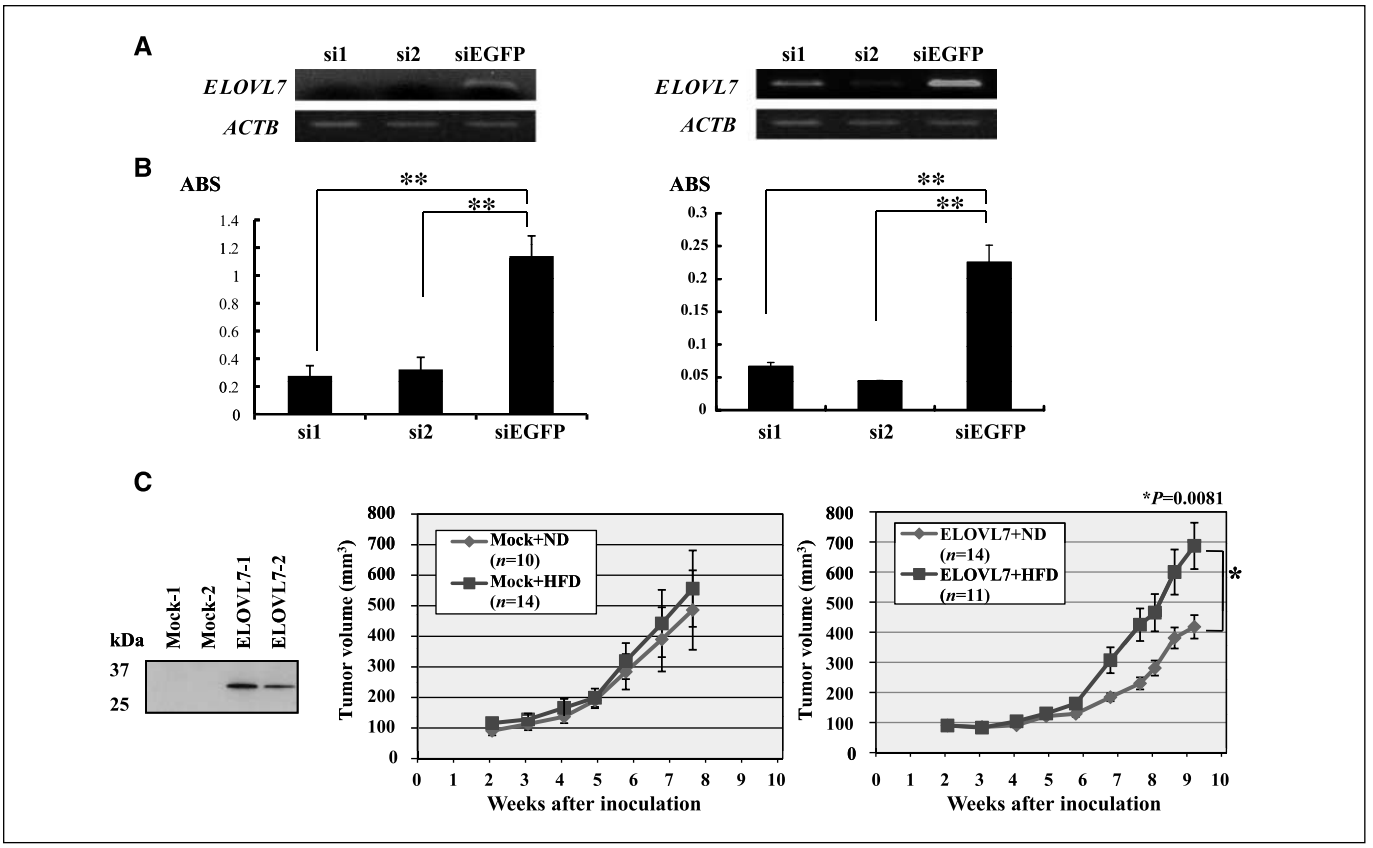

Figure 2. ELOVL7 was involved in prostate cancer cell growth. *A,* knockdown effect on ELOVL7 in 22Rv1 cells (*left*) and LNCaP cells (*right*). Semiquantitative RT-PCR was performed using cells transfected with shRNA-expressing vectors to *ELOVL7* (*si1* and *si2*) as well as a negative control vector (*siEGFP*). *ACTB* was used as a loading control. *B,* MTT assay of 22Rv1 cells (*left*) and LNCaP cells (*right*) transfected with indicated shRNA-expressing vectors to *ELOVL7* (*si1* and *si2*) and a negative control vector (*siEGFP*). *Columns,* mean; *bars,* SD. *ABS on Y-axis,* absorbance at 490 nm, and at 630 nm as a reference. **, $P < 0.01$. *C,* high-fat diet promoted ELOVL7-expressing prostate cancer cells *in vivo*. Western blot analysis using HA-tag antibody (*left*) showed that two clones of LNCaP-ELOVL7 (ELOVL7-1 and ELOVL7-2) constitutively expressed exogenous ELOVL7. LNCaP-mock cells (*middle*) were inoculated into male nude mice that were bred with normal diet (*ND*; $n = 10$) or high-fat diet (*HFD*; $n = 14$). LNCaP-ELOVL7-1 cells (*right*) were inoculated into male nude mice that were bred with normal diet ($n = 14$) or high-fat diet ($n = 11$). High-fat diet breeding significantly promoted the growth of LNCaP-ELOVL7-1 cells *in vivo* compared with normal diet breeding ($P = 0.0081$ by Student's *t* test).

identified an ~3.8-kb *ELOVL7* transcript in the prostate, kidney, adrenal gland, and several tissues (Fig. 1*B*), but semiquantitative RT-PCR analysis showed that *ELOVL7* expression in prostate cancer cells was much higher than that in normal kidney or prostate (Supplementary Fig. S1*A*), implicating its distinct expression in prostate cancer cells. To further investigate the expression of ELOVL7 protein in prostate cancer cells, we generated a polyclonal antibody to ELOVL7 and performed immunohistochemical analysis using clinical prostate cancer tissues. As shown in Fig. 1*C*, strong immunochemical signal for ELOVL7 was detected predominantly in the cytoplasm of prostate cancer cells in all of 12 prostate cancer tissues we examined, whereas we observed weak signals in noncancerous prostate epithelial cells and PINs. Immunohistochemical analysis on various normal organs observed ELOVL7 expression in the kidney and the adrenal gland as well as the prostate (Supplementary Fig. S1*B*), which was concordant with the result from Northern blot analysis.

Androgen stimulated ELOVL7 expression through SREBP1. A number of lipogenic factor expressions are regulated by androgens (23, 24). ELOVL7 is possibly involved in elongation of long-chain fatty acids and belongs to a class of lipogenic enzyme. To examine whether androgen stimulation could transactivate ELOVL7 expression, we stimulated LNCaP cells with synthetic androgen R1881, and checked ELOVL7 expression at several time points. Semiquantitative RT-PCR (Fig. 1*D, left*) showed that androgens stimulated ELOVL7 expression as well as prostate-specific antigen (PSA). The expressions of many lipogenic enzymes are regulated by androgens through sterol-regulatory element binding protein-1 (SREBP-1; refs. 24, 25). Then, we knocked down SREBP-1 in prostate cancer cells by siRNA duplex (siSREBP1) and checked ELOVL7 expression. In Fig. 1*D* (*right*), introduction of siSREBP1 in prostate cancer cells decreased ELOVL7 expression as well as SREBP-1 expression, suggesting that ELOVL7 expression could be regulated by androgen through SREBP-1 as well as other lipogenic factors.

Knockdown of ELOVL7 expression attenuated prostate cancer cell growth. To examine the biological roles of ELOVL7 in prostate cancer cells, we constructed several shRNA expression vectors targeting *ELOVL7*, and transfected them into LNCaP and 22Rv1 cells, which expressed ELOVL7 endogenously. When si1 and si2 were transfected into 22Rv1 cells (*left*) and LNCaP cells (*right*), a significant knockdown effect of *ELOVL7* expression was observed by semiquantitative RT-PCR (Fig. 2*A*). MTT assay (Fig. 2*B*) and colony formation assay (Supplementary Fig. S2*A*) showed that introduction of si1 and si2 in 22Rv1 cells (*left*) and LNCaP cells (*right*) drastically attenuated their cell growth, whereas siEGFP did not.

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Fatty diet promoted the growth of ELOVL7-expressing prostate cancer cells *in vivo*. To further investigate the oncogenic function of ELOVL7 in prostate cancer cells, we generated LNCaP-derived clones (LNCaP-ELOVL7-1 and LNCaP-ELOVL7-2) that constitutively expressed ELOVL7 at higher level (Fig. 2*C*). We compared the growth of LNCaP-ELOVL7-1 cells and LNCaP-Mock cells *in vitro*, but there was no significant growth-promoting effect by ELOVL7 overexpression *in vitro* (data not shown). Because high-fat dietary intake or lipid overload can promote prostate cancer growth or progression (3–6), we examined how ELOVL7 expression could be involved in possible oncogenic effect in the presence of lipid overload. We inoculated prostate cancer cells overexpressing ELOVL7 (LNCaP-ELOVL7-1) or mock cells (LNCaP-Mock) in nude male mice and bred them with normal diet or high-fat diet. As shown in Fig. 2*C*, LNCaP-Mock cells did not reveal any significant growth promotion by breeding with high-fat diet. On the other hand, high-fat diet breeding significantly promoted the growth of LNCaP-ELOVL7-1 cells *in vivo* ($P = 0.0081$; Fig. 2*C*). When another clone ELOVL7-2 cells were inoculated to mice bred with or without high-fat diet, we also observed similar result (Supplementary Fig. S2*B*). These findings are consistent with previous experimental evidences (7–9), suggesting that ELOVL7 might be a key molecule that might explain the linking between fatty diet intake and prostate carcinogenesis.

Knockdown of ELOVL7 affected fatty acid composition in prostate cancer cells. Members of ELOVL family (ELOVL1–6) catalyze the elongation reaction of long-chain fatty acids (C≥18) with some specificity for different chain length of saturated or

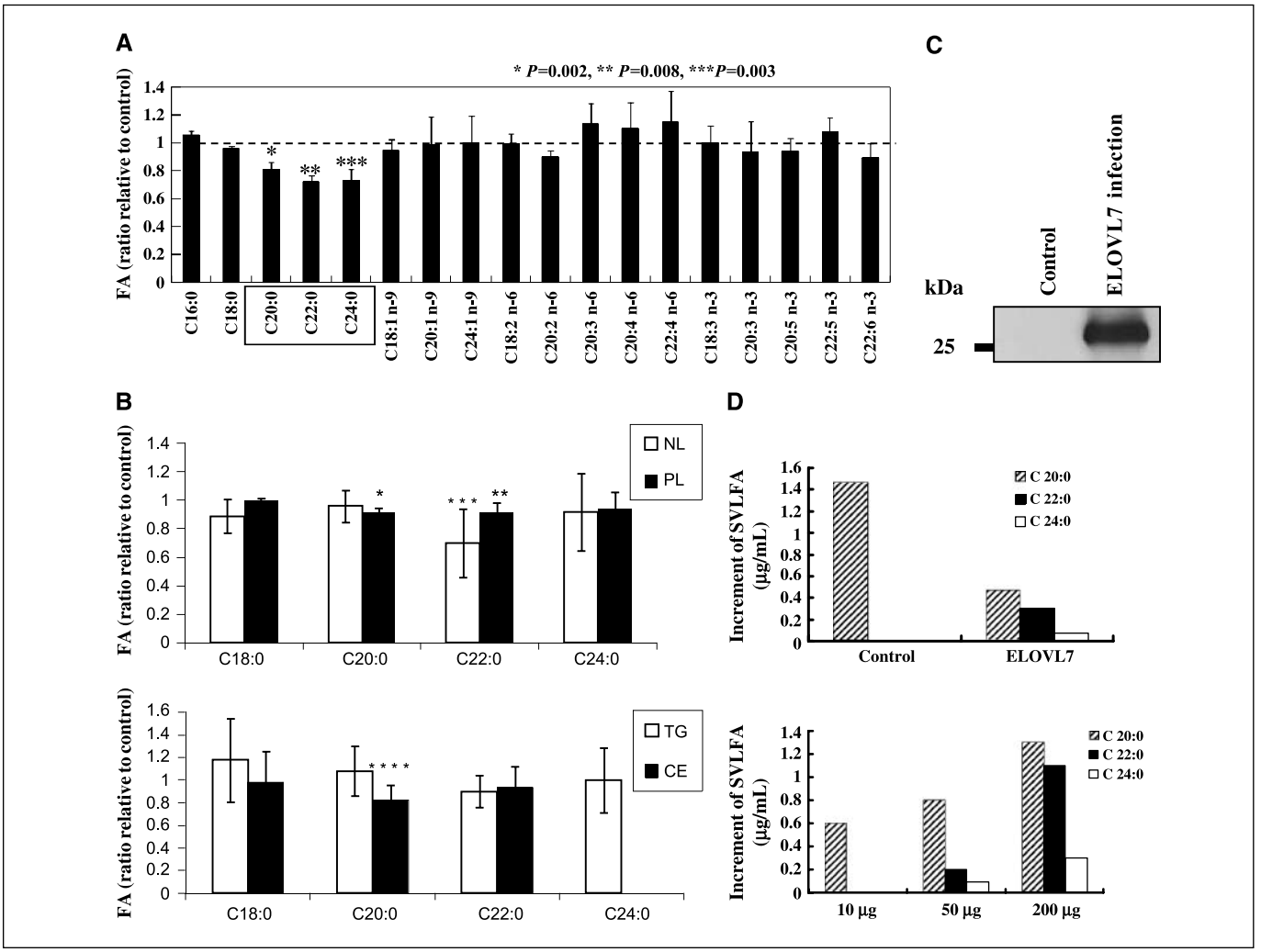

Figure 3. ELOVL7 was involved in the production of SVLFAs. *A,* the total fatty acid composition of C4-2B cells in ELOVL7 knockdown was analyzed by GC/MS. *Y*-axis shows the relative ratio of long-chain fatty acids (siELOVL7 versus siEGFP). ELOVL7 knockdown resulted in the specific reduction of SVLFAs ($P = 0.002$ for C20:0; $P = 0.008$ for C22:0; $P = 0.003$ for C24:0). *B,* GC/MS analysis after the purification on silica beads displayed decrease trend of SVLFAs in phospholipid (*PL*) and neutral lipid (*NL*) fractions (C20:0 in phospholipid: *, $P = 0.006$; C22:0 in phospholipid: **, $P = 0.014$; and C22:0 in neutral lipid: ***, $P = 0.072$ by Student's test) in C4-2B cells by ELOVL7 knockdown (*top*). In neutral lipid fraction [triglycerides (*TG*) and cholesterol ester (*CE*)], C20:0 fraction in cholesterol ester was significantly decreased in ELOVL7 knockdown (****, $P = 0.0489$; *bottom*). C24:0 in cholesterol ester fraction was not detected by GC/MS analysis. *Y*-axis showed the relative ratio of each fatty acid of siELOVL7-treated prostate cancer cells versus that of siCONTROL-treated cells, and these experiments were performed in quadruplicate. *C,* Western blot analysis revealed that ELOVL7 protein was present in the microsome fraction of Sf21 cells infected with ELOVL7-expressing baculovirus. *D, in vitro* fatty acid elongation activity using an arachidoyl (C20:0)-CoA as a substrate and the microsome fraction as an enzyme source. Elongation activity was measured as an increment of each fatty acid level before and was measured by GC/MS analysis (*Y*-axis, μg/mL) after the 5-min reaction. The microsome (50 μg) prepared from ELOVL7-expressing cells produced C22:0 and C24:0, whereas the microsome from uninfected cells did not at all (*top*). An increment of each fatty acid level in 10, 50, and 200 μg of the enzyme source microsome fraction was measured (*bottom*). Efficiency of the fatty acid chain elongation of the microsome fraction of ELOVL7-expressing cell was dose dependent.

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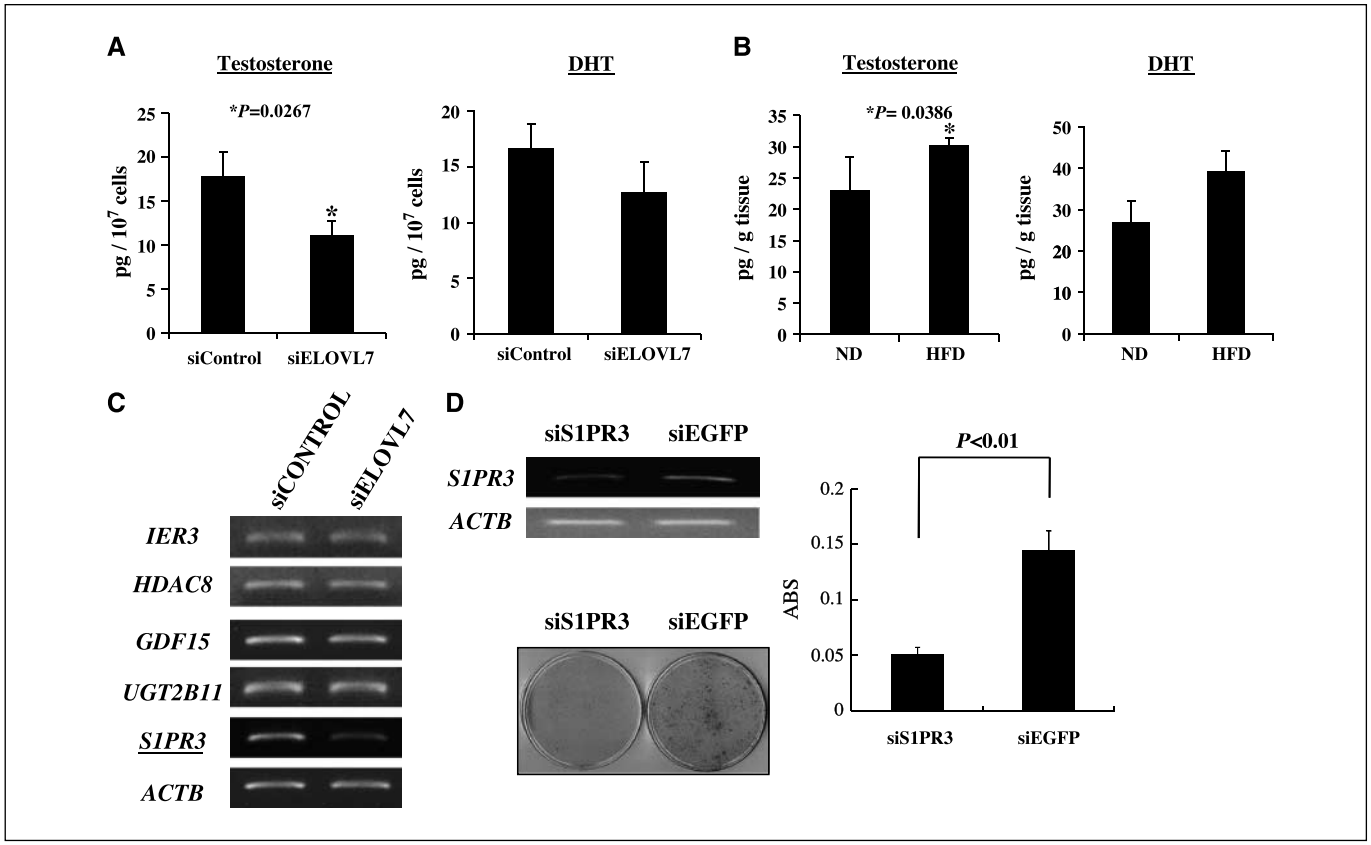

Figure 4. ELOVL7 affected *de novo* androgen synthesis and lipid signaling. *A,* ELOVL7 knockdown significantly decreased testosterone level in C4-2B cells ($P = 0.0267$) and there was also a decreasing trend in DHT levels in C4-2B cells in ELOVL7 knockdown ($P = 0.11289$). *B,* the tumors in the high-fat diet group contained significantly more testosterone than the tumors in normal diet group ($P = 0.0386$). There was also an increasing trend in DHT levels in the tumors in high-fat diet group compared with DHT in normal diet group ($P = 0.1659$). *C,* RT-PCR analysis validated that ELOVL7 knockdown decreased *S1PR3* expression in C4-2B cells among five candidates (*IER3, HDAC8, GDF15, UGT2B11*, and *S1PR3*) as the down-streaming genes of ELOVL7. *D,* RT-PCR showed the shRNA to S1PR3 knocked down *S1PR3* expression in C4-2B cells, compared with a negative control vector (*siEGFP*). S1PR3 knockdown in C4-2B cells significantly attenuated the cell growth, which was evaluated by colony formation assay and MTT assay ($P < 0.01$, by Student's *P* test).

unsaturated fatty acids (15, 26). To examine the specificity of ELOVL7 as a fatty acid elongase, we knocked down ELOVL7 in LNCaP cells and analyzed their fatty acid composition by GC/MS. In Fig. 3*A*, ELOVL7 knockdown resulted in the significant reduction of SVLFAs (C20:0, C22:0, C24:0) compared with the control, whereas it did not affect the level of monounsaturated and polyunsaturated ones. We also more efficiently knocked down ELOVL7 in C4-2B cells by synthesized siRNA duplexes and we observed more drastic reduction of SVLFAs (C20:0, C22:0, C24:0) specifically (Supplementary Fig. S3). To precisely identify which lipid classes were affected by ELOVL7, we purified the phospholipids and neutral lipid fractions on silica beads after ELOVL7 knockdown, and analyzed their fatty acid composition by GC/MS. In Fig. 3*B* (*top*), GC/MS analysis displayed a decreased trend of SVLFAs in both phospholipid and neutral lipid fractions (C20:0 in phospholipid, $P = 0.006$; C22:0 in phospholipid, $P = 0.014$; and C22:0 in neutral lipid, $P = 0.072$) in C4-2B cells by siELOVL7. But no significant reduction of C24:0 in both phospholipid and neutral lipid fractions was observed, probably because C24:0 in other lipid fractions, such as glycolipids, might be affected by ELOVL7 knockdown. Focusing on neutral lipid, we purified the main esterified components of the neutral lipid fraction, triglycerides, and cholesterol ester using TCL, and analyzed SVLFAs content in triglycerides and cholesterol ester fractions. As a result, C20:0 fraction in cholesterol ester was significantly decreased in ELOVL7 knockdown ($P = 0.0489$; Fig. 3*B, bottom*).

***In vitro* fatty acid elongation.** To further validate the fatty acid elongase activity of ELOVL7, recombinant ELOVL7 protein was generated in insect cells and their microsome fraction was purified. Western blot analysis with anti-6×His antibody revealed that the expressed ELOVL7 protein was present in the microsome fraction (Fig. 3*C*). However, ELOVL7 recombinant protein could not be purified due to its insolubility. Hence, we used this microsome fraction as an enzyme source for the *in vitro* fatty acid elongase reaction. Elongation activity was measured as an increment of each fatty acid level before and after the 5-minute reaction. Because nearly a half of fatty acids in the microsome fraction were composed of stearic acid (C18:0), arachidoyl-CoA (C20:0) was used as a substrate for this *in vitro* reaction. In Fig. 3*D* (*top*), the microsome (50 μg) prepared from ELOVL7-expressing cells showed high activity of fatty acid elongase that produced C22:0 and C24:0, whereas the microsomes from uninfected cells did not. We repeated this *in vitro* assay using 10, 50, and 200 μg of the enzyme source microsome, and observed that this elongation reaction was reproducible and the efficiency of fatty acid chain elongation of the microsome fraction of ELOVL7-expressing cell was dose dependent (Fig. 3*D, bottom*).

ELOVL7 affected *de novo* androgen synthesis in prostate cancer cells. Cholesterol ester is the stored source mainly for steroid synthesis in the steroidogenic cells such as the adrenal

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gland and the testis, and we hypothesized that prostate cancer cells could use the stored cholesterol ester for *de novo* steroid synthesis to produce their most critical growth factor, androgens. By the highly sensitive LC-MS/MS analysis, we measured testosterone and DHT levels of the cells or the tissues when ELOVL7 expression was modulated in prostate cancer cells. When ELOVL7 was knocked down, testosterone and DHT level was significantly decreased in prostate cancer cells (Fig. 4*A*). The progenolone level was also significantly decreased in prostate cancer cells when ELOVL7 was knocked down (data not shown). Furthermore, because high-fat diet promoted the growth of ELOVL7-overexpressing prostate cancer cells *in vivo* (see Fig. 2*C*), we measured SVLFAs, testosterone, and DHT in these tumors and we observed significant increase of testosterone and an increased trend in DHT in the tumors of the high-fat diet group (Fig. 4*B*), as well as SVLFAs content (Supplementary Fig. S4). We did not observe any significant difference of testosterone and DHT content in Mock cells treated with high-fat diet and normal diet.

ELOVL7 regulates the expression of sphingosine 1-phosphate receptor 3. To investigate into other possible downstream pathways of ELOVL7, we performed the gene expression analysis by the genome-wide cDNA microarrays. The genome-wide gene expression patterns were compared between prostate cancer cells transfected with siELOVL7 and prostate cancer cells transfected with siCONTROL. The gene expression patters were also compared between the ELOVL7-overexpressing tumors bred with high-fat diet and those with normal diet. As a result, we identified five genes (*IER3, HDAC8, GDF15, UGT2B11*, and *S1PR3*) whose expressions were down-regulated with 20% and more in ELOVL7 knockdown and also were up-regulated with 20% and more in the tumors of the mice bred with high-fat diet. Among them, RT-PCR validated significant down-regulation of *S1PR3* (Fig. 4*C*) in ELOVL7 knockdown. S1PR3 is one of the functional receptors for sphingosine 1-phosphate (S1P), which is a bioactive lipid mediator released from ceramides composed of long-fatty acids, and this pathway is reported to regulate diverse biological functions including cell proliferation and migration (27, 28). Thus, to examine the involvement of S1PR3 in prostate cancer cell growth, we knocked down S1PR3 expression in LNCaP cells (Fig. 4*C*), and S1PR3 knockdown significantly affected LNCaP cell growth or survival. These findings indicated S1P-S1P receptor pathway could be associated with ELOVL7 and prostate carcinogenesis, and knockdown of ELOVL7 might affect S1P production and lead to the feedback down-regulation of their receptor, S1PR3.

Discussion

In this study, we identified a novel lipogenic enzyme ELOVL7 as an overexpressed molecule in prostate cancer cells. Six ELOVL family members (ELOVL1–6) were characterized in mammals thus far and some of them display tissue-specific expression or specific fatty acid substrate selectivity (15, 26). According to our genome-wide expression data of prostate cancer cells and other organs (17), ELOVL7 was expressed at the highest level in the prostate and prostate cancer cells among seven ELOVL family members. Such expression patterns can lead to the speculation that ELOVL7 could be involved in specific metabolic pathways of long-chain fatty acids and other lipids that could play some important roles in prostate carcinogenesis.

Notably, in our microarray studies (17, 18), several genes associated with lipid or cholesterol metabolism were up-regulated in prostate cancers, as well as ELOVL7, and there is a growing body of evidence that lipid metabolism and the lipid metabolism–associated genes could play some significant roles in prostate cancer development and progression through their involvement in metabolic pathways or antiapoptotic effect (29–31). Among them, fatty acid synthase (FASN) has been shown to be overexpressed in a wide range of cancers and it is responsible for synthesis of palmitate (C16:0) by using acetyl-CoA as a primer and malonyl-CoA as a two carbon donor (see Fig. 5; refs. 29, 30). Acetyl-CoA carboxylase (ACC) was also overexpressed in cancer cells concordantly with FASN, and it catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA (C3), fueling malonyl-CoA for *de novo* synthesis of long-chain fatty acids and subsequent chain elongation by FASN (32). They are likely to function together with ELOVL7 on the same pathway involving fatty acid metabolism (Fig. 5), and the products of ELOVL7 could be incorporated into cholesterol esters and used for *de novo* steroidogenesis, energy

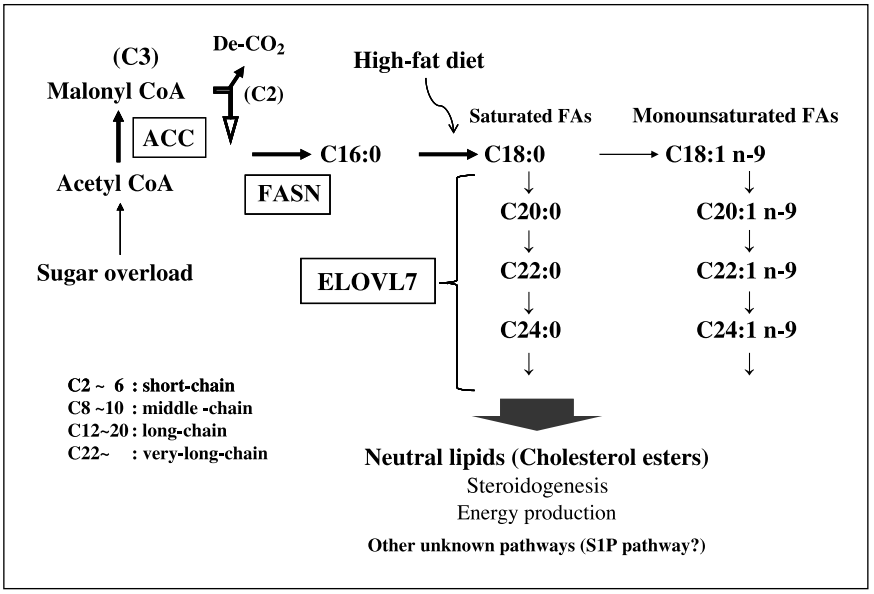

Figure 5. Schematic diagram of saturated fatty acid biosynthetic pathway mediated by ELOVL7. ACC catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA (*C3*) and fuels malonyl-CoA for *de novo* synthesis of long-chain fatty acids and subsequent chain elongation. FASN synthesizes long-chain fatty acids by using acetyl-CoA as a primer and malonyl-CoA as a two-carbon (*C2*) donor, and the predominant product of FASN is C16:0. ELOVL7 is responsible for the chain elongation of (very) long-chain saturated fatty acids (C18:0 ~), and the products of ELOVL7 are incorporated into the neutral lipids such as cholesterol esters and the phospholipids, and used for *de novo* steroidogenesis, energy production, and other unknown pathways probably involving lipid signaling such as S1P pathway in prostate cancer cells.

production, and other unknown pathways involving with prostate cancer growth. Overexpressing FASN and ACC have been suggested as a relevant drug target (29–32), and some cholesterol synthesis inhibitors are now expected to be potential drugs for cancer prevention or treatment because high levels of cholesterol was reported to promote prostate cancer cell proliferation (12, 13, 33). Targeting ELOVL7 enzyme or SVLFA metabolic pathways controlled by ACC-FASN-ELOVL7 (Fig. 5) could be a promising approach for novel therapeutic and preventive strategies against prostate cancers.

Long-chain fatty acids in the phospholipids are essential for membrane stabilization and raft formation where many oncogenic or growth signaling molecules are anchored (33). At first, we investigated for raft formation associated with ELOVL7, but knockdown of ELOVL7 did not affect the raft formation in prostate cancer cells. Next, we focused on cholesterol ester in the neutral lipids, because cholesterol ester is the stored source mainly for steroid synthesis in steroidogenic cells and prostate cancer cells could use the stored cholesterol ester for *de novo* steroid synthesis to produce androgens. Ours and other recent reports (34, 35) have shown that prostate cancer cells expressed CYP11A1 (data not shown), which is the bottleneck enzyme of *de novo* steroid synthesis from cholesterol in steroidogenic cells, indicating possibility of *de novo* steroid synthesis from cholesterol in prostate cancer cells. We here showed that ELOVL7 expression could affect *de novo* steroid and androgen synthesis in prostate cancer cells, although molecular mechanism leading to the enrichment of SVLFAs in the cholesterol ester fraction is unknown. In addition, ELOVL7 can possibly activate other lipid-associated signaling pathways through SVLFA synthesis. Our microarray analysis suggested the S1P-S1P receptor pathway could be associated with ELOVL7 and prostate carcinogenesis. S1P is released from ceramide, which is composed of long-chain fatty acids (28) and ELOVL7, might produce S1P and other derivatives. Although how ELOVL7 can regulate S1PR3 is unknown, S1PR3 expression is also likely to be regulated with SREBP1, and this pathway could be tightly regulated by lipogenic pathways including ELOVL7. The detail mechanism and other molecules or pathways involved by ELOVL7 in prostate cancer should be further investigated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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