NOVEL BIOSYNTHESIS PATHWAYS OF FOOD BASED ODD-NUMBERED CARBON CHAIN AND BRANCHED CHAIN FATTY ACIDS

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Zhen Wang

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NOVEL BIOSYNTHESIS PATHWAYS OF FOOD BASED ODD-NUMBERED CARBON CHAIN AND BRANCHED CHAIN FATTY ACIDS

Zhen Wang, Ph. D.

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Normal odd-numbered carbon chain fatty acids (n-OCFA) and branched chain fatty acids (BCFA) are both important components of dairy, beef and seafood. n-OCFA pentadecanoic acid (n-15:0) and heptadecanoic acid (n-17:0) are biomarkers of dairy, seafood and dietary fiber intake, because the consumption of n-15:0 and n-17:0 from these foods is reflected in human tissue FA composition. Difference in the ratios of n-15:0 to n-17:0 between foods and human tissues suggests that endogenous FA interconversion alters their ratio. BCFA are rare in most internal tissues, but present in skin sebaceous glands (SG), meibomian glands, and vernix caseosa in high levels. Little is known about the endogenous metabolism of n-OCFA and BCFA once ingested. Thus, we explored the substrate specificity of the human fatty acid interconversion enzymes, elongases ELOVL1-7, and desaturases SCD and FADS 1-2 toward n-OCFA and BCFA.

The first study investigated the elongation of n-OCFA n-15:0 and n-17:0. ELOVLx transient transfected MCF-7 cells were treated with n-13:0, n-15:0 and n-17:0. ELOVL6 is the most active enzyme catalyzing n-13:0 → n-15:0 →

n-17:0; ELOVL7 has modest activity toward n-15:0 (n-15:0 → n-17:0); no elongation activity was detected for any ELOVL toward n-17:0 → n-19:0. The second study focused on elongations of representative BCFA *anteiso*-15:0 and *iso*-18:0. ELOVL6 mediates *anteiso*-15:0 → *anteiso*-17:0, while ELOVL3 is active toward *iso*-18:0 → *iso*-20:0. Substrate competition studies between BCFA and n-FA for ELOVLx mediated elongations revealed n-16:0 is preferred over *anteiso*-15:0 for ELOVL6, while n-18:0 is preferred over *iso*-18:0 in ELOVL3 transfected cells. The third study characterized the FADS2 mediated Δ6-desaturation in stable FADS2 MCF-7 cells towards BCFA *iso*-16:0 → *iso*-6Z-16:1, *iso*-18:0 → *iso*-6Z-18:1, *iso*-17:0 → *iso*-6Z-17:1 and *anteiso*-17:0 → *anteiso*-6Z-17:1, and towards n-OCFA n-17:0 → n-6Z-17:1. FADS2, which is highly expressed in human SG, is responsible for desaturating BCFA and n-OCFA found on the skin. These novel pathways of n-OCFA and BCFA have implications in understanding their metabolism and inform the association between metabolic disease and gene-regulated FA composition shift.

BIOGRAPHICAL SKETCH

Zhen Wang was born in Zhengzhou, Henan, China. After got her Bachelor of Engineering degree in Food Science and Engineering from China Agricultural University, Beijing, China, Zhen went to University of Minnesota-Twin Cities, Minnesota, US, in 2012, and studied Food Science towards Master of Science degree. During that period, Zhen spent 2 years in Dr. Roger Ruan's lab doing degree-related project on edible microalgae that are high in protein, oil and polyunsaturated acid (PUFA), and published a paper on it. From this experience, Zhen developed her research interest in oil and fatty acid composition of food and their health impact on humans. Therefore, Zhen applied to the Ph. D. program in the department of Food Science at Cornell University after she graduated, and luckily got accepted into Dr. J. Thomas Brenna's lab in 2014, and started working on food based fatty acid metabolism and the relevant health effects. Zhen met his husband, Donghao Wang, in the same lab at Cornell, and they got married in the great summer time of 2016 at Ithaca, with the witness of their advisor, parents and friends. During the five years of her Ph.D. study, Zhen has written up 3 manuscripts for publication as first author, and contributed to 6 papers as a co-author. She has also done a summer research (R&D) intern at Nestle Purina, learned the methodology of mass spectrometry and its application in lipid and fatty acid identification. Zhen completed the requirements of her Ph.D. program in May 2019.

To
My Mother Lingyun Ziao
My Father Yuqing Wang
and
My husband Donghao

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AD: Atopic dermatitis

BCAA: Branched chain amino acids

BCFA: Branched chain fatty acids

BSA: Bovine serum albumin

C: Carbon atom number

CACI: Covalent adduct chemical ionization

CE: Cholesterol ester

C. elegans: *Caenorhabditis elegans*

CMV: Cytomegalovirus

CNS: Central nervous system

DB: Double bond

EC: Even- numbered carbon chain

EGF: Epidermal growth factor

EI: Electron ionization

ELOVL: ELOngases of Very Long chain fatty acids

ER: Endoplasmic reticulum

FA: Fatty acids

FACES: Fatty acid chain elongation system

FADS: Fatty acid desaturases

FAME: Fatty acid methyl esters

FASN/FAS: Fatty acid synthase

FBS: Fetal bovine serum

FID: Flame ionization detector

GC: Gas chromatography

GI: Gastrointestinal

GWAS: Genome-wide association study

HACD: 3-hydroxyacyl-CoA dehydratases

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

KAR: 3-ketoacyl-CoA reductase

LCFA: Long chain fatty acids

MBCFA: monounsaturated branched chain fatty acids

MS: Mass spectrometry

MEM-α: Minimum essential medium alpha

MUFA: Monounsaturated fatty acids

NADPH: Nicotinamide adenine dinucleotide phosphate

NASH: Nonalcoholic steatohepatitis

NEC: Necrotizing enterocolitis

n-ECFA: Normal even-carbon-numbered FA

n-FA: Normal/linear chain fatty acids

n-OCFA: Normal odd- numbered carbon chain FA

OC: Odd- numbered carbon chain

ORF: Open reading frame

P: Product

PBS: Phosphate-buffered saline

PL: Phospholipid

PUFA: Polyunsaturated fatty acids

RBC: Red blood cells

S: Substrate

SBCFA: Saturated branched chain fatty acids

SCD: Stearoyl-CoA desaturase

SFA: Saturated fatty acids

SG: Sebaceous glands

SNPs: Single nucleotide polymorphisms

TAG: Triglycerides

TER: 2,3-transenoyl-CoA reductase

WT: Wild type

VLCFA: Very long chain fatty acids

CHAPTER 1

INTRODUCTION

Classification of fatty acids

Fatty acids (FA) are important components of many lipid classes in human diet, such as triglycerides (TAG), phospholipid (PL) and cholesterol ester (CE); they can also come from human de novo biosynthesis and participate in functional activities in multiple organisms/tissues such as liver, heart and brain. Major FA found in nature can be classified by carbon atom (C) numbers on the main chain: short chain fatty acids with less than 6C; medium chain fatty acids with 6-12C; long chain fatty acids (LCFA) with 14-20C; and very long chain fatty acids (VLCFA) with 22C or more [1]. Depending on the exact carbon numbers, FA are classified into even-numbered carbon chain (EC) or odd-numbered carbon chain (OC) FA. FA could also be categorized by degree of unsaturation into saturated fatty acids (SFA) with no double bond (DB), monounsaturated fatty acids (MUFA) with only one DB, and polyunsaturated fatty acids (PUFA) with more than one DB in carbon chain [1]. Moreover, based on the terminal end structure of FA, they are specified into two kinds, branched chain fatty acids (BCFA) with one or more methyl branches usually on the methyl terminal end, or normal/linear chain fatty acids (n-FA) with linear methyl terminal end. The different classifications and nominations of FA will be applied in the following text with more explanations.

Fatty acid elongation

Most FA found in nature are normal even-carbon-numbered FA (n-ECFA), either saturated or unsaturated. Common FA found in human organisms/tissues are mainly LCFA including palmitic acid n-16:0, stearic acid n-18:0, oleic acid n-18:1 and linoleic acid n-18:2 [2]. Thus, this dissertation will focus on LCFA. The n-ECFA metabolism including elongation and desaturation will be introduced below.

The metabolism of n-ECFA in mammals have been studied intensively before [1-4]. Briefly, as for elongation, FA comprise of up to 16C are generated via fatty acid synthase (FASN) activity. Acetyl-CoA (2C) and propionyl-CoA (3C) serve as the primers of biosynthesis for normal EC and OCFA, respectively [4, 5]. FA carbon chain can be extended by 2 carbon units donated from malonyl-CoA in one elongation step [4]. FA are able to undertake elongation in a cyclic manner to synthesize new longer chain FA by FASN in cytosol until the ultimate product palmitic acid n-16:0 forms [4]. A certain amount of FA biosynthesized by FASN and FA consumed from the diet could be further elongated to LCFA and VLCFA by ELOngases of Very Long chain fatty acids (ELOVL), which mainly occurs in endoplasmic reticulum (ER) [4].

FA elongated in the ER are mediated continuously by four individual enzymes in elongation cycle including four separate reactions: condensation, reduction, dehydration and reduction [1, 4]. In mammals, condensation is a reaction

catalyzed by seven ELOVL (ELOVL1-7) that combines malonyl-CoA and fatty acyl-CoA, yielding 3-ketoacyl-CoA; reduction is catalyzed by 3-ketoacyl-CoA reductase (KAR), acts on 3-ketoacyl-CoA and produces 3-hydroxyacyl-CoA; dehydration of 3-hydroxyacyl-CoA generates trans-2-enoyl-CoA, which is mediated by four 3-hydroxyacyl-CoA dehydratases (HACD1-4); second-time-reduction is accomplished by 2,3-transenoyl-CoA reductase (TER) to generate the new acyl-CoA with two more carbon units compared to the initial acyl-CoA in the condensation step [1, 4]. Besides, nicotinamide adenine dinucleotide phosphate (NADPH) attends in both reduction processes and serves as reductant [1, 4]. In yeast, there are alternative individual enzymes mediating these four reactions [1]. This stepwise FA elongation system can be repeated to synthesize longer chain fatty acids. And the condensation reaction catalyzed by the substrate-specific ELOVL is the rate-limiting step [3]. The entire elongation cycle is shown in **Figure 1.1** [1].

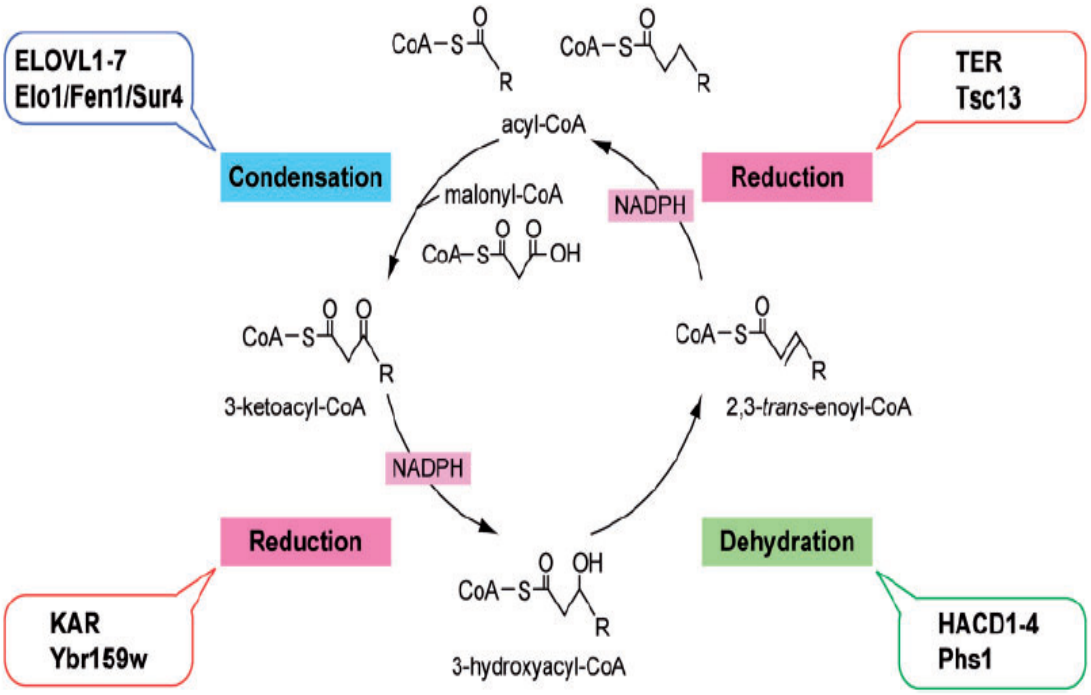

Figure 1.1 The FA elongation system. [1]

Until now, seven ELOVL (ELOVL1-7) are known to catalyze mammalian fatty acid elongation. ELOVL1, ELOVL3, ELOVL6 and ELOVL7 are known to act on SFA and MUFA, ELOVL2 and ELOVL5 mediate the PUFA elongations, and ELOVL4 elongates FA with 24 or more carbon regardless of the desaturation degree [6-13]. ELOVL has substrate specificity towards FA. The ELOVL substrate specificities towards n-FA, including n-SFA, n-MUFA and n-PUFA, have been well investigated and summarized in previous studies [1-3], which is presented in **Figure 1.2** [2] and modified based on work in our lab [14]. Specifically, in LC or VLC SFA and MUFA elongations, ELOVL6 has catalytic activity towards 16C FA; ELOVL3 has activities towards 18-22C FA with the highest activity towards 18C FA; ELOVL1 is able to mediate 18-26C FA elongations with the predominant activity towards VLCFA; ELOVL4 has major activity towards 24C or more VLCFA; ELOVL7 is the last one found in mammals and share overlapping elongation activities with ELOVL1 or 3 [2, 6, 10, 15].

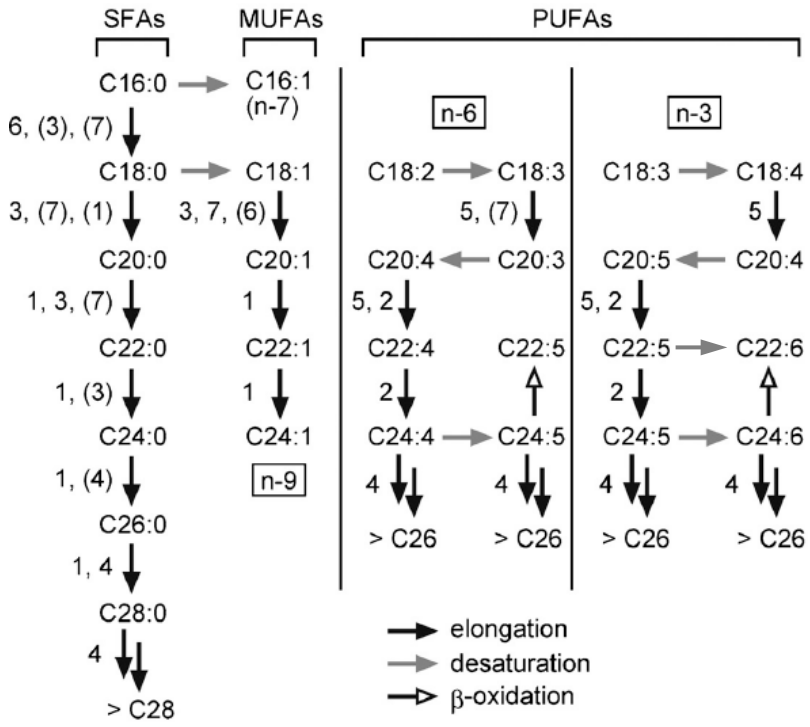

Figure 1.2 Elongation pathways of LCFA and VLCFA specifically catalyzed by ELOVL1-7. [2, 14]

ELOVL1-7 are expressed in mammalian various tissues/organisms. ELOVL6 expression has been found in human, bovine and mice tissues [16, 17], and associated with insulin sensitivity and skin & adipose tissue problems [4, 18, 19]. ELOVL3 are also found in skin and brown adipose tissues and associated with hair and skin dysfunctions [20-22]. ELOVL1 plays an important role in membrane integrity and support the normal function of central nervous system (CNS) parts [4]. ELOVL4 is highly expressed in human eye retina, suggesting its association with eye diseases [6]. ELOVL7 is involved in prostate cancer cell growth and suppression of ELOVL7 could attenuate prostate cancer development [10]. ELOVL2 and 5 contribute to PUFA synthesis which is related to sexual organ function and hormone secretion [23, 24].

Fatty acid desaturation

FA are desaturated by adding 1 or more of double bond (DB) to specific positions in carbon chain to produce MUFA or PUFA. Mammalian FA desaturation is catalyzed by multiple desaturases including five isoforms of stearoyl-CoA desaturase (SCD1-5) and three in fatty acid desaturases family (FADS1-3).

SCD is able to introduce a *cis*-configuration DB to Δ9 position, which is called Δ9-desaturation. Among five SCD isoforms, SCD1 was the first one isolated [25], and expressed in both humans and other mammals such as mouse and pig [26, 27], so the interpretations of SCD in this dissertation are largely based

on studies of SCD1. SCD catalyze the rate-limiting step in MUFA biosynthesis in ER, mediating the accumulations of important n-ECFA monoenes such as palmitoleic acid (16:1n-7) and oleic acid (18:1n-9) that are high in human plasma fatty acid composition [3].

FADS family are all located on chromosome 11 at 11q12–q13.1 [28]. FADS1 catalyzes Δ5- and Δ7- desaturation; FADS2 accounts for Δ4-, Δ6- and Δ8-desaturation; while FADS3 is reported for Δ13- desaturation [14, 27, 29-31]. Among them, FADS2 Δ6- desaturase enzymatic activity is of importance since it participates in multiple pathways of PUFA biosynthesis. Specifically, to date FADS2 expression is found both in ER and mitochondria [14]. It is known to desaturate ten n-ECFA substrates: eight PUFA, two MUFA (oleic acid 18:1n-9 → 18:2n-9 & gondoic acid 20:1n-9 → 20:2n-9) and one SFA (palmitic acid 16:0 → sapienic acid 16:1n-10) [27]. We also see FADS2 competes with SCD on palmitic acid desaturation when excessive palmitic acid is available [27].

The mammalian desaturation pathways of saturated LC or VLC n-ECFA catalyzed by specific SCD or FADS family are illustrated in **Figure 1.3** [3].

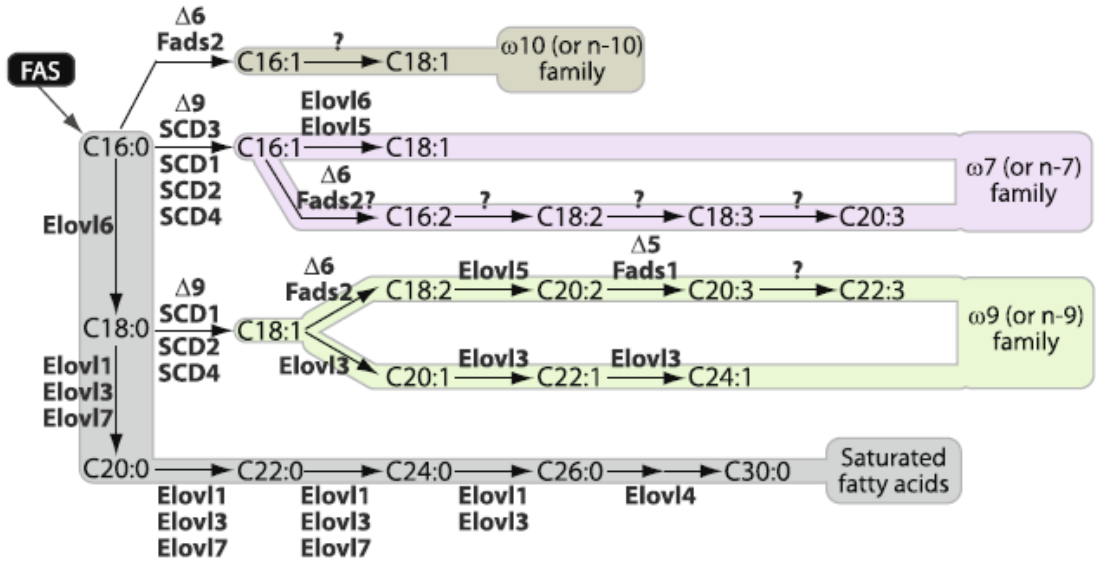

Figure 1.3 Desaturation pathways of saturated LCFA and VLCFA specifically catalyzed by SCD, FADS1 and FADS2. [3]

FADS2 enzymatic activities are found in many human tissues/organisms, primarily in brain, liver, testis and skin [32]. Distinctively, FADS2 is highly expressed in sebaceous glands (SG) of human skin where SCD expression is barely detected [32]. Human SG are featured by the unique sebaceous follicles because these follicles are not found in other mammals [33]. Human sebaceous follicles are likely to be concentrated on the skin with the potential to develop acne, such as face, back and chest skin; and acne is a skin disease unique to human [33-35]. Moveover, human sebum lipids especially wax esters comprise abundant FA that are rare in other human tissues or other mammals, such as SFA and MUFA of BCFA and n-OCFA, as well as the sapienic acid 16:1n-10 [32, 36-38]. Previous studies established that sapienic acid is the predominant unsaturated FA in human SG lipids [27, 39, 40]. Sapienic acid biosynthesized from palmitic acid is a good example of FADS2 Δ6-desaturation in human SG [32]. However, no one has characterized the FADS2 substrate specificities towards other unique FA components of human SG, and no reports on the association between the unique FA composition changes and acne development in human SG.

Odd-numbered carbon chain fatty acids

Odd-numbered carbon chain fatty acids in this dissertation refer to linear chain normal FA with odd number of carbons, namely odd chain fatty acids or n-OCFA, but not include BCFA with odd number of carbons. Unlike n-ECFA, they were mostly overlooked at the very beginning because they are at

relatively low levels in food and human tissues. In recent decades, long chain saturated n-OCFA, especially tridecanoic acid (n-13:0), pentadecanoic acid (n-15:0) and heptadecanoic acid (n-17:0), have drawn increasing interests because they exists in almost all ruminant food and milk, and are also detected in non-ruminant seafood and dietary fibers [41-44]. Besides, they are found in human adipose tissue, serum, plasma, red blood cells (RBCs) and liver, indicating they can be transported and incorporated into most human tissues [42, 45-50].

n-OCFA n-15:0 and n-17:0 are widely regarded as biomarkers of specific food intake. For example, the increase of n-15:0 and n-17:0 consumption from dairy fat could reflect in various human tissues FA composition, thus n-15:0 and n-17:0 are believed to be good biomarkers of dairy food intake [45-50]. They are also suggested to be biomarkers of seafood and dietary fiber intake because similar associations between intake and human tissues FA composition were found [43, 44]. Additionally, a series of cohort and case-control studies found that n-15:0, n-17:0 levels were positively associated with insulin sensitivity and negatively associated with Type 2 diabetes, when n-ECFA having the opposite correlations [47, 51-54]. Thus, n-OCFA have the potential to be the index of health for certain populations [51-54]. However, the ratio of n-15:0/n-17:0 in U.S. dairy sources is approximately 2:1 [5, 55, 56], contradictory to that in human plasma [5, 44, 51, 57] and seafood [43, 58-60] which is 1:2, indicating there is probably de novo metabolism of n-OCFA in human body after

consumption [5, 61]. Due to the uncertainty of endogenous metabolism of n-OCFA in humans, we should use n-OCFA with caution when interpreting dietary fat intake and health status.

The speculated pathways of n-OCFA in human are inspired by their metabolic pathways in bacteria and discussed here. The first origin of n-OCFA could be biosynthesis from n-ECFA by α-oxidation [61]. α-oxidation is defined as intermediate hydroxylation and then removal of one carbon from the terminal carboxylic end of FA [5]. Research showed that in adipocytes, n-16:0 was oxidized at α carbon into n-15:0; and in rat serum n-18:0 into n-17:0 [62-65]. Some researchers suggested that α-oxidation of these n-ECFA was promoted by the rumen microbial population or human gut microbiota [5, 62]. Secondly, they could be synthesized by chain elongation from shorter-chain n-OCFA or by chain shortening/β-oxidation of VLCFA, just like n-ECFA endogenous metabolism. The only difference is that n-OCFA elongation starts with propionyl-CoA as compared to acetyl-CoA [42, 44]. However, the rate-limiting elongases specifically catalyzing n-OCFA have not been elucidated before.

Branched chain fatty acids

Branched chain fatty acids (BCFA) are FA with one or more methyl branches. In nature, most BCFA are saturated; while few monounsaturated along with the major saturated BCFA are also found in bacteria and human skin-like tissues. In this dissertation, BCFA refer to the common saturated BCFA if not

specified.

BCFA can be categorized into mono-, di- and multi-methyl BCFA, among which the majority are mono-methyl. Fatty acids terminating with an isopropyl or isobutyl group are named as *iso*- or *anteiso*-BCFA, respectively, in mono-methyl BCFA group. As shown in **Figure 1.4**, n- represents FA hydrocarbon chains are linear with no branching, for example, n-16:0 has linear chain and is called palmitic acid or hexadecanoic acid; *iso*- represents FA have a bifurcated methyl branch, for example, a systematic name for *iso*-16:0 or *iso*-palmitic acid is 14-methyl pentadecanoic acid; *anteiso*- represents FA have a methyl/ethyl branch, for example *anteiso*-16:0 is 13-methyl-pentadecanoic acid.

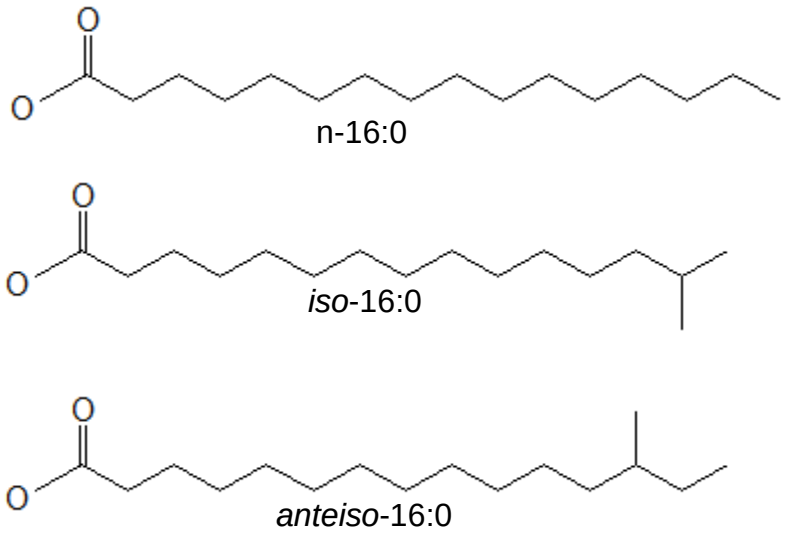

Figure 1.4 Nomination and structure of representative branched chain fatty acids (BCFA).

BCFA are common components of food, present at least 1-2% in ruminant fat such as beef, cheese and commercial cow's milk [66-69], as well as in non-ruminant sources such as fish [43, 67]. The mean intake of BCFA from these food sources in the American diet is about 500 mg/d [68, 69]. They are also components of food safety related bacteria such as *Listeria monocytogenes* [70, 71], and GI tract microbiota bacteria such as *Bacillus subtilis* and *Bifidobacterium* [72], contributing to bacteria membrane fluidity [73, 74]. BCFA are reported at 0.58%-1.5% w/w in human milk fat, but their levels are different between populations, probably due to their difference in diet; it also suggests the maternal microbiota is likely to be a source of de novo BCFA biosynthesis [75].

BCFA are rare in internal mammalian tissues, but they are present at high levels in skin and vernix caseosa. In human skin, BCFA are synthesized in sebaceous glands [36, 37] and meibomian glands [76, 77]. Saturated and monounsaturated BCFA comprise 5.4% and 6.6%, respectively, of total fatty acids in human skin lipids [36]. Vernix caseosa is the unique waxy white substance produced by human fetal skin in the last weeks of gestation and coating the skin of term newborns until delivery [78]. It has been called humans first meal, because vernix suspended in amniotic fluid is swallowed by late term human fetuses and are present in meconium [78, 79]. Vernix caseosa is very rich in BCFA, which is at 30% of its total fatty acids [80]. Accordingly, BCFA was found high in gut of the normal term gestation

newborns and selectively absorbed and metabolized by human alimentary canal [78, 79]. A study using neonatal rats further revealed dietary BCFA can be incorporated into the serum, liver and phospholipid (PL) fraction of the ileum by a structure-selective manner [81]; and the substitution of 20 wt% of fat as BCFA altered the prenatal gastrointestinal (GI) microbial ecology, enhanced BCFA utilization, and reduced the incidence of necrotizing enterocolitis (NEC) with an increased anti-inflammatory cytokine IL-10 expression [81]. Additional human intestinal cell studies confirmed the structure-selective incorporation of BCFA into human fetal cell lipid fractions in which *anteiso*-17:0 is the most preferred [82]; they also showed BCFA suppressed LPS-induced IL-8 expression in which *anteiso*-BCFA work better than *iso*-BCFA but less efficient than PUFA, and BCFA-enriched vernix-monoacylglycerol has similar anti-inflammatory effect [83, 84]. Therefore, BCFA in human are at least important to the skin health and prenatal GI tract development, and may be a key modulating factor in preventing intestinal inflammation.

The physiological effects of BCFA are likely to be associated with their unique branched terminal end structure. When at same carbon number, the melting point of FA are in the order of n-SFA > BCFA > n-PUFA. BCFA in bacteria membrane have been investigated a lot for its function during temperature adaption. As bacteria growth temperature is lowered, the proportion of lower-melting point FA increases to ameliorate the effects of temperature changes

on physical state of membrane phospholipids [74]. BCFA especially *anteiso*-15:0 were found increasing when bacteria are in cold stress, verified the contribution of lower-melting-point BCFA to membrane fluidity [85, 86]. BCFA are thought to be synthesized from the catabolic products of the branched chain amino acids (BCAA) valine, leucine and isoleucine in bacteria [73]. These amino acids undergo transamination and oxidative decarboxylation to produce *iso*-butyryl CoA, *iso*-valeryl CoA, and alpha-methylbutyryl CoA, respectively [86], which are then elongated to even-numbered *iso*-BCFA, odd-numbered *iso*-BCFA, and odd-numbered *anteiso*-BCFA, respectively. The BCFA synthesis pathways from BCAA in bacteria are summarized in **Figure 1.5** [86]. Oku et al. further found that valine and isoleucine are primary precursors of BCFA but leucine is the preferential precursor of n-FA in rat skin by giving ^{14}C labeled BCAA diets [87], which could explain that even-numbered *iso*-BCFA and odd-numbered *anteiso*-BCFA are abundant but odd-numbered *iso*-BCFA are rare in mammalian tissues [36, 37, 76, 77].

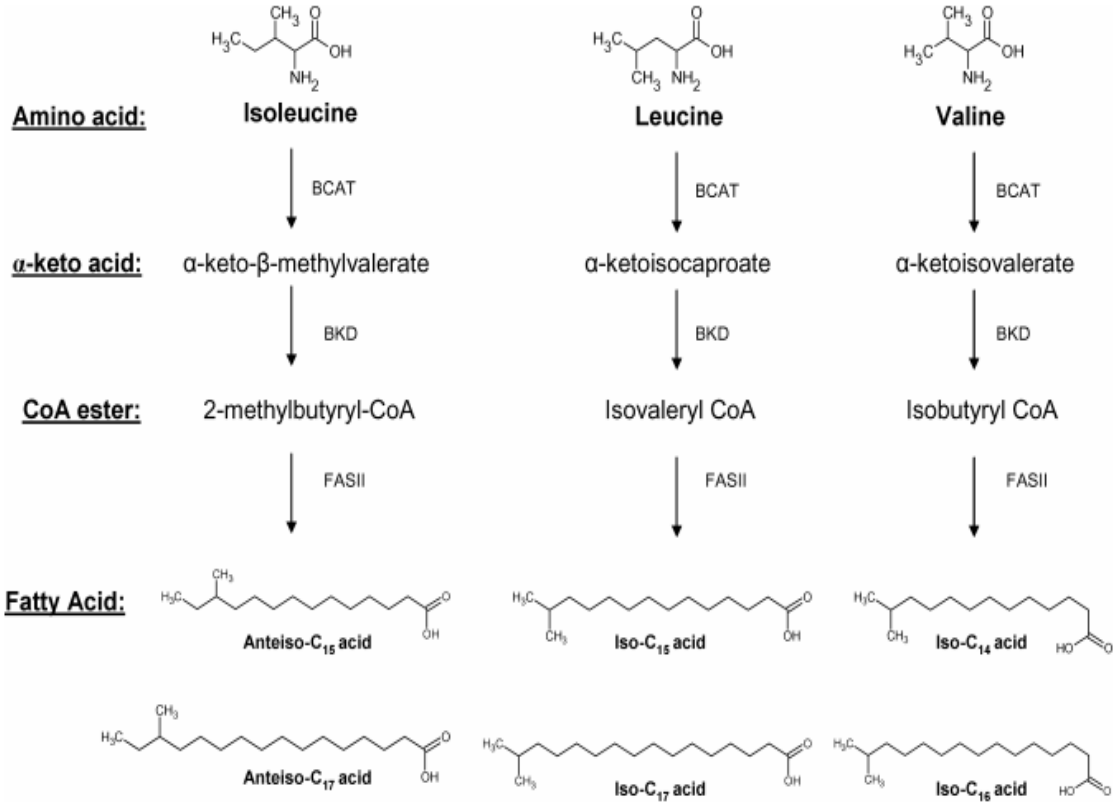

Figure 1.5 Pathways of branched chain fatty acid (BCFA) synthesis from branched chain amino acids (BCAA) in bacteria. [86]

The de novo interconversions of BCFA and relevant metabolic enzymes have not been carefully investigated in human tissues. They have been reports for the model organism, the roundworm *Caenorhabditis elegans* (*C. elegans*). The BCFA and n-FA biosynthesis in *C. elegans* have been summarized in **Figure 1.6** [88]. In BCFA biosynthesis pathways, longer chain BCFA are synthesized from BCAA precursors firstly by FAS, then *iso*-13:0 is catalyzed by ELO-5 and ELO-6 specially encoding elongases to *iso*-15:0 and *iso*-17:0 [88, 89]. While in n-FA biosynthesis pathways of *C. elegans*, ELO-1 and ELO-2 are the major elongases and FAT1-7 are responsible for generating MUFA and PUFA [88].

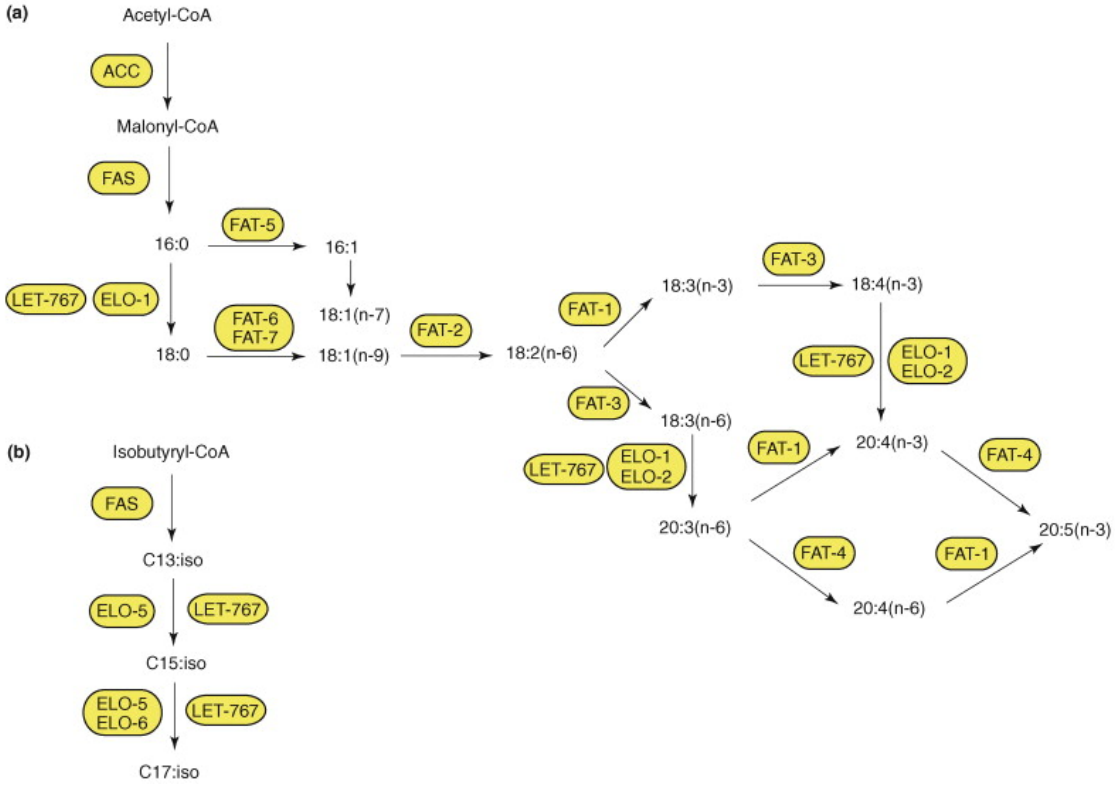

Figure 1.6 Biosynthesis of normal fatty acids (n-FA) and branched chain fatty acids (BCFA) in *C. elegans*. [88]

(a) Normal fatty acids; (b) Branched chain fatty acids.

Since BCFA are high in human skin and newborn vernix caseosa, and exist mainly as saturates and monounsaturates, we propose BCFA in human tissues can be de novo synthesized in a similar way as that in bacteria and *C. elegans*, catalyzed by substrate-specific elongases ELOVL1-7 and desaturases including SCD and FADS.

Summary

n-OCFA and BCFA are both important components of the American diet including dairy, beef and non-ruminant seafood. They are at high levels in human skin and GI tract, and also detected in blood and liver. n-OCFA level changes are used as biomarker of food intake in nutritional studies; and BCFA level changes are associated with multiple metabolic diseases such as necrotizing enterocolitis and skin dysfunctions. However, we have very limited knowledge on their biosynthesis in humans.

This dissertation is aimed to illustrate the interconversion of common n-OCFA and BCFA which are associated with human metabolic conditions, by using human cell models, to establish the substrate specificity of elongase and desaturase enzymatic activities in n-OCFA and BCFA biosynthesis.

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CHAPTER 2

The ELOVL6 gene product catalyzes elongation of n-13:0 and n-15:0 odd chain saturated fatty acids in human cells*

Abstract

Normal odd chain saturated fatty acids (OCSFA), particularly tridecanoic acid (n-13:0), pentadecanoic acid (n-15:0) and heptadecanoic acid (n-17:0), are normal components of dairy, beef and seafood. The ratio of n-15:0 to n-17:0 in ruminant foods (dairy and beef) is 2:1, while in seafood and human tissues it is 1:2, and their appearance in plasma is often used as a marker for ruminant fat intake. Human elongases encoded by ELOVL1, ELOVL3, ELOVL6, and ELOVL7 catalyze biosynthesis of the dominant even chain saturated fatty acids (ECSFA), however, there are no reports of elongase function on OCSFA. ELOVL transfected MCF7 cells were treated with n-13:0, n-15:0, or n-17:0 (80 μM) and products analyzed. ELOVL6 catalyzed elongation of n-13:0 → n-15:0 and n-15:0 → n-17:0; and ELOVL7 had modest activity toward n-15:0 (n-15:0 → n-17:0). No elongation activity was detected for n-17:0 → n-19:0. Our data expand ELOVL specificity to OCSFA, providing the first molecular evidence demonstrating ELOVL6 as the major elongase acting on OCS n-13:0 and n-15:0 FA. Studies of food intake relying on OCS as biomarker should consider endogenous human metabolism when relying on OCSFA ratios to indicate specific food intake.

* Zhen Wang, Dong Hao Wang, Yuliya Goykhman, Yuanyuan Yan, Peter Lawrence, Kumar S. D. Kothapalli, and J. Thomas Brenna. The ELOVL6 gene product catalyzes elongation of n-13:0 and n-15:0 odd chain saturated fatty acids in human cells. British Journal of Nutrition, Oct 2018, in press.

Introduction

Normal odd chain saturated fatty acids (OCSFA), particularly tridecanoic acid (n-13:0), pentadecanoic acid (n-15:0) and heptadecanoic acid (n-17:0), are normal components of ruminant products, specifically dairy and beef [1, 2]. They are also found in non-ruminant sources such as seafood [3]. In recent years, n-15:0 and n-17:0 are considered biomarkers of dairy fat intake, mainly because their concentrations in serum and adipose tissue correspond with dairy intake [4-9]. Not only serum and adipose tissue, they are also found to incorporate in other human tissues such as plasma, red blood cells (RBCs) and liver [2]. Recent studies showed that n-15:0 and n-17:0 are biomarkers of not only dairy but also seafood [3] and dietary fiber intake [10], which again indicates OCSFA is applicable in estimation of food intake. Additionally, n-15:0 and n-17:0 are positively associated with insulin sensitivity and inversely associated with Type 2 diabetes in both cohort [6, 11] and case-control studies [12-14], which contrasts with links between incident diabetes and prevalent even chain saturated (ECS) FA intake such as stearic acid (n-18:0). Circulating OCSFA and/or their relative concentrations (e.g. ratios) may be useful as a physiological index of health with careful attention to their origin

and endogenous metabolism [2, 12, 14, 15].

Concentrations of n-13:0, n-15:0 and n-17:0 in general milk products in the U.S. are about 0.1%, 1.2% and 0.6% of total FA, respectively [1]. They are likely to be originated primarily via ruminal bacteria. The ratio of n-15:0 to n-17:0 in U.S. dairy fat is 2:1 [16-18]. In contrast, is approximately 1:2 in both freshwater and marine fishes [3, 19-21] as well as in human plasma [10, 12, 15, 18]. Comparable amount of OCSFA are found in vegan RBCs [22]. Because dairy fat is the predominant source of OCSFA in the U.S., it is likely that endogenous fatty acid interconversion alters their ratio via elongation, most importantly as n-15:0→n-17:0 [2, 18, 23]; they may also arise by de novo synthesis. Current endogenous synthesis pathways of OCSFA are well summarized in previous reviews [2, 18, 23]. Among them, one theory proposes an α-oxidation of ECSFA by intermediate hydroxylation/removal of one carbon from carboxylic end [18]. However, few details are available on the gene products responsible for OCSFA biosynthesis, unlike well-known biochemical routes to ECSFA.

In ECSFA metabolism, FA elongation follows a four-step cycle comprised of condensation, reduction, dehydration and second time reduction which occurs in endoplasmic reticulum (ER) [24]. Mammalian FA elongases ELOVL1-7 (elongation of very long chain fatty acids) work in the first and rate-limiting condensation step and all have substrate specificities and tissue specific

expression distribution. Among them, ELOVL1, 3, 6 and 7 preferentially act on saturated and monounsaturated FA (SFA and MUFA); ELOVL2 and 5 are known to work on polyunsaturated FA (PUFA); while ELOVL4 elongate very long chain FA (VLCFA) with more than 24 carbons regardless of unsaturation [24-26]. Currently established substrate specificities of ELOVL towards ECSFA are as follows: ELOVL6, n-12:0→n-14:0→n-16:0→n-18:0 [25, 27]; ELOVL1, 3 and 7, n-18:0→n-20:0→→→n-26:0; ELOVL4, n-26:0→→30:0 [24, 25]. ELOVL6 catalyzes n-16:0→n-18:0 in ruminant mammary cells [28] and is expressed ubiquitously in bovine mammary epithelial cells [29]. ELOVL6 genetic variants are reportedly associated with insulin sensitivity in a Spanish population [30].

Unlike the well-studied ECSFA, endogenous metabolism of OCSFA once ingested is not well characterized with respect to the relevant genes encoding enzymes that catalyze their interconversion. As circulating n-15:0 and n-17:0 are regarded as biomarkers of dairy, dietary fiber and seafood intake, we aimed to establish specificity of the ELOVL responsible for elongation of OCSFA of quantitative significance in the human diet. We tested the hypothesis that ELOVL6 is specific to n-13:0→n-15:0→n-17:0 compared to the other ELOVL known to operate on straight chain fatty acids. We adopted an approach analogous to previous successful studies that established numerous novel functions for PUFA biosynthetic genes by transient or stable transfection of the open reading frame (ORF) into MCF7 cells and other models [31-38]. To

test ELOVLx (ELOVL1, 3, 6 and 7) function we constructed expression vectors and transiently transfected them individually into MCF7 cells as a human cell host.

Materials and Methods

Chemicals, solvents and reagents

Fatty acids (n-13:0, n-15:0 and n-17:0) were purchased from Sigma-Aldrich (St. Louis, MO). Solvents are HPLC grade for fatty acid extraction and were purchased from Sigma-Aldrich (St. Louis, MO) and Burdick & Jackson (Muskegon, MI). Cell culture media, fetal bovine serum (FBS) and other cell culture reagents were obtained from Life Technologies (NY), Corning (MA) and Thermo Fisher Scientific (MA).

ELOVL6 sequence and phylogenetic analysis

The amino acid (AA) sequences of ELOVL6 from various vertebrate species are obtained from GenBank accession numbers (**Table S2.1**). The AA sequence of human ELOVL6 was aligned with several other vertebrate ELOVL6 sequences using ClustalX2.1 software [39]. The phylogenetic tree was constructed using the neighbor-joining method [40] with MEGA7 [41]. Confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping test method with 1000 replicates [42].

ELOVL expression vector constructs

The open reading frame of ELOVL transcripts (ELOVL1, ELOVL3, ELOVL6 and ELOVL7) were cloned into a pcDNA3.1(+) expression vector (Thermo Fisher Scientific, MA) containing cytomegalovirus (CMV) promoter. The specific ELOVL gene synthesis and cloning was carried out by GenScript service, NJ. The GenBank accession numbers of ELOVL mRNA (NM) and protein (NP) are provided in **Table S2.1**. Plasmid DNA used for transfection assays was extracted and purified using Plasmid Midi Kit (QIAGEN, MD). The extracted DNA was verified by DNA sequencing and stored at -20°C. DNA sequencing was performed at Cornell University life sciences core laboratories center using the Applied Biosystems automated 3730 DNA analyzer.

Mammalian cell culture, transfection, and fatty acid supplementation

MCF7 human breast cancer cells were grown at 37°C in a humidified environment with 5% CO_2, using minimum essential medium alpha (MEM-α) with 10% FBS and 10mM buffer [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEPES] as described previously [32, 34].

MCF7 cells were seeded at 1× 10^6 cell density into 60mm cell culture dishes. After 48h, when they reach 60-80% confluency, cells were washed with 1× PBS and ELOVL (ELOVL1, 3, 6 and 7) transcripts were transfected individually using Polyplus (NY) jetPRIME transfection reagent. Empty vector was used as control. According to the jetPRIME reagent kit protocol, 4µg of Vector (control) or ELOVL DNA was transfected into cells along with 200µl jetPRIME buffer, 8µl jetPRIME reagent, and 5ml growth media. After 24h, the

transfected MCF7 cells were supplemented with 80 μM of bovine serum albumin (BSA) bound OCSFA substrates (n-13:0, n-15:0 and n-17:0). Briefly, to make BSA bound substrates, n-13:0, n-15:0 and n-17:0 were dissolved in absolute ethanol to make 100mM FA stock. FA stock (200μl) was then mixed with FA free BSA in 1× PBS (4.4% w/w) and incubated overnight at 37°C. BSA bound OCSFA were filtered using 0.22 μm syringe, and diluted to 80 μM with non-FBS MCF7 media then added to cells. After additional 24 h incubation, cells were washed twice with 1 × PBS, harvested by trypsinization and supernatant removed after centrifuging.

RNA isolation and cDNA synthesis

RNA was isolated from harvested MCF7 cell pellets using E.Z.N.A. Total RNA Kit I (Omega Bio-tek Inc, GA). The RNA quantity and quality was verified by micro spectrophotometer Nanodrop 2000 (Thermo Scientific). cDNA was synthesized from 1μg of RNA using High Capacity cDNA Reverse Transcription Kit (Life Technologies, NY). Synthesized cDNA was then used as template for RT-PCR reactions [43].

RT-PCR

Gene specific primers ELOVL1-7 were designed using PrimerQuest software (Integrated DNA Technologies, Coralville, IA), primer sequences and annealing temperatures are shown in **Table S2.2**. RT-PCR amplification reactions were performed using EmeraldAmp GT PCR Master Mix (Clontech,

CA) using gradient thermal cycler (Eppendorf, NY). PCR products were separated by 2% agarose gel electrophoresis stained with ethidium bromide, and bands were visualized under UV light. GAPDH was used as control gene.

Fatty acid extraction and analysis

Fatty acid methyl esters (FAME) from the harvested MCF7 cell pellets were prepared according to the modified one-step method of Garces and Mancha [44]. FAME were structurally identified by gas chromatography (GC) - chemical ionization, electron ionization (EI) mass spectrometry (MS) and EIMS/MS using a Saturn 2000 mass spectrometer attached to a Varian Star 3400 gas chromatograph [45]. FAME were quantified by GC-flame ionization detector (GC-FID) (Hewlett-Packard). An equal weight FAME mixture, GLC462 (Nu-Check Prep, Inc.), was used to calculate response factors of all fatty acids. Percent conversion of substrates (S) to products (P) was calculated as: [(P) / (S + P)] * 100, and normalized to the control group.

Statistical analysis

All treatments were performed using two biological replicates; the mean of three technical replicate GC-FID analyses were used for each biological replicate and no data was excluded. In numerous earlier studies, we used an analogous approach with MCF7 transfection and fatty acid treatment with 2-3 biological replicates for functional characterization of PUFA biosynthetic genes [31-34]; this approach is comparable to functional characterization studies

conducted by others [35-38]. Values generated using averages across the two biological replicates are expressed as mean ± SD. Statistical analysis of comparisons between multiple groups was performed using OriginPro 8 Software (OriginLab Corporation, MA). One-way analysis of variance (ANOVA) with post-hoc Tukey Honest Significant Difference (HSD) test was used to analyze significant differences between groups. Differences when $P<0.05$ are considered significant.

Results

Amino acid sequence and phylogenetic analysis of ELOVL6

The human ELOVL6 cDNA (NM_024090.2) consist of a 798 base pair (bp) ORF, encoding a protein of 265 AA (NP_076995.1). As shown in **Figure 2.1**, human ELOVL6 shares >90% AA sequence identity with other vertebrate ELOVL6 sequences. The human ELOVL6 shared 98.11%, 96.98%, 96.60% and 93.16% identity, with monkey, mouse, rat and cattle sequences respectively. All vertebrate ELOVL6 possessed five transmembrane domains (I-V) found among elongases [46] as well as the conserved histidine box HXXHH motif characteristic of elongase families [47-49]. A phylogenetic tree was constructed by comparing the AA sequences of ELOVL6 from various vertebrates (**Figure 2.2**). As expected, the human ELOVL6 grouped with primates, while rodents (rat and mouse) and fish (catfish and zebrafish) grouped together.

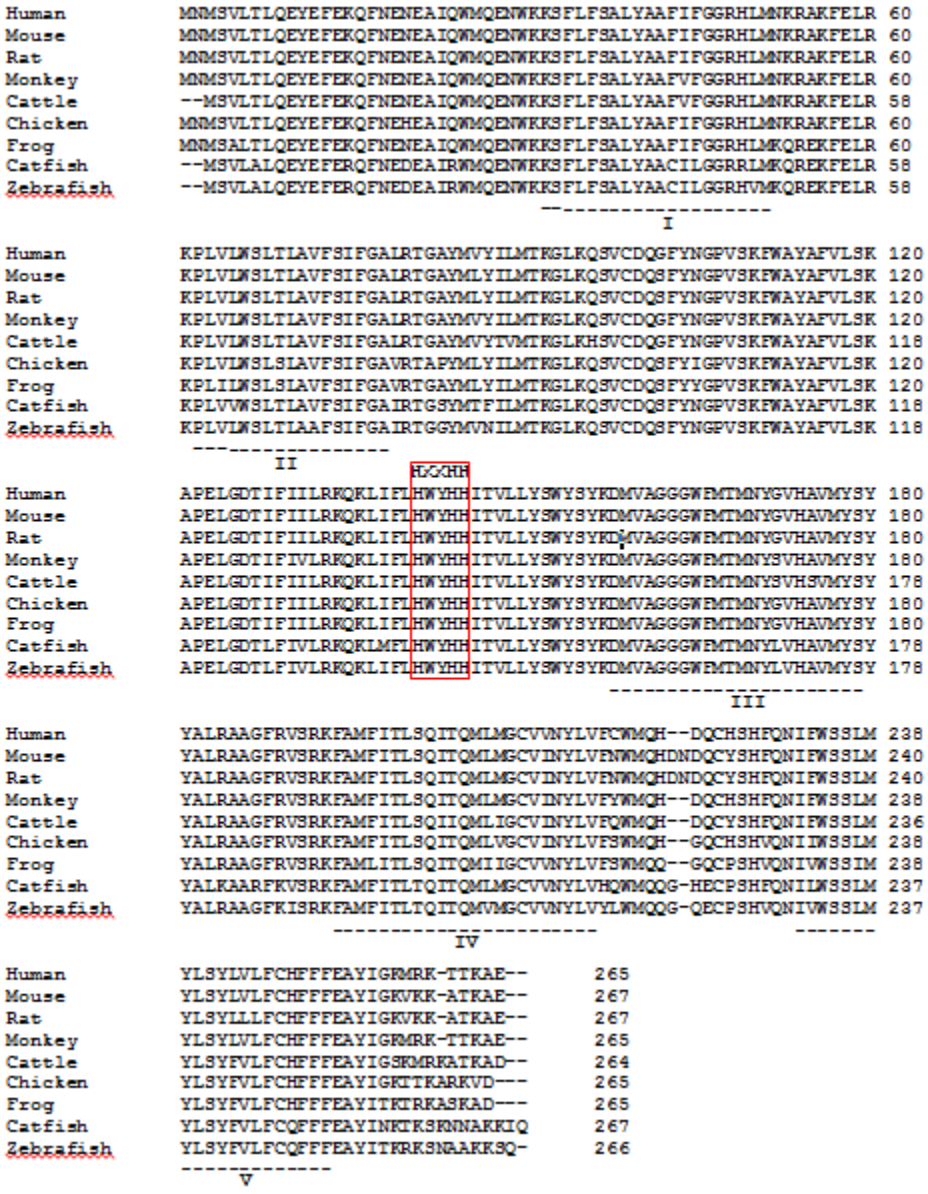

Figure 2.1 Alignment of amino acid (AA) sequences of ELOVL6 from human and other vertebrates.

The AA sequences of various species obtained from GenBank accession numbers were aligned using ClustalX 2.1. The well conserved histidine motif HXXHH is depicted in the box. The dotted lines with Roman numerals indicate the putative transmembrane regions [46].

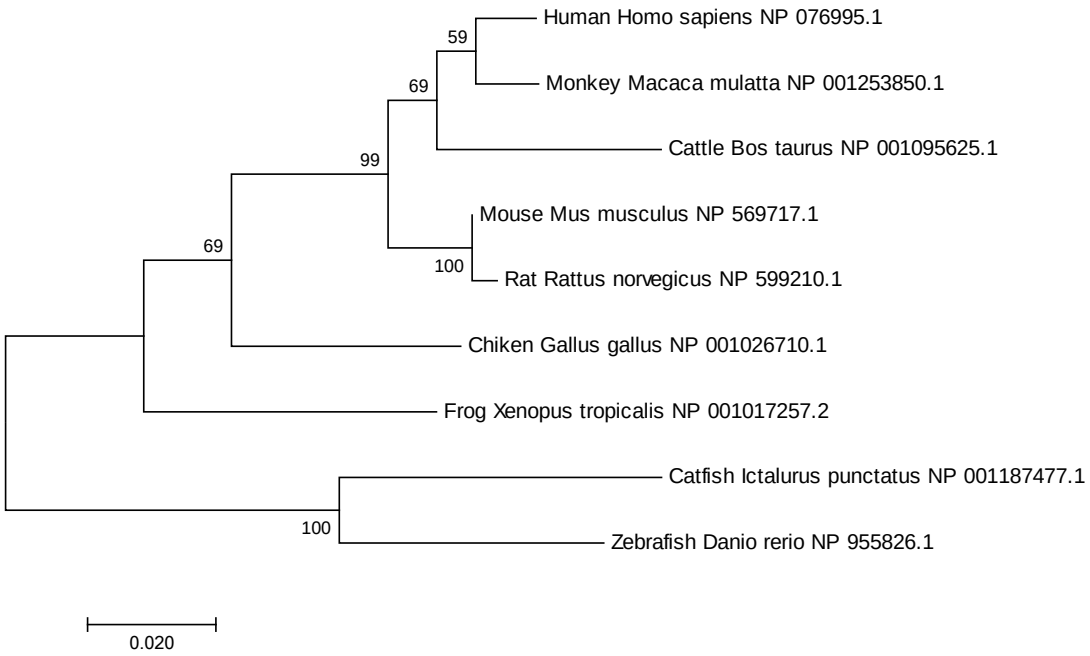

Figure 2.2 Phylogenetic tree of ELOVL6 from human and other organisms.

The tree was constructed using the Neighbor-Joining method with 1000 bootstrap replicates in MEGA7. The numbers represent the frequencies (%); and horizontal branch length is proportional to AA substitution rate per site. Each species was followed by its NCBI Reference Sequence (NP).

Distribution of SFA in MCF7 cells

MCF7 cells are high in ECSFA n-16:0 (16.63 ± 0.43%) and n-18:0 (12.98 ± 0.52%), but contain only trace amount of OCSFA n-17:0 (0.22 ± 0.02%) and n-13:0 and n-15:0 are at undetectable amounts (unpublished data). **Table 2.1** summarizes when MCF7 cells are dosed with 80 μM of specific OCSFA, cells readily uptake OCSFA in the order of n-17:0 > n-15:0 > n-13:0.

Table 2.1 Uptake Efficiency of OCSFA by MCF7 cells.

OCSFA	**n-13:0**		**n-15:0**		**n-17:0**	
	Mean	SD	Mean	SD	Mean	SD
Uptake Efficiency[1]	3.276	0.002	20.334	0.136	27.733	0.175

[1]Uptake Efficiency (%) = wt (BCFA uptake) / wt (total fatty acids excluding OCSFA)×100%
MCF7 cells have trace amount of basal OCSFA; control cells readily uptake OCSFA when treated with 80 μM of albumin bound individual OCSFA, and was in the order of n-17:0 > n-15:0 > n-13:0.
Data from two biological replicates.

Transient Transfection ELOVL gene expression

Figure S2.1 shows ELOVL (ELOVL1 to ELOVL7) gene expression in wild type (WT), control (empty pcDNA3.1(+) vector) and ELOVLx transiently transfected cells. The cells were incubated with A) 80µM n-13:0 B) 80µM n-15:0 and C) 80µM n-17:0. ELOVL1 expression was higher in ELOVL1 transfected cells; similarly ELOVL3, ELOVL6 and ELOVL7 expression are higher in ELOVL3, 6 and 7 transfected cells. These results show transfections were successful.

Elongation, n-13:0→n-15:0

MCF7 cells transiently expressing ELOVL1, ELOVL3, ELOVL6 and ELOVL7 and control (empty vector) were incubated with 80 µM of albumin bound n-13:0 OCSFA. MCF7 cells have native elongase activity, so the gain of function is compared to control (**Figure 2.3**). The cells expressing ELOVL6 showed significantly increased activity towards n-13:0 (131.19 ± 5.52 (%)). No gain of activity was seen with other elongases.

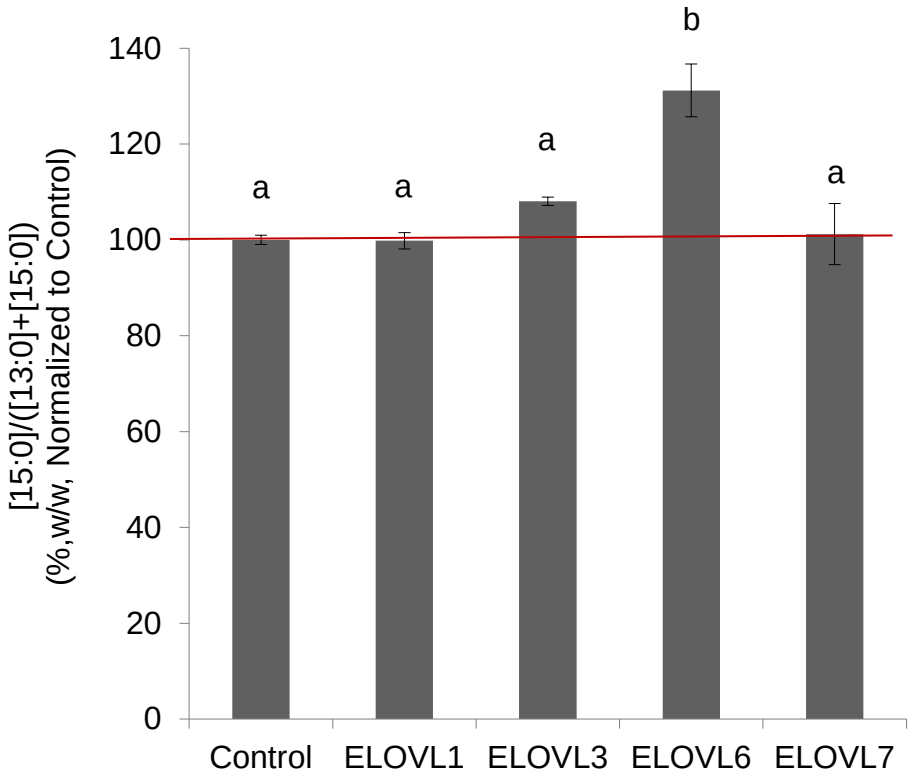

Figure 2.3 ELOVLx activity, n-13:0→n-15:0.

Percent conversion of fatty acid substrate n-13:0 into elongation product n-15:0 (ratios shown, calculated as [n-15:0]/[n-13:0]+ [n-15:0]) was measured in MCF7 cells and normalized to control group. ELOVL1, 3 and 7 showed no activity towards n-13:0 (n-13:0→n-15:0). ELOVL6 had significantly higher catalytic activity towards n-13:0 (n-13:0→n-15:0). Data from two biological replicates. a-b: $P<0.05$.

Elongation, n-15:0→n-17:0

MCF7 cells transiently expressing ELOVL1, ELOVL3, ELOVL6 and ELOVL7 and control (empty vector) were incubated with 80 μM of albumin bound n-15:0 OCSFA. MCF7 cells have native elongase activity, so the gain of function is compared to control (**Figure 2.4**). The cells expressing ELOVL6 showed significantly increased activity towards n-15:0 (129.51 ± 1.74 (%)), followed by moderate activity in ELOVL7 cells (113.99 ± 1.30 (%)). No gain of activity was seen with ELOVL1. Elongation activity was lower than control in the ELOVL3 cells though not different than ELOVL1 cells.

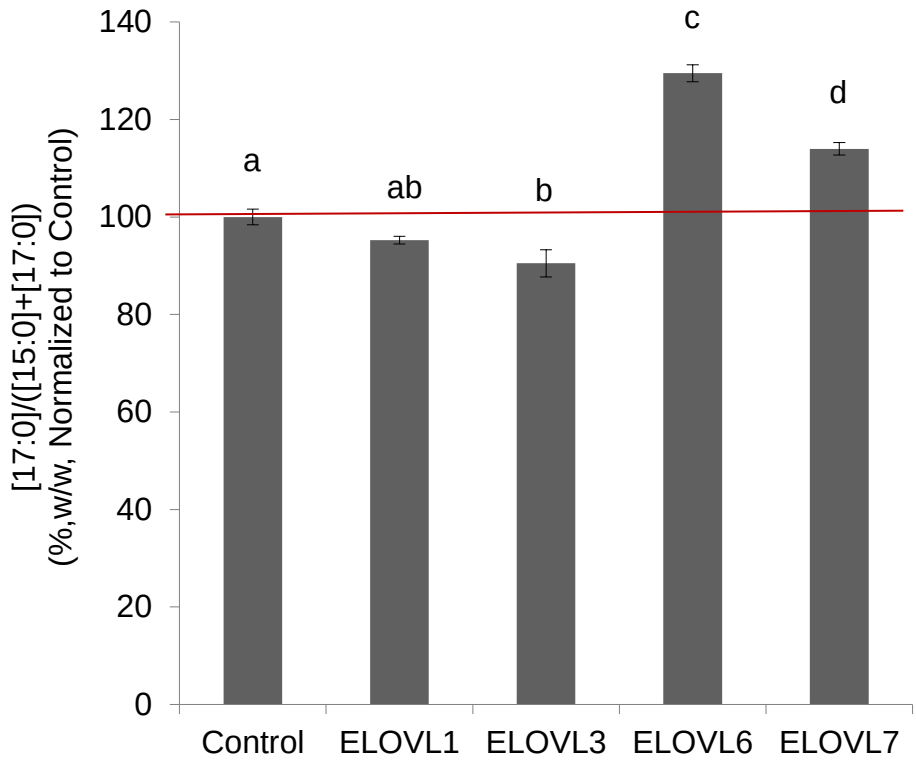

Figure 2.4 ELOVLx activity, n-15:0→n-17:0.

Percent conversion of fatty acid substrate n-15:0 into elongation product n-17:0 (ratios shown, calculated as [n-17:0]/([n-15:0] + [n-17:0]) was measured in MCF7 cells and normalized to control group. ELOVL1 showed no activity towards n-15:0 (n-15:0→n-17:0). ELOVL3 reduced activity towards n-15:0 (n-15:0→n-17:0) compared to control though it was not different than ELOVL1. ELOVL6 had significantly higher catalytic activity towards n-15:0 (n-15:0→n-17:0). ELOVL7 showed moderate activity towards n-15:0 (n-15:0→n-17:0). Data from two biological replicates. a-d: $P<0.05$.

Interconversion, n-17:0→n-15:0 but not n-19:0

MCF7 cells transiently expressing ELOVL1, ELOVL3, ELOVL6 and ELOVL7 and control (empty vector) were incubated with 80 μM of albumin bound n-17:0 OCSFA. All MCF7 cells (ELOVL1, ELOVL3, ELOVL6 and ELOVL7 and control) readily uptake n-17:0 but none of the cells showed elongation activity towards n-17:0; n-19:0 is not detected. While n-15:0 is not detectable in untreated cells, incubation with n-17:0 results in n-15:0 appearance at approximately equal amounts ([15:0]/([15:0]+[17:0]) ~ 3%) regardless of ELOVLx transfection, suggesting that β-oxidation is a route of n-17:0 conversion.

Discussion

Our results confirm the hypothesis that ELOVL6 is the primary ELOVL with activity toward OCSFA, specifically catalyzing n-13:0→n-15:0 and n-15:0→n-17:0. Further, ELOVL7 has moderate activity toward n-15:0→n-17:0. ELOVL1 had no activity toward any OCSFA, and no ELOVL catalyzed n-17:0→n-19:0, consistent with the trace amount of n-19:0 in human tissue. ELOVL3 significantly reduced activity compared to control but not compared to ELOVL1 which was not different than control. MCF7 cells have native elongation activity, thus it is plausible that ELOVL3 may have inhibited that native conversion, possibly by non-active substrate binding. The magnitude of the effect is small, however.

Our AA sequence alignments of ELOVL6 showed a high degree of conservation among evolutionarily distant related genomes (human to zebrafish). The histidine rich motif and all five transmembrane regions are conserved from human to zebrafish. The pattern of ELOVL6 homology among several vertebrate species even with distant phylogeny points to similar metabolic conservation.

ELOVL6 belongs to a highly conserved microsomal enzyme family that is involved in fatty acid biosynthesis. ELOVL6 activity characterized by cloning of mammalian ELOVL6 ORF into expression vector and expressing the vector in mammalian cells shows that ELOVL6 specifically catalyzes the elongation of even chain saturated and monounsaturated fatty acids with chain length 12, 14, and 16 carbons [46, 50]. ELOVL6 is found to be ubiquitously expressed in mice and bovine tissues [29, 46], and plays a role in energy metabolism and insulin sensitivity [27], nonalcoholic steatohepatitis [51], breast cancer [52], pulmonary fibrosis [53] and squamous cell carcinoma of the lung [54].

The endogenous synthesis of OCSFA via α-oxidation of ECSFA has been previously reported [18, 55, 56]. In an adipocyte differentiation study, both kinetic and ^{13}C palmitate labelled experiments showed significant increase in the synthesis of OCSFA via α-oxidation [55]. The differentiating adipocytes converted ^{13}C palmitate (n-16:0) to n-15:0, showing that OCSFA were endogenously synthesized from n-16:0 by α-oxidation in this cell type. This

conversion happened only in the cells and not in cell culture media [55]. Similarly, Su et al. showed differentiating adipocytes when incubated with [9,-10-^{3}H]n-16:0 resulted in the production of radiolabeled [^{3}H]n-15:0 via α-oxidation [57]. Casteels et al. [58] proposed α-oxidation mechanism might play a role in the formation of OCSFA in the brain. An *in-vivo* experiment with rats infused with n-18:0 showed ~70% ($p < 0{\cdot}001$) increase in the n-17:0 levels in the serum compared to a control rat group [59]. Our results show that specific ELOVL6 or 7 may operate on these nascent products α-oxidation n-13:0 and n-15:0 but not on n-17:0 to further alter OCSFA profile (**Figure 2.5**).

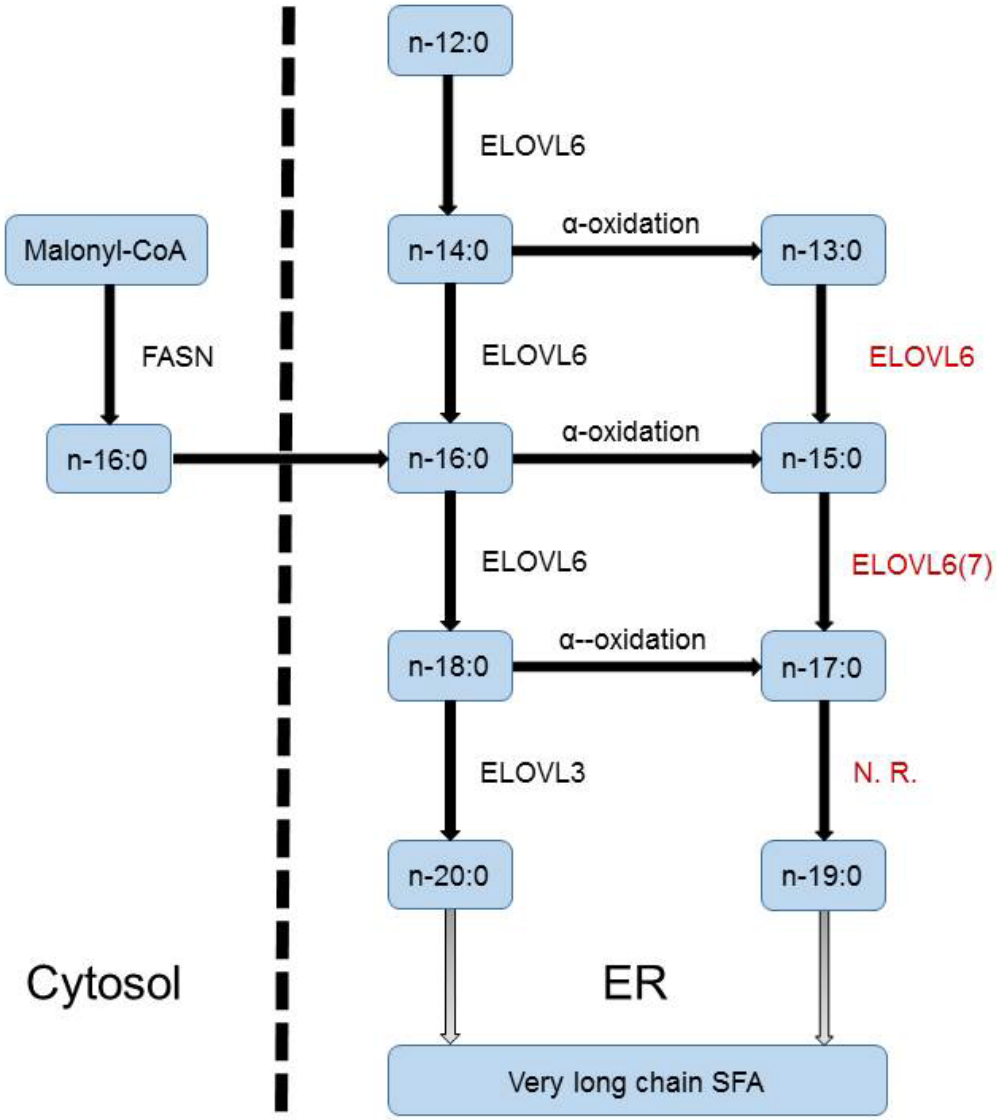

Figure 2.5 Even chain saturated fatty acid (ECSFA) and Odd chain saturated fatty acid (OCSFA) biosynthesis in vertebrates.

ELOVL6 catalyzes elongation of n-12:0 to n-18:0, whereas ELOVL3 catalyzes elongation of n-18:0 to n-20:0. Depicted in red font color: ELOVL6 elongates OCSFA n-13:0→n-15:0 and n-15:0→n-17:0. ELOVL7 (shown as 7) has moderate activity (n-15:0→n-17:0). Palmitic acid (n-16:0), the major product of fatty acid synthase (FASN), and α-oxidation of ECSFA to OCSFA are also shown. FASN, fatty acid synthase; ER, endoplasmic reticulum; N.R., no reaction.

Here we found overlapping activity for ELOVL6 and ELOVL7; both had substrate specificity for n-15:0. Elongases in several species are known to share common overlapping functions [49, 60, 61]. Previously it has been shown that mammalian ELOVL1, ELOVL3 and ELOVL7 share common substrates [25, 49, 62, 63]. ELOVL1 elongates SFA of chain lengths n-18:0 to n-26:0, with the highest activity toward n-22:0 FA, whereas, ELOVL3 and ELOVL7 were found to elongate n-16:0 to n-22:0 FA, with the highest activity toward n-18:0 FA [62-64].

The relatively small biological replicate size (n=2) in our study has consistently yielded reliable results in our previous work [31-34]. For functional characterization studies, others have used a single assay to support conclusions based on identification of a novel fatty acid product by chromatography with subsequent calculation of a conversion ratio [35-38], thus greater confidence in matching biological duplicates of the present results is warranted. Our uses of duplicates with consistently small standard deviations in FAME analysis, as well as validation of ELOVLx transfection efficiency via RT-PCR, support a similar level of confidence in the present results. Finally, we note that our data on normal polyunsaturated fatty acids [31-34] and ECSFA (data not shown) faithfully replicates that of others.

In conclusion, we provide the first molecular evidence demonstrating ELOVL6 is the major elongase acting on OCSFA, with specificity toward n-13:0 and n-

15:0. Modest activity was found for ELOVL7 toward n-15:0. The present study expands ELOVL substrate specificity range to OCSFA. Nutrition studies considering these fatty acids as markers of specific food intake should consider interconversion of OCSFA in light of these findings, particularly genome-wide association studies (GWAS) and targeted gene studies as they associate circulating OCSFA levels with single nucleotide polymorphisms (SNPs) and other polymorphisms.

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APPENDIX

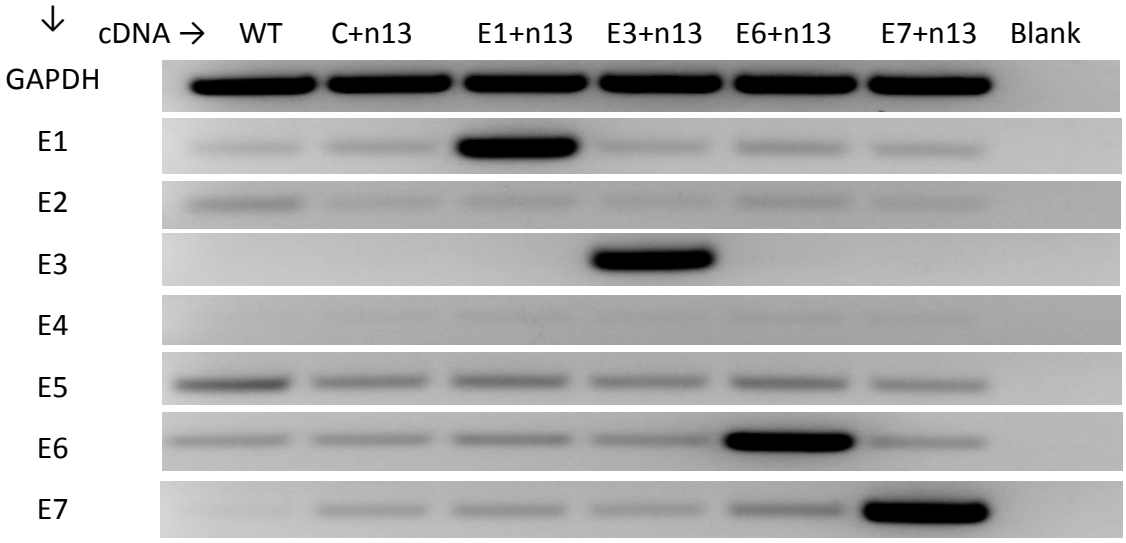
Table S2.1 GenBank accession numbers of human ELOVLx mRNA (NM) used for transfection studies. Protein coding sequences (NP) of vertebrate ELOVL6 used for phylogenetic analysis. AA, amino acids; NM, mRNA; NP, protein.

	Name	**NCBI Reference Sequence**
Human mRNA	*Homo sapiens* ELOVL1	NM_022821.3
	Homo sapiens ELOVL3	NM_152310.2
	Homo sapiens ELOVL6	NM_024090.2
	Homo sapiens ELOVL7	NM_024930.2
Vertebrate AA	*Homo sapiens* (human) ELOVL6	NP_076995.1
	Mus musculus (house mouse) ELOVL6	NP_569717.1
	Rattus norvegicus (Norway rat) ELOVL6	NP_599210.1
	Macaca mulatta (Rhesus monkey) ELOVL6	NP_001253850.1
	Bos Taurus (cattle) ELOVL6	NP_001095625.1
	Gallus gallus (chicken) ELOVL6	NP_001026710.1
	Xenopus tropicalis (tropical clawed frog) ELOVL6	NP_001017257.2
	Ictalurus punctatus (channel catfish) ELOVL6	NP_001187477.1
	Danio rerio (zebrafish) ELOVL6	NP_955826.1

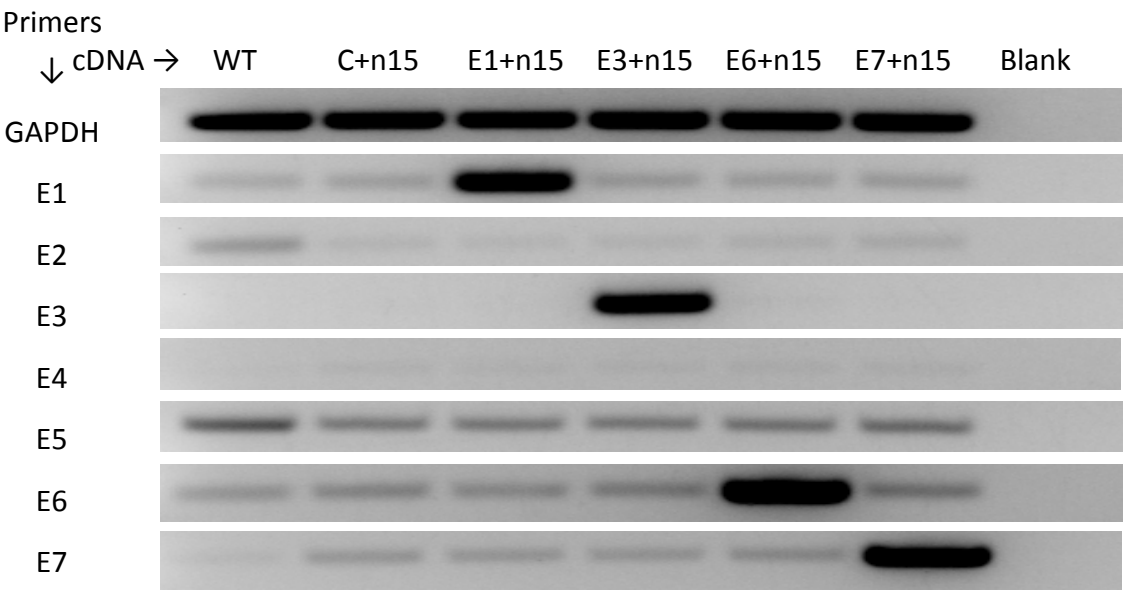
Table S2.2 Primer sequences and annealing temperatures used for testing ELOVLx transfection efficiency by RT-PCR.

Primer	F/R	Sequence	Annealing Temp.
GAPDH	Forward	5’-AAC GGA TTT GGT CGT ATT GGG C-3’	61
	Reverse	5’-TTG ACG GTG CCA TGG AAT TTG C-3’	
ELOVL1	Forward	5’-TAC CAA GAG GTG ATG AAG CAC GCA-3’	67
	Reverse	5'-AAC GAA GTA CAC GTA GGT CAG GAG-3’	
ELOVL2	Forward	5’-GGA CAA TAT GTT TGG ACC GCG A-3’	61
	Reverse	5’-ACC CAG CCA TAT TGA GAG CAG A-3’	
ELOVL3	Forward	5’-TTA TTC ACT GGT ACC ACC ACA G-3’	59
	Reverse	5’-GAA CAC CAA AGT TCA TGG TGA C-3’	
ELOVL4	Forward	5’-AAA CGT AGT GTC CAC GGC ACT CAA-3’	67
	Reverse	5’-ACC CAG CCA CAC AAA CAG GAG ATA-3’	
ELOVL5	Forward	5’-ACT ATG GTT TGT CGT CAG TCC CTT-3’	66
	Reverse	5'-ACA ACC AAC CAA GAG GGA ATG TGC-3’	
ELOVL6	Forward	5’-TGA GGA AGC CAT TAG TGC TCT GGT-3’	67
	Reverse	5’-AAA CTG ACT GCT TCA GGC CTT TGG-3’	
ELOVL7	Forward	5’-GTG ATC TTA CAT CGA GGA CTG TGC-3’	65
	Reverse	5'-TGA GCT TTG GTC CCA AGG AAG TGA-3’	

A


Primers
↓
cDNA →
WT
C+n13
E1+n13
E3+n13
E6+n13
E7+n13
Blank
GAPDH
E1
E2
E3
E4
E5
E6
E7

B


Primers
↓ cDNA →
WT
C+n15
E1+n15
E3+n15
E6+n15
E7+n15
Blank
GAPDH
E1
E2
E3
E4
E5
E6
E7

C

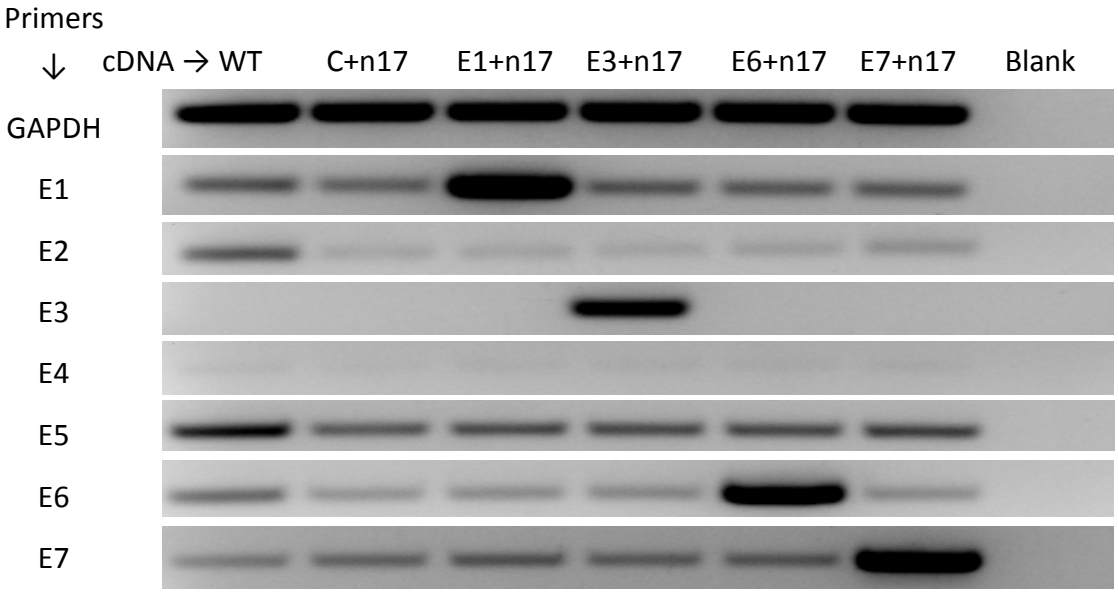


Figure S2.1 ELOVL1-ELOVL7 gene expression of Wild Type, control and ELOVLx cells incubated with A) 80µM n-13:0; B) 80µM n-15:0; C) 80µM n-17:0.
PCR was performed for 28 cycles. GAPDH is used as housekeeping control. All transfections were found to be successful as the expression levels of transfected ELOVLx are higher in all treatment groups. E1, ELOVL1; E2, ELOVL2; E3, ELOVL3; E4, ELOVL4; E5, ELOVL5; E6, ELOVL6; E7, ELOVL7; WT, Wild Type; C, Control; n13= n-13:0; n15= n-15:0; n17= n-17:0; Blank, was water only as template.

CHAPTER 3

Identification of genes mediating branched chain fatty acid elongation*

Abstract

Saturated branched chain fatty acids (BCFA) terminating with an isopropyl or sec-butyl groups, are common bioactive food components consumed from beef, fish, and dairy products, generally lowering melting point when they substitute for *normal* (linear) saturated fatty acids. Little is known about their endogenous metabolism and the enzymes mediating their interconversions. We report for the first time the substrate specificity of the ELOVLx (elongases) towards representative BCFA, *anteiso*-15:0 and *iso*-18:0, and assessed competition between BCFA and normal saturated FA (n-SFA). Transient transfection of ELOVLx into the human breast cancer MCF-7 cells show ELOVL6 mediating elongation of *anteiso*-15:0→*anteiso*-17:0, while ELOVL3 is active toward *iso*-18:0→*iso*-20:0. Competition studies between BCFA and n-SFA for ELOVLx mediated elongations reveal n-16:0 is preferred over *anteiso*-15:0 for ELOVL6, while n-18:0 is preferred over *iso*-18:0 in ELOVL3 cells. Thus, ELOVLx that primarily operate on linear saturated fatty acids operate on BCFA rather than the ELOVLx that operate on lower melting, polyunsaturated, fatty acids. The competition between BCFA and n-SFA for ELOVLx mediated elongation may have implications in the skin, sebaceous, and meibomian glands where both normal and branched chain fatty acids are present at comparable levels.

* Zhen Wang, Dong Hao Wang, Hui Gyu Park, Yuanyuan Yan, Yuliya Goykhman, Peter Lawrence, Kumar S. D. Kothapalli, and J. Thomas Brenna. Identification of genes mediating branched chain fatty acid elongation. Submitted for publication, Sep 2018.

Introduction

Saturated branched chain fatty acids (BCFA) of chain length greater than C11 terminating with a prop-2-yl (*iso*) or sec-butan-2-yl (*anteiso*) group are common bioactive food components [1-4]. Dairy and beef [1], fish [2], and cow's milk [4] fat all contain 1% or more of BCFA; and these food products in the American diet contribute to a mean intake more than 500 mg/d of BCFA in the U.S. [3, 4]. In humans, BCFA are primarily found in skin surface lipids [5], synthesized by the sebaceous [6] and meibomian glands [7, 8]. They are components of many bacterial membranes where they serve similar biophysical roles as *cis* unsaturated FA to lower melting point and increase the fluidity of membrane and excreted lipids [9]. Vernix caseosa, the thick lipid-laden material produced by human fetal skin in the last weeks of gestation is the human substance which is richest in BCFA, at about 30% of fatty acids [10]. Vernix has an early nutritional role because vernix particles suspended in amniotic fluid are swallowed by the late term human fetus and are present in meconium; BCFA thus are present throughout the GI tract in normal term gestation newborns [10, 11]. Experimental provision of BCFA to preterm

neonatal rat pups showed a reduction in necrotizing enterocolitis, with an increase in the anti-inflammatory cytokine IL-10, a dramatic shift in the relative abundance of *bacillus subtilis* and *pseudomonas aeruginosa* in a BCFA-fed group compared to the control, and incorporation of BCFA into membrane lipids of the intestinal tissue [12]. In Caenorhabditis elegans, post-embryonic development ceases when *iso*-17:0 biosynthesis is blocked by RNAi mediated disruption of the elongase elo-5 [13, 14] demonstrating that metabolic effects of BCFA may be highly structure dependent. Despite their importance, little is known of BCFA metabolism once ingested.

FA are often modified by elongation upon entering the cell. Understanding of FA metabolism and functional genomics requires details on genes and enzymes mediating the molecular transformations. Interconversion of fatty acids via either elongation or chain shortening in mammals proceeds by net 2 carbon addition or deletion from the fatty acyl chain [15]. Mammalian fatty acid elongation mainly takes place in the endoplasmic reticulum (ER). Specifically, fatty acids consumed in the diet or synthesized by fatty acid synthase (FASN) in cytosol, are elongated in the ER by coupled individual enzymes of the fatty acid chain elongation system (FACES) sequentially catalyzing condensation, reduction, dehydration and the second reduction reactions [16]. Among these four steps, condensation catalyzed by ELOVL (ELOngases of Very Long chain fatty acids) is the initial and rate-limiting step [15]. ELOVL show substrate specificity: of the seven known human ELOVLx (ELOVL1-7), ELOVL1,

ELOVL3, ELOVL6 and ELOVL7 act primarily on saturated and monounsaturated fatty acids, ELOVL2 and ELOVL5 act on polyunsaturated fatty acids (PUFA), and ELOVL4 acts on fatty acids of chain length greater than C24 regardless of unsaturation [15, 17-24]. Others have comprehensively investigated substrate specificity of ELOVL1-7 with normal (linear chain) fatty acids [25]. To the best of our knowledge no data is available on the ELOVLx gene activity towards BCFA.

Here we used *anteiso*-15:0 (12-methyltetradecanoate) and *iso*-18:0 (16-methylheptadecanoate) as representative of the two major classes of BCFA to evaluate the substrate specificity of ELOVLx towards BCFA, and to investigate the competition between BCFA and normal saturated fatty acids (n-SFA) for the same ELOVLx mediated elongation. The MCF-7 human mammalian breast cancer cell line was used as a model because our lab has deep understanding of how these cells behave when transfected or treated with fatty acids and they have no native FADS2 encoded activities [26-29]. ELOVLx are transiently transfected into MCF-7 cells and shifts in product to substrate proportions are evaluated as the index of activity.

Materials and Methods

Chemicals and reagents

Fatty acids (*anteiso*-15:0, palmitic acid (n-16:0), *iso*-18:0 and stearic acid (n-18:0)) were purchased from Sigma-Aldrich (St. Louis, MO) and Larodan

(Malmo, Sweden). HPLC grade solvents for fatty acid extraction are from Sigma-Aldrich (St. Louis, MO) and Burdick & Jackson (Muskegon, MI). Cell culture media, PBS, FBS and other cell culture reagents are obtained from Life Technologies (Carlsbad, CA), Corning (Corning, NY) and Thermo Fisher Scientific (Waltham, MA).

ELOVL expression vector construction

ELOVL gene synthesis and cloning into expression vector were performed by GenScript service, Piscataway Township, NJ. PcDNA3.1 expression vector (Thermo Fisher Scientific, Waltham, MA) containing cytomegalovirus (CMV) promoter was used to clone seven open reading frame of ELOVL transcripts (ELOVL1-7). The GenBank accession numbers of ELOVL mRNA (NM) are given in **Table S3.1**. Plasmid DNA for transfection assays was extracted from E.coli using Plasmid Midi Kit (QIAGEN, Germantown, MD), verified by DNA sequencing and stored at -20°C. DNA sequencing was carried out at University of Texas at Austin sequencing facility using Agilent BioAnalyzer or at Cornell University life sciences core laboratories center using the Applied Biosystems automated 3730 DNA analyzer.

Mammalian cell culture, gene transfection, and fatty acid supplementation

General aspects of cell culture, fatty acid substrate treatment, and fatty acid analysis conditions are similar to those presented previously from our

laboratory [26] and briefly outlined here.

MCF-7 human breast cancer cells at Passage 22-45 were grown at 37°C in a humidified environment with 5% CO_2, using minimum essential medium alpha (MEM-α) with 10% fetal bovine serum (FBS) and 10mM buffer [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEPES] [28]. Fatty acid profiles of these cells are stable over this number of passages.

MCF-7 cells are seeded at a density of $1\times10^6/cm^2$. After 48h, cells were washed with 1× PBS and then transfected with specific ELOVLx transgene vectors or empty vector (control) using Polyplus jetPRIME transfection reagent (NY) following the manufacturer's recommendations. After 24h, the transfected MCF-7 cells were treated with 0 to 100μM of bovine serum albumin (BSA) bound BCFA and/or n-SFA substrates. Briefly, to make BSA bound substrates, *anteiso*-15:0, n-16:0, *iso*-18:0 and n-18:0 were dissolved in absolute pure ethanol to make 100mM fatty acid (FA) stock. FA stock (200μl) was mixed with FA free BSA in 1× PBS (4.4% w/w), followed by 30 min sonication and then incubated overnight at 37°C. BSA bound BCFA were filtered using 0.22μM syringe, and diluted to 0 to 100μM with non-FBS MCF-7 media then added to cells. After another 24 h, cells were washed twice with 1 × PBS, harvested by trypsinization and supernatant removed after centrifuging.

Fatty acid extraction and analysis

Fatty acid methyl esters (FAME) from harvested MCF-7 cell pellets were prepared according to the modified one-step method of Garces and Mancha [30]. Fatty acid composition were structurally identified by gas chromatography (GC) – covalent adduct chemical ionization tandem mass spectrometry (CACI/MS/MS) for double bonds [31, 32], and by GC - electron ionization mass spectrometry (EIMS) and EIMS/MS for branched carbon chain using a Saturn 2000 mass spectrometer attached to a Varian Star 3400 gas chromatograph [33]. EIMS spectra for *anteiso*-15:0, *anteiso*-17:0, *iso*-18:0 and *iso*-20:0, are shown in **Figure S3.1**. FAME were quantified by GC-flame ionization detector (GC-FID) (Shimadzu QS2010 Plus, Austin, TX or Hewlett-Packard, Palo Alto, CA). An equal weight FAME mixture, GLC462 (Nu-Chek Prep, Inc.), was used to calculate response factors of all fatty acids [34].

Substrate incorporation and product generation are expressed as substrate (% of total FA) and product (% of total FA) and their peak areas are normalized to 20:3n-6. 20:3n-6 was used for normalization because its area percent was stable between ELOVLx and Control treatments as in our previous studies [26]. Significant changes in the BCFA level(s) between ELOVL and Control (empty pcDNA3.1(+) vector) samples established the substrate specificity of a particular ELOVL. The catalytic activity of ELOVLx was evaluated by looking for the enhanced conversion of substrates (S) to products (P) calculated as: (P) / (S + P), normalized to the Control group and expressed as a %. Substrate competition between BCFA and n-SFA was addressed by assessing

the inhibition of substrate incorporation S / 20:3n-6, product generation P / 20:3n-6 and/or the corresponding conversion P / (S + P) (% of total FA/% of total FA).

Statistical analysis

The cells transfected with empty vector are considered controls. Data was analyzed by Student's t-test to compare the differences between the two groups (ELOVLx transfection treatment vs control), and by one-way ANOVA with post-hoc Tukey Honest Significant Difference (HSD) test in multiple group comparisons. In competition studies, cells with 0μM fatty acid dose are considered control; other doses are considered treatment. Statistical analyses are performed using Microsoft Excel 2010 (Microsoft Corporation, WA) and OriginPro 8 Software (OriginLab Corporation, MA). All values are presented as mean ± standard deviation (SD). Differences when $p<0.05$ are considered significant.

Results

MCF-7 cells readily take up BCFA

Native MCF-7 breast cancer cells have high amounts of n-SFA n-16:0 (25.14 ± 0.20 (%)) and n-18:0 (13.50 ± 0.04 (%)) but negligible BCFA. Control (empty vector) and ELOVLx transiently transfected cells dosed with 80μM substrates readily take up 17.13 ± 1.48 (%) of *anteiso*-15:0 and 27.89 ± 1.51 (%) of *iso*-18:0, respectively.

Elongation of *anteiso*-15:0 → *anteiso*-17:0

Figure 3.1 shows the ELOVLx activity towards *anteiso*-15:0 → *anteiso*-17:0. The percent conversion of fatty acid substrate anteiso-15:0 into elongation product anteiso-17:0, was calculated as [anteiso-17:0] / ([anteiso-15:0] + [anteiso-17:0]), and normalized to control group. When cells transiently expressing ELOVL1-7 and control were incubated with 80μM *anteiso*-15:0, ELOVL6 showed highest catalytic activity (175.97 ± 0.47 (%), $p<0.05$) towards *anteiso*-15:0 → *anteiso*-17:0; ELOVL5 showed moderate activity (157.35 ± 7.89 (%), $p<0.05$); other ELOVLx showed no significant activities.

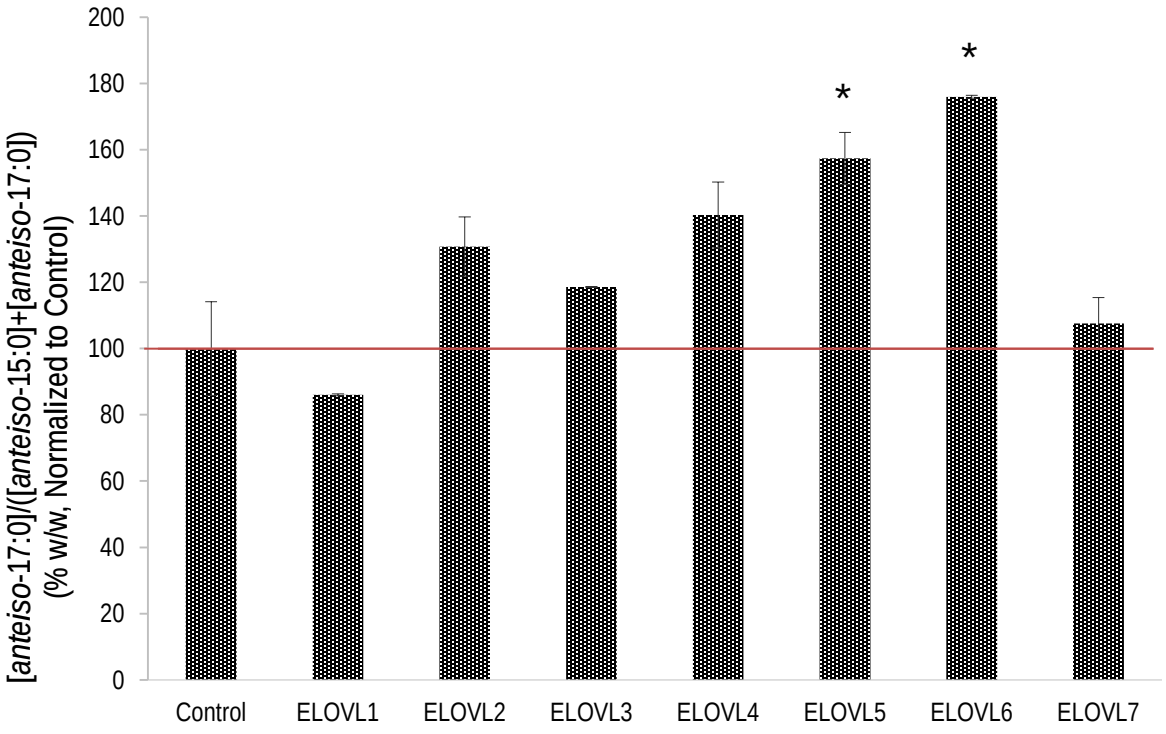


Figure 3.1 ELOVLx activity, *anteiso*-15:0→*anteiso*-17:0.

Percent conversion of fatty acid substrate *anteiso*-15:0 into elongated product *anteiso*-17:0 (ratios calculated as [*anteiso*-17:0]/[*anteiso*-15:0]+ [*anteiso*-17:0]) was measured in ELOVLx transiently transfected MCF-7 cells and normalized to control (empty vector) group. ELOVL6 showed highest catalytic activity towards *anteiso*-15:0→*anteiso*-17:0, ELOVL5 had moderate activity, and other ELOVL show no activity. *<0.05, **<0.01, ***<0.001.

Competition between *anteiso*-15:0 and n-16:0 for ELOVL6 mediated elongation

Figure 3.2 shows conversion of *anteiso*-15:0 → *anteiso*-17:0 and n-16:0 → n-18:0 in control and ELOVL6 transfected cells. The conversion of anteiso-15:0 → anteiso-17:0 and n-16:0 → n-18:0 was enhanced from 0.241 ± 0.003 (control) to 0.322 ± 0.006 (ELOVL6 transfection) and from 0.340 ± 0.001 (control) to 0.388 ± 0.004 (ELOVL6 transfection), respectively, in cells dosed with 80µM substrates. As ELOVL6 acts on n-16:0 and *anteiso*-15:0 we investigated *anteiso*-15:0 competition with n-16:0 for ELOVL6 mediated elongation.

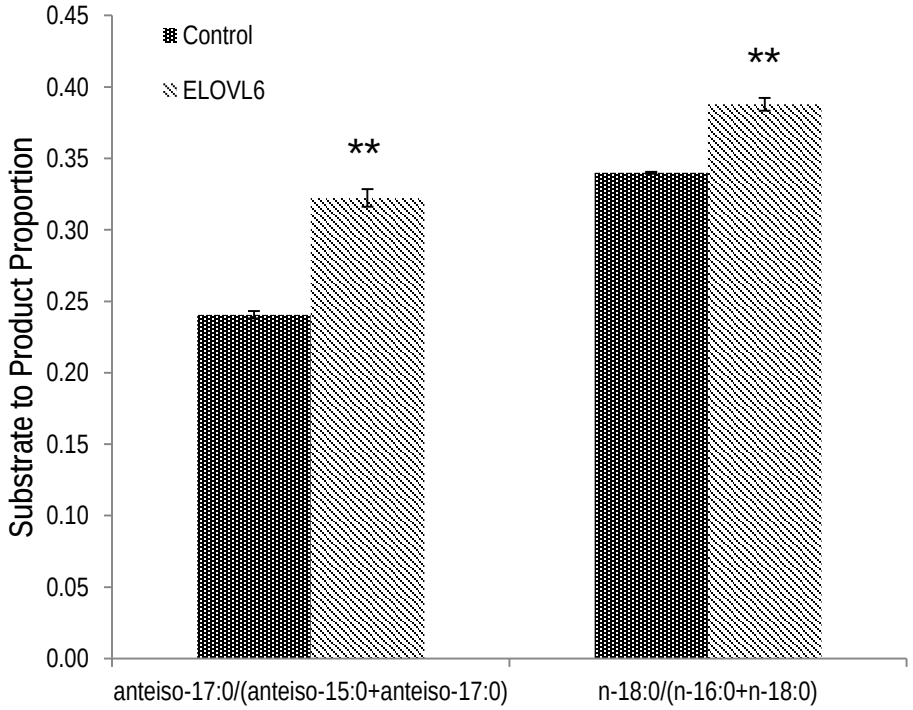
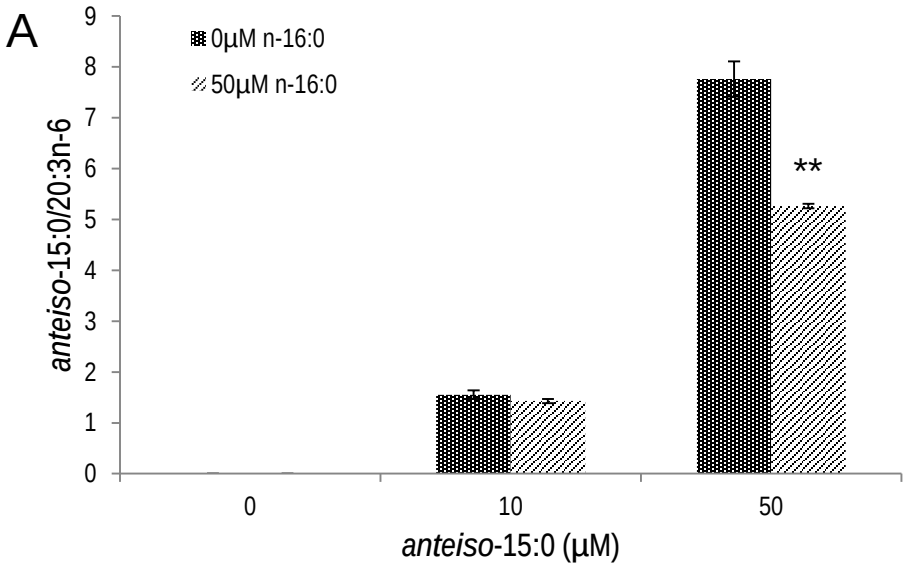


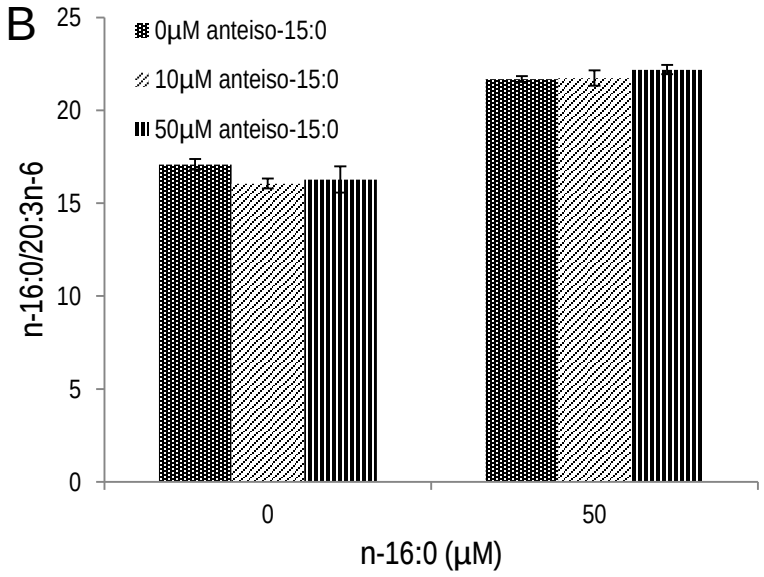
Figure 3.2 ELOVL6 activity toward *anteiso*-15:0 and n-16:0.

Substrate to product proportions were calculated in Control and ELOVL6 transfected MCF-7 cells dosed with 80µM *anteiso*-15:0 or 80µM n-16:0. ELOVL6 acts on both *anteiso*-15:0 and n-16:0 substrates as seen by significant increase in the product *anteiso*-17:0 and n-18:0 for ELOVL6 mediated elongation. *<0.05, **<0.01, ***<0.001.

Competition between FA is evaluated by two ways, (a) substrate incorporation in the cells and corresponding product generation, and (b) conversion of substrate to product. Competition between *anteiso*-15:0 and n-16:0 is illustrated in **Figure 3.3**, **Figure 3.4** and **Figure S3.2**. Untreated cells have high basal n-16:0 and negligible *anteiso*-15:0. Competition for ELOVL6 mediated elongation was investigated by holding n-16:0 (0μM, 50μM) and varying *anteiso*-15:0 (0μM, 10μM, 50μM) doses. The 50μM *anteiso*-15:0 dose was significantly lower in cells dosed with 50μM n-16:0 compared to 0μM n-16:0 (**Figure 3.3A**), whereas the incorporation of *anteiso*-15:0 as low as 10μM was not affected by n-16:0 coadministration (**Figure 3.3A**). The n-16:0 at doses of 0μM and 50μM was not affected by increasing *anteiso*-15:0 doses (**Figure 3.3B**). ELOVL6 catalyzes *anteiso*-15:0 → *anteiso*-17:0 conversion, thus we probed the generation of n-18:0 and *anteiso*-17:0 in cells holding n-16:0 (0μM, 50μM) and varying *anteiso*-15:0 (0μM, 10μM, 50μM) doses. The amount of *anteiso*-17:0 was significantly lower in cells at doses of 10μM and 50μM of *anteiso*-15:0 and 50μM n-16:0 compared to 0μM n-16:0 (**Figure 3.3C**). In cells with no exogenous n-16:0 (0μM), the increasing doses 10μM and 50μM of *anteiso*-15:0 significantly lowered n-18:0 generation (**Figure 3.3D**); whereas the generation of n-18:0 in 50μM n-16:0 dosed cells was not affected by increasing *anteiso*-15:0 doses (**Figure 3.3D**). In addition, the conversion of *anteiso*-15:0 → *anteiso*-17:0 was suppressed in cells at doses of 10μM and 50μM of *anteiso*-15:0 and 50μM n-16:0 compared to 0μM n-16:0 (**Figure 3.4A**); whereas the conversion of n-16:0 → n-18:0 was not affected by

anteiso-15:0 doses (**Figure 3.4B**). Consequently, n-16:0 competes with *anteiso*-15:0 not only for substrate incorporation and product generation, but also for conversion. n-16:0 is preferred over *anteiso*-15:0 for ELOVL6 mediated elongation.


A
0μM n-16:0
50μM n-16:0
anteiso-15:0/20:3n-6
9
8
7
6
5
4
3
2
1
0
**
0
10
50
anteiso-15:0 (μM)


B
0μM anteiso-15:0
10μM anteiso-15:0
50μM anteiso-15:0
n-16:0/20:3n-6
25
20
15
10
5
0
0
50
n-16:0 (μM)

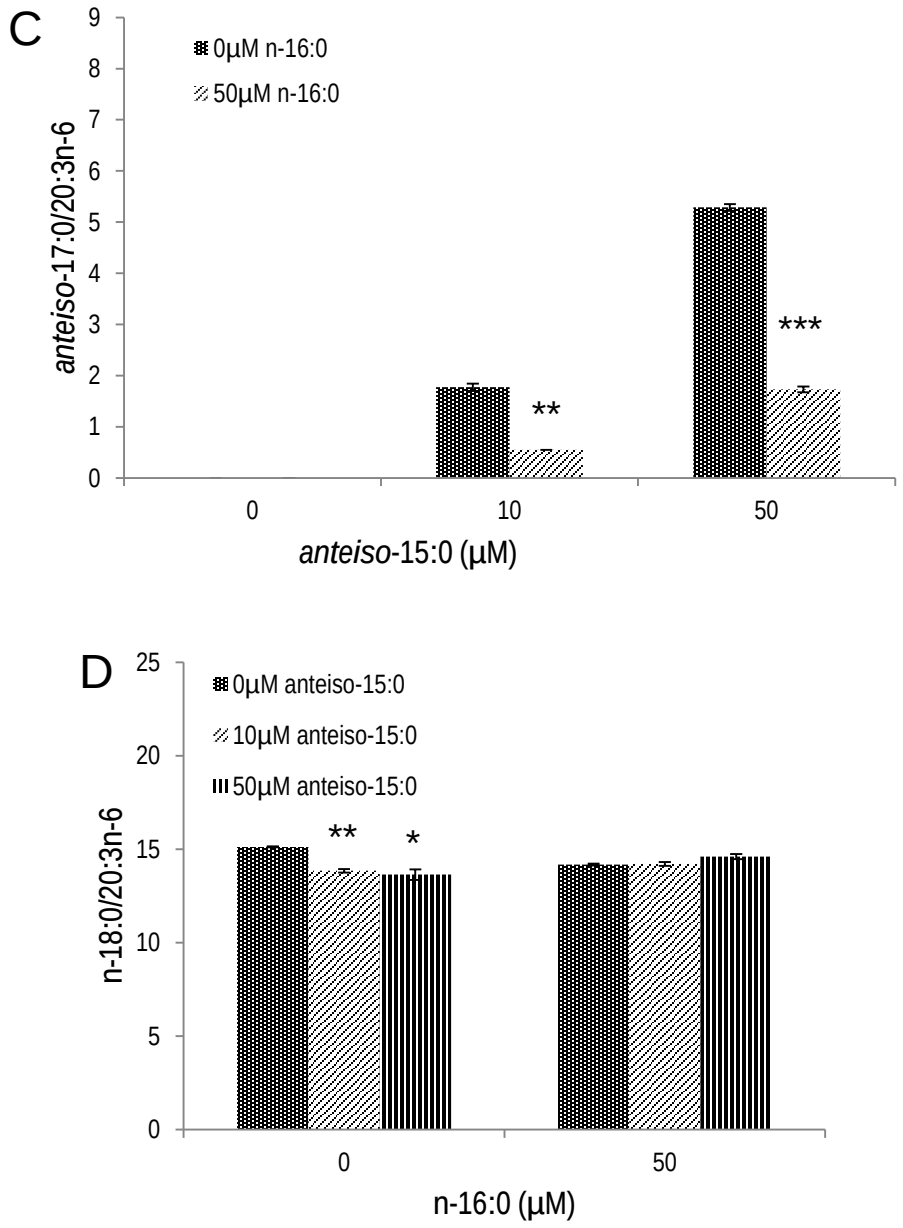


Figure 3.3 Competition between *anteiso*-15:0 and n-16:0 for ELOVL6 mediated elongation.

A. The 50μM n-16:0 compared to 0μM n-16:0 reduced *anteiso*-15:0 incorporation significantly in cells with 50μM *anteiso*-15:0; B. The increasing *anteiso*-15:0 doses did not affect n-16:0 incorporation; C. *anteiso*-17:0 was significantly lower in cells at doses of 10μM and 50μM of *anteiso*-15:0 and 50μM n-16:0 compared to 0μM n-16:0; D. The increasing *anteiso*-15:0 doses significantly lowered n-18:0 accumulation in cells with no exogenous n-16:0 (0μM). All FA levels normalized to 20:3n-6. *<0.05, **<0.01, ***<0.001.

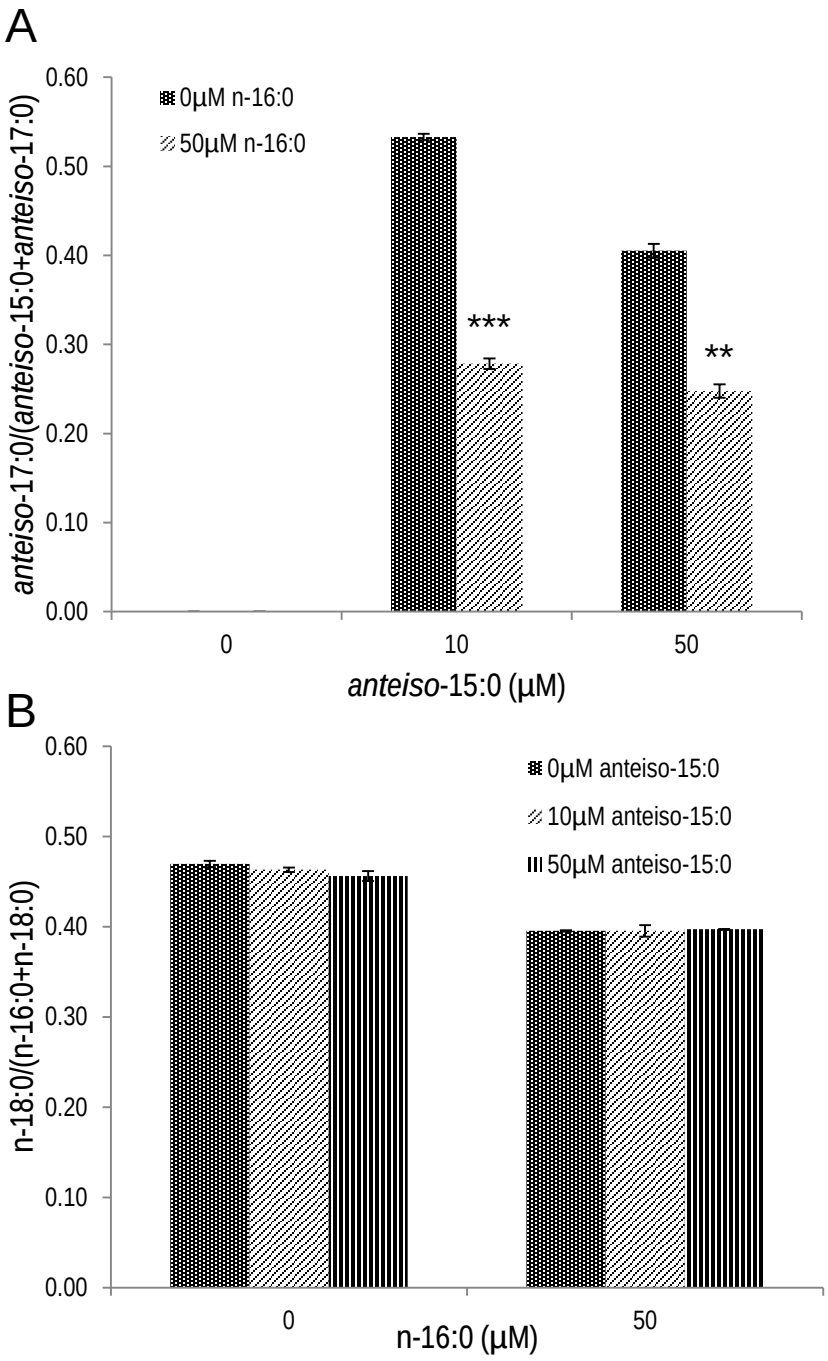


Figure 3.4 Conversion proportions of substrate to product in ELOVL6 mediated elongation competition study between *anteiso*-15:0 and n-16:0.

A. Conversion of *anteiso*-15:0 to *anteiso*-17:0 is suppressed in *anteiso*-15:0 (10µM and 50µM) and 50µM n-16:0 dosed cells compared to 0µM n-16:0. B. Conversion of n-16:0 to n-18:0 was not affected by *anteiso*-15:0 dosage. *<0.05, **<0.01, ***<0.001.

Elongation of *iso*-18:0 → *iso*- 20:0

Figure 3.5 shows the ELOVLx activity towards *iso*-18:0 → *iso*-20:0. Percent conversion of fatty acid substrate *iso*-18:0 into elongation product *iso*-20:0, was calculated as [*iso*-20:0] / ([*iso*-18:0] + [*iso*-20:0]), and normalized to the control group. When cells transiently expressing ELOVL1-7 and control were incubated with 80μM *iso*-18:0, ELOVL3 showed highest catalytic activity (343.31 ± 4.39 (%), $p<0.001$) towards *iso*-18:0→ *iso*-20:0. ELOVL2 and ELOVL7 showed moderate activities as 150.19 ± 1.23 (%) ($p<0.001$) and 213.90 ± 15.69 (%) ($p<0.01$) respectively, whereas, ELOVL4, 5 and 6 showed minor activities as 122.78 ± 0.43 (%), 131.61 ± 2.07 (%), 126.25 ± 0.84 (%) ($p<0.01$), respectively. ELOVL1 showed no activity.

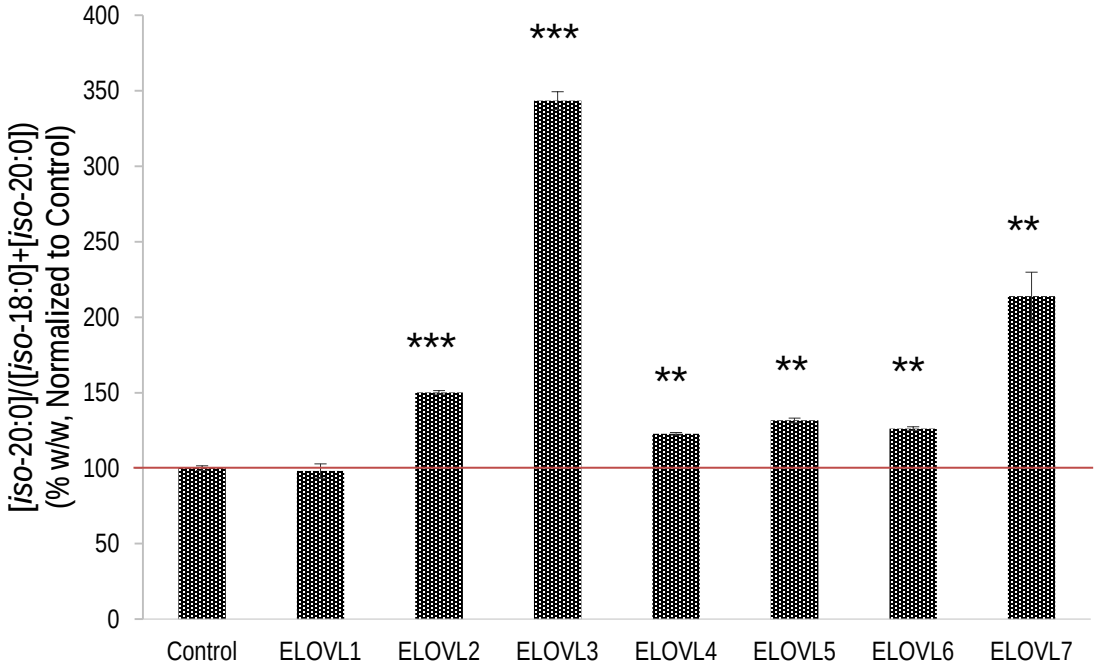


Figure 3.5 ELOVLx activity, *iso*-18:0→*iso*-20:0.

Percent conversion of fatty acid substrate *iso*-18:0 into elongated product *iso*-20:0 (ratios calculated as [*iso*-20:0]/[*iso*-16:0]+ [*iso*-18:0]+ [*iso*-20:0]) measured in ELOVLx transiently transfected MCF-7 cells and normalized to control (empty vector) group. ELOVL3 has highest catalytic activity towards *iso*-18:0→ *iso*-20:0. ELOVL2, 4, 5, 6 and 7 have moderate to minor activities, and ELOVL1 shows no activity. *<0.05, **<0.01, ***<0.001.

Competition between *iso*-18:0 and n-18:0 for ELOVL3 mediated elongation

The conversion efficiency from substrate to product was investigated by incubating ELOVL3 cells with 0 to 100 μM of n-18:0 or *iso*-18:0 (**Figure S3.3**). n-18:0 shows no significant differences between various substrate concentrations (**Figure S3.3A**); while *iso*-18:0 has highest conversion efficiency in cells incubated with 10 and 20 μM *iso*-18:0 (**Figure S3.3B**). Therefore, 10 μM groups were chosen to investigate competition of *iso*-18:0 and n-18:0 for ELOVL3 mediated elongation. The conversions of *iso*-18:0 → *iso*-20:0 and n-18:0 → n-20:0 are enhanced from 0.015 ± 0.002 to 0.077 ± 0.002 and from 0.023 ± 0.001 to 0.042 ± 0.001, respectively, in ELOVL3 cells treated with 10μM substrates, showing ELOVL3 action on both substrates (**Figure 3.6**). We investigated *iso*-18:0 competition with n-18:0 for ELOVL3 mediated elongation.

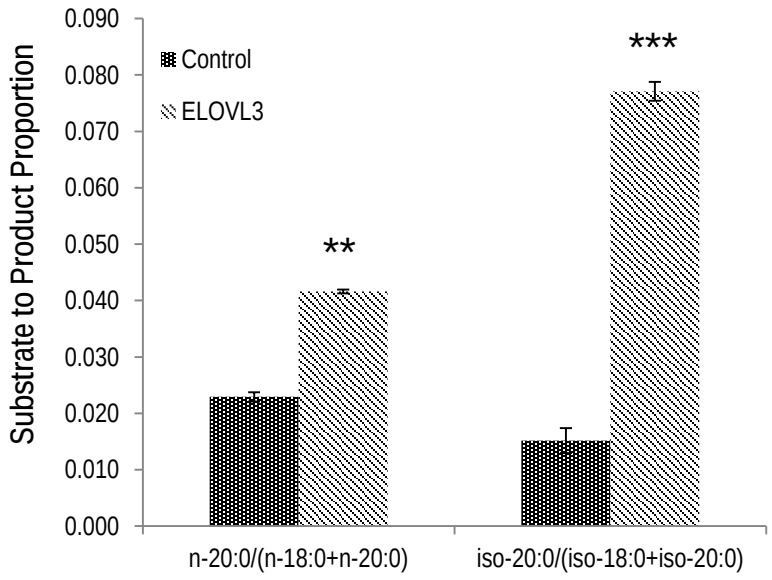
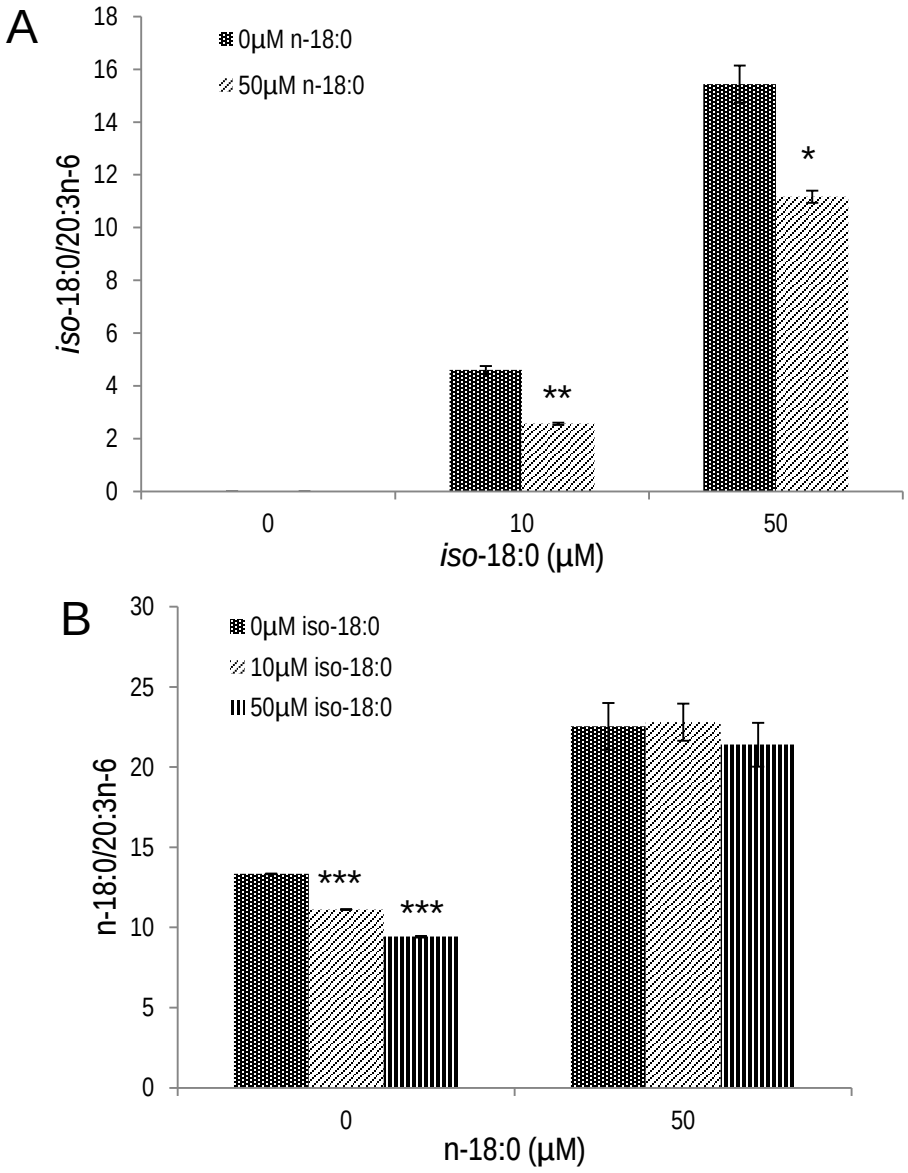


Figure 3.6 ELOVL3 activity toward n-18:0 and *iso*-18:0.

Substrate to product proportions were calculated in Control and ELOVL3 transfected MCF-7 cells dosed with 10µM *iso*-18:0 or 10µM n-18:0. ELOVL3 acts on both n-18:0 and iso-18:0 substrates as seen by significant increase in the product n-20:0 and *iso*-20:0 in ELOVL3 transfected cells compared to control cells. *<0.05, **<0.01, ***<0.001.

Competition between *iso*-18:0 and n-18:0 is illustrated in **Figure 3.7** and **Figure 3.8**. Untreated cells have high basal n-18:0 but negligible *anteiso*-18:0. Competition for ELOVL3 mediated elongation was investigated by holding n-18:0 (0μM, 50μM) and varying iso-18:0 (0μM, 10μM, 50μM) doses. The *iso*-18:0 at doses of 10μM and 50μM was significantly lower in cells dosed with 50μM n-18:0 compared to 0μM n-18:0 (**Figure 3.7A**). In cells with no exogenous n-18:0 (0μM), the increasing doses 10μM and 50μM of *iso*-18:0 significantly lowered n-18:0 incorporation (**Figure 3.7B**), whereas, the incorporation of n-18:0 as high as 50μM into cells was not affected by *iso*-18:0 coadministration (**Figure 3.7B**). ELOVL3 catalyzes *iso*-18:0→ *iso*-20:0 conversion, so we probed the generation of n-20:0 and *iso*-20:0 in cells holding n-18:0 (0μM, 50μM) and varying *iso*-18:0 (0μM, 10μM, 50μM) doses. *iso*-20:0 was significantly lower in cells at doses of 10μM and 50μM of *iso*-18:0 and 50μM n-18:0 compared to 0μM n-18:0 (**Figure 3.7C**). In cells with no exogenous (0μM) or 50μM of n-18:0, the increasing doses 10μM and 50μM of *iso*-18:0 didn't affect n-20:0 generation (**Figure 3.7D**). Additionally, the conversion of *iso*-18:0 to *iso*-20:0 was not affected by n-18:0 dosage in both concentrations of *iso*-18:0 tested (**Figure 3.8A**); similarly the conversion of n-18:0 to n-20:0 was not affected by *iso*-18:0 doses (**Figure 3.8B**). Thus, n-18:0 competes with *iso*-18:0 for substrate incorporation and product generation, but not for conversion. n-18:0 is preferred over *iso*-18:0 for ELOVL3 mediated elongation.


A
0μM n-18:0
50μM n-18:0
iso-18:0/20:3n-6
18
16
14
12
10
8
6
4
2
0
**
*
0
10
50
iso-18:0 (μM)
B
0μM iso-18:0
10μM iso-18:0
50μM iso-18:0
n-18:0/20:3n-6
30
25
20
15
10
5
0
***
***
0
50
n-18:0 (μM)

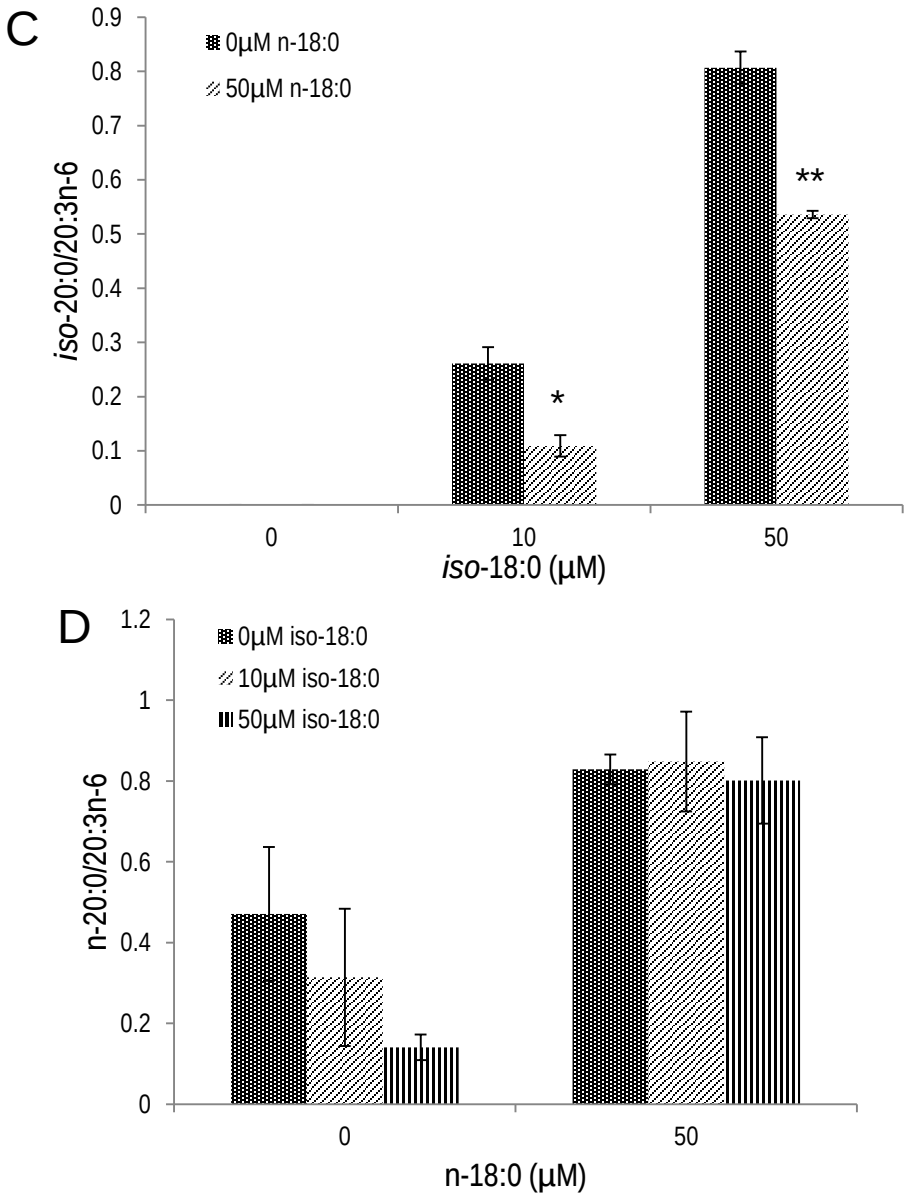


Figure 3.7 Competition between *iso*-18:0 and n-18:0 for ELOVL3 mediated elongation.

A. The 50μM n-18:0 compared to 0μM n-18:0 reduced *iso*-18:0 significantly; B. The increasing *iso*-18:0 doses significantly lowered n-18:0 incorporation in cells with no exogenous n-18:0 (0μM); C. *iso*-20:0 was significantly lower in cells at doses of 10μM and 50μM of *iso*-18:0 and 50μM n-18:0 compared to 0μM n-18:0; D. The increasing *iso*-18:0 didn't affect n-20:0 accumulation in cells with no exogenous (0μM) or 50μM of n-18:0. All FA levels normalized to 20:3n-6. *<0.05, **<0.01, ***<0.001.

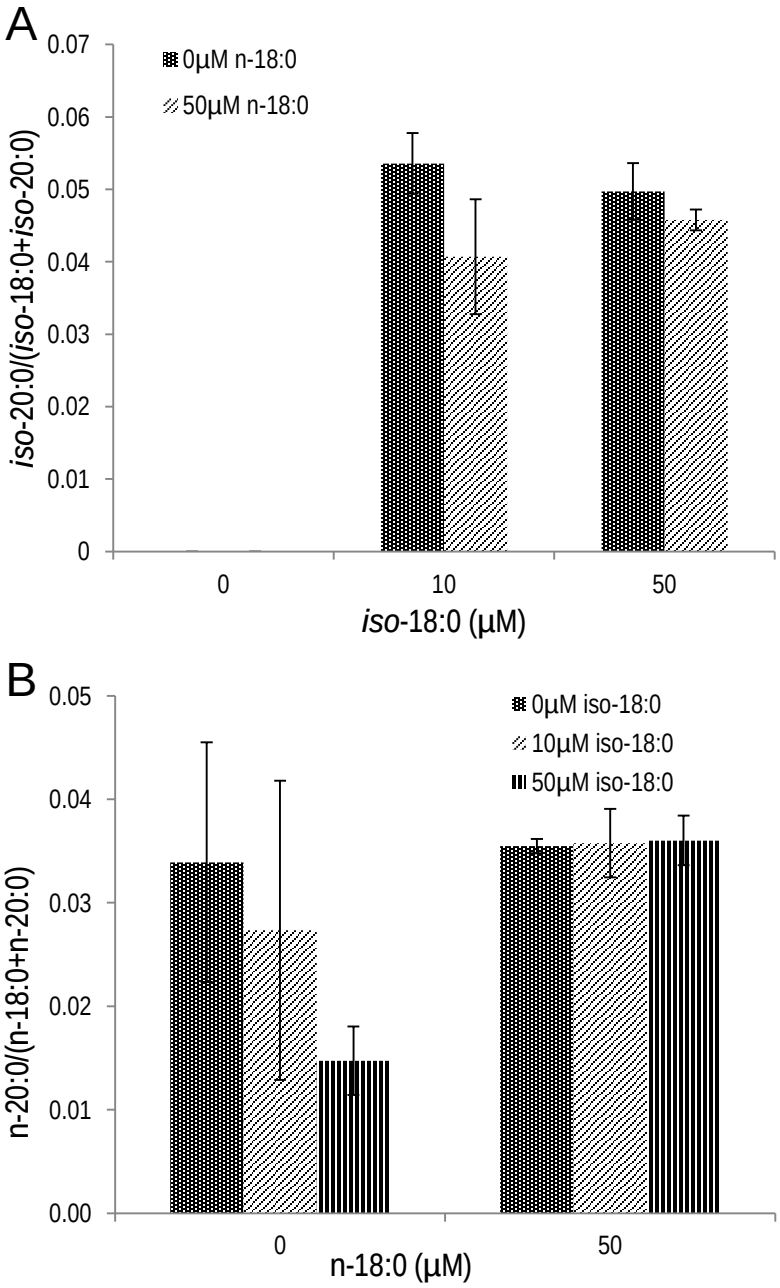


Figure 3.8 Conversion proportions of substrate to product in ELOVL3 mediated elongation competition study between n-18:0 and *iso*-18:0.

A. Conversion of *iso*-18:0 to *iso*-20:0 was not affected by n-18:0 dose; B. Conversion of n-18:0 to n-20:0 was not affected by *iso*-18:0 dosage.

Discussion

The methyl group near the terminal end of BCFA has long been known to influence their biophysical properties, specifically lowering phase transition temperature of BCFA-containing phospholipids and yielding higher membrane fluidity compared to the corresponding linear chain isomers [9]. BCFA properties in mammalian tissues are therefore similar to *cis* unsaturated fatty acids. In wild type MCF-7 cells, negligible amounts of BCFA are found, consistent with observation in vivo in which BCFA are very low in internal tissue. However, cells grown in media with BCFA present readily take up *iso* and *anteiso* BCFA.

To the best our knowledge, this is the first study to identify the ELOVL principally responsible for elongation of BCFA in human cells, and moreover to show that n-SFA compete for these ELOVLx. Even though the biophysical properties of BCFA are more comparable to that of *cis* unsaturated fatty acids than saturated fatty acids [9], elongation activity was by ELOVL6 and 3 which are active against normal (linear) saturated fatty acids and not ELOVL2/5 active against unsaturates of similar chain length. In the present study, we verified the ELOVL6 activity for the elongation of n-16:0 → n-18:0 and ELOVL3 elongation n-18:0 → n-20:0 found in other studies [15, 35-37]. Here we extend those activities to show that ELOVL6 specially catalyzes elongation of *anteiso*-15:0 → *anteiso*-17:0, whereas ELOVL3 mediates *iso*-18:0 → *iso*-20:0. Elongases in several species are known to share overlapping functions

[23, 38, 39]. Previously it has been shown that mammalian ELOVL3 and ELOVL7 share overlapping functions towards n-SFA [37, 40]. ELOVL3 and ELOVL7 were found to elongate n-16:0 to n-22:0 FA, with the highest activity toward n-18:0 FA [37, 40]. Here we found ELOVL3 has major activity towards *iso*-18:0, whereas ELOVL2 and ELOVL7 showed low activities. Thus, ELOVL2, ELOVL3 and ELOVL7 share overlapping functions towards *iso*-18:0. We also recently discovered ELOVL6 actions on odd chain saturated fatty acids n-13:0 and n-15:0 (Wang, et al., 2018, in press).

Previously we have shown n-16:0 competes with polyunsaturated fatty acid precursors 18:2n-6 and 18:3n-3 for FADS2 mediated Δ6-desaturation [26] and have also shown exogenously added BCFA readily displacing n-SFA in human cells [41]. Based on our previous and present results, we hypothesized that BCFA compete with n-SFA for ELOVLx mediated elongation. Here we investigated the competition between BCFA and n-SFA by treating ELOVL6 cells with *anteiso*-15:0 and n-16:0, and ELOVL3 cells with *iso*-18:0 and n-18:0 in. We choose n-16:0 as it is the most abundant n-SFA in MCF-7 cells. We looked at substrate incorporation (S / 20:3n-6), product generation (P / 20:3n-6) and conversion proportion of substrate to product (P / (S+P)) to reveal competition between *anteiso*-15:0 and n-16:0, and between *iso*-18:0 and n-18:0. Our data show that ELOVL6 mediated elongation prefers n-16:0 over *anteiso*-15:0 in substrate incorporation, product generation and conversion. In contrast, n-18:0 is preferred over *iso*-18:0 in ELOVL3 cells only for substrate

incorporation and product generation, but not for conversion. Also, the competition between n-SFA and BCFA became negligible at higher n-SFA dosage, thus dosage-dependent.

ELOVL6 specially catalyzes elongation of n-SFA and monounsaturated fatty acids (MUFA) with 12, 14 and 16 carbons thereby regulating de novo lipogenesis [20, 42-44]. ELOVL6 gene polymorphisms were associated with risk of insulin resistance and type 2 diabetes mellitus in European and Han Chinese populations [44, 45]. Moreover, both ELOVL6 and its upstream epidermal growth factor (EGF) gene are significantly associated with gout in a Chinese Han population [46]. Our present study expands the ELOVLx activity range to BCFA, which might provide clues to address the ELOVL6 genetic variations in association with prevalence of these diseases.

The competition between n-SFA (n-16:0 and n-18:0) and BCFA suggests BCFA may influence metabolic conditions characterized by relatively high de novo lipogenesis and excess n-SFA production. Increased cellular n-16:0 and n-18:0 produced by lipogenesis from high carbohydrate and alcohol intake are associated with inflammation, insulin resistance and increased diabetes risk [47]. ELOVL6 converting 16:0 → 18:0 is highly expressed in nonalcoholic steatohepatitis (NASH), breast cancer and liver cancer [48-50]. From **Figure 3.3D** and **Figure 3.7B**, increasing BCFA dosage reduced production of n-18:0. Previous studies showed inhibiting ELOVL6 activity in mice enhanced insulin

sensitivity and ameliorated diabetes [44, 51, 52]. As BCFA substrates compete with n-SFA substrates for ELOVL6 mediated elongation, we speculate that in diabetics increasing BCFA intake may displace n-SFA and influence the disease phenotype.

ELOVL3 is expressed in skin and connective tissues such as sebaceous glands, meibomian glands, and hair follicles, and brown adipocytes [42, 53-56], of which BCFA and n-SFA dominate FA composition [7, 53, 57, 58]. ELOVL3-ablated mice showed skin dysfunction and no obvious FA with more than 20 carbons [55]. Both ELOVL3 and ELOVL6 are downregulated in the stratum corneum of atopic dermatitis (AD) patients, which had significant effects on the proportion of skin lipids, especially long-chain fatty acids [59]. Human skin is unique in its fatty acid profile, with the highest monounsaturated being sapienic acid (16:1n-10) produced by action of FADS2 on 16:0 [26]. Sapienic acid and BCFA are both likely to modulate melting point and related biophysical properties of sebum and other skin lipids. Further research is required to establish how these changes influence skin function.

In conclusion, our data expands the range of ELOVLx substrate specificity to BCFA, particularly revealing ELOVL6 for *anteiso*-15:0 → *anteiso*-17:0 elongation and ELOVL3 for *iso*-18:0 → *iso*-20:0 elongation. We also tested the competition between BCFA and n-SFA for same ELOVLx mediated elongation, namely, *anteiso*-15:0 and n-16:0 for ELOVL6 and *iso*-18:0 and n-

18:0 for ELOVL3 mediated elongations. BCFA is less favored than n-SFA in substrate preference for ELOVLx mediated elongation. The competition between BCFA and common n-SFA (n-16:0 and n-18:0) for ELOVLx mediated elongation provide clues to metabolic conditions that are related to local excess of n-SFA such as excess carbohydrate levels as in diabetes.

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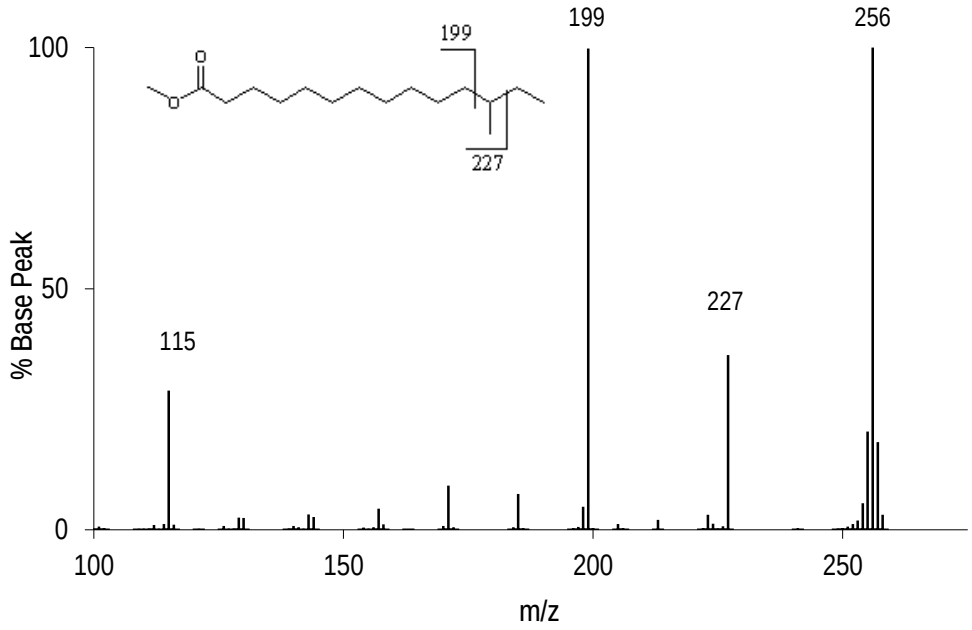
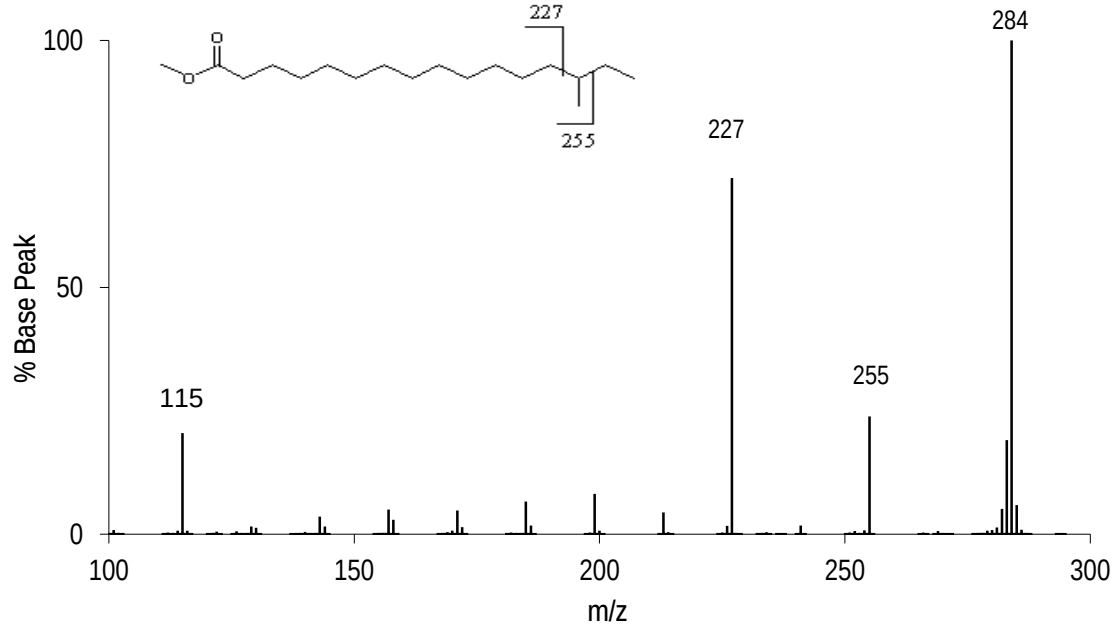
APPENDIX

A. EIMS2 *anteiso*-15:0



B. EIMS2 *anteiso*-17:0



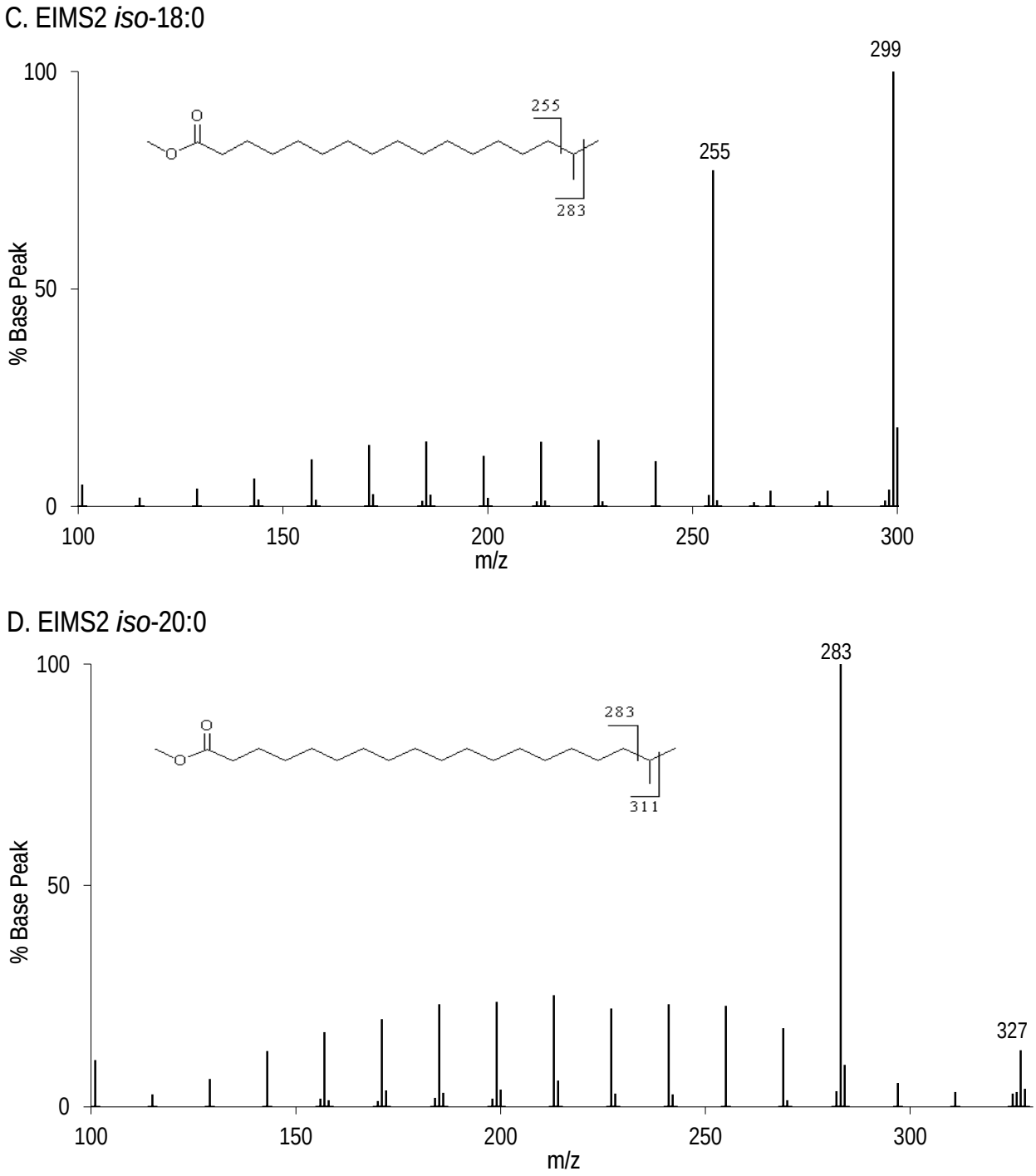


Figure S3.1 Identification of *iso*-18:0, *iso*-20:0, *anteiso*-15:0 and *anteiso*-17:0 with electron ionization mass spectrometry (EIMS). A. EIMS showing the diagnostic ions (m/z 199, 227) characteristic of *anteiso*-15:0; B. EIMS showing the diagnostic ions (m/z 227, 255) characteristic of *anteiso*-17:0; C. EIMS spectrum showing the diagnostic ions (m/z 255, 283) characteristic of *iso*-18:0; D. EIMS showing the diagnostic ions (m/z 283, 311) characteristic of *iso*-20:0.

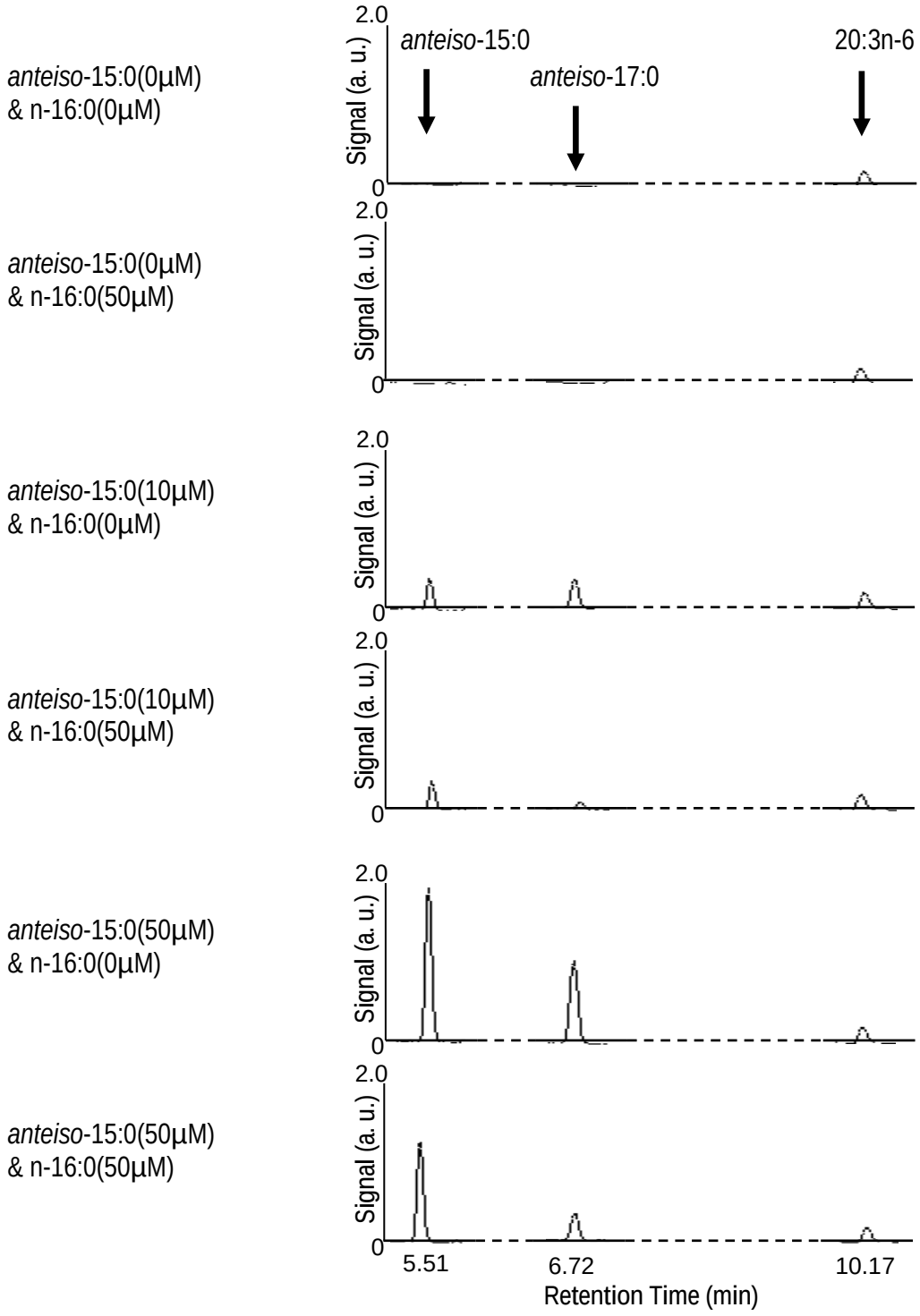


Figure S3.2 GC-FID chromatography of substrate and product as well as normalization FA 20:3n-6 in ELOVL6 transfected MCF-7 cells in competition study. Both of substrate *anteiso*-15:0 and the corresponding product *anteiso*-17:0 are marked out and normalized to FA 20:3n-6. The y axis scale is

reflects identical number of counts in each panel, showing that 20:3n-6 signal is stable from treatment to treatment.

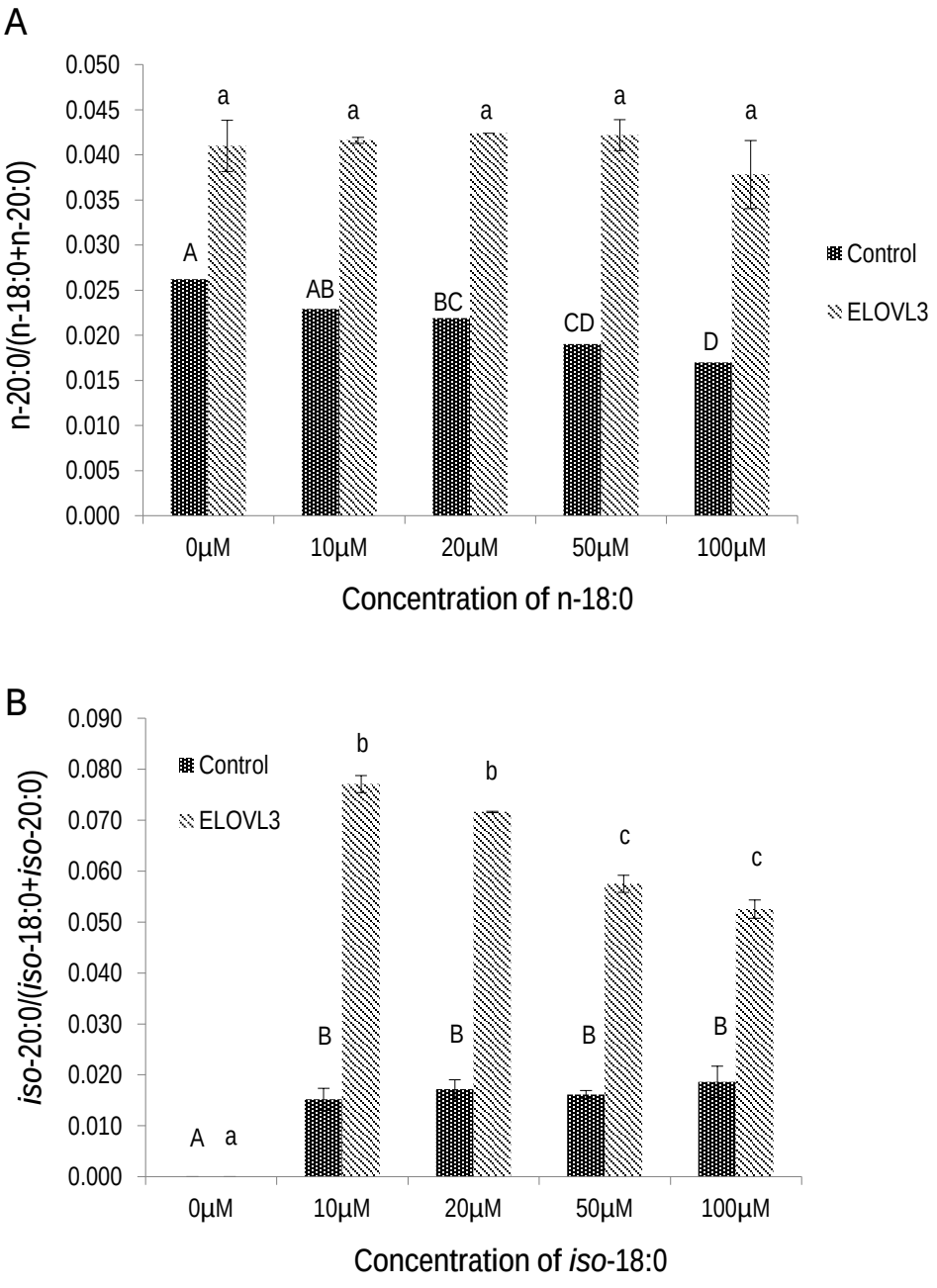


Figure S3.3 Substrate to product proportions in transfected MCF-7 cells dosed with n-18:0 and *iso*-18:0. A. Control and ELOVL3 transfected MCF-7 cells dosed with various concentrations of n-18:0. n-18:0 shows no significant difference between various substrate concentration incubations; B. Control and ELOVL3 transfected MCF-7 cells dosed with various concentrations of *iso*-18:0. *iso*-18:0 has highest conversion proportions in cells incubated with 10 and 20μM *iso*-18:0. A-D: $p<0.05$; a-c: $p<0.05$.

Human mRNA	NCBI Reference Sequence
Homo sapiens ELOVL1	NM_022821.3
Homo sapiens ELOVL2	NM_017770.3
Homo sapiens ELOVL3	NM_152310.2
Homo sapiens ELOVL4	NM_022726.3
Homo sapiens ELOVL5	NM_021814.4
Homo sapiens ELOVL6	NM_024090.2
Homo sapiens ELOVL7	NM_024930.2

Table S3.1 NCBI reference human ELOVL mRNA sequence accession numbers

CHAPTER 4

The role of fatty acid desaturase 2 gene in branched chain and odd chain fatty acid metabolism*

Abstract

Branched chain fatty acids (BCFA) and linear chain/normal odd chain fatty acids (n-OCFA) are major fatty acids in human skin lipids, especially sebaceous gland (SG) wax esters. Skin lipids contain variable amounts of monounsaturated BCFA and OCFA, in some reports over 20% of total fatty acids. Fatty acid desaturase 2 (FADS2) codes for a multifunctional enzyme that catalyzes Δ4-, Δ6- and Δ8- desaturation towards eleven fatty acid substrates. FADS2 is expressed in human SG, and produces the unique normal even chain fatty acid (n-ECFA) sapienic acid (16:1n-10/n-6Z-16:1) from palmitic acid (n-16:0) via Δ6-desaturation. We hypothesized FADS2 operates on BCFA and n-OCFA found on the skin. MCF-7 human epithelial cancer cells stably expressing FADS2 treated with BCFA were all Δ6-desaturated according to BCFA *iso*-16:0 → *iso*-6Z-16:1, *iso*-18:0 → *iso*-6Z-18:1, *iso*-17:0 → *iso*-6Z-17:1 and *anteiso*-17:0 → *anteiso*-6Z-17:1. The n-OCFA n-17:0 was converted to both n-6Z-17:1 by FADS2 Δ6-desaturation and n-9Z-17:1 by SCD Δ9-desaturation. We thus establish novel FADS2-coded enzymatic activity towards BCFA and n-OCFA. Aberrations in activity of sebaceous FADS2 may play a role in acne or other skin conditions that have been associated with skin lipids.

* Zhen Wang, Hui Gyu Park, Dong Hao Wang, Riki Kitano, Kumar S. D. Kothapalli, and J. Thomas Brenna. The role of fatty acid desaturase 2 gene in branched chain and odd chain fatty acid metabolism. In preparation, Oct 2018.

Introduction

Saturated Fatty acid desaturases (FADS) along with stearoyl CoA desaturase (SCD) are known for their site-specific introduction of cis double bonds (DB) into fatty acid hydrocarbon chains in mammals and biosynthesis of unsaturated fatty acids [1, 2]. FADS1, FADS2 and FADS3, are the three family members of FADS gene cluster localized on human chromosome 11(11q12-q13.1) [3]. FADS2 classical transcript codes for a multifunctional enzyme which catalyze Δ4-, Δ6- and Δ8- desaturation towards multiple linear chain/normal (n-) fatty acid substrates including eight polyunsaturates, two monounsaturates and one saturate (palmitic acid 16:0 → sapienic acid 16:1n-10/n-6Z-16:1); while FADS1 has shown Δ5- and Δ7- desaturation activities [1, 4-7]. SCD catalyze Δ9- desaturation to biosynthesize monounsaturated fatty acids (MUFA), particularly oleic acid (18:0 → 18:1n-9/n-9Z-18:1) and palmitoleic acid (16:0 → 16:1n-7/n-9Z-16:1). SCD has two isoforms (SCD1 and SCD5) in humans and 4 isoforms (Scd1 to Scd4) in mice [8, 9]. Among them, SCD1 is the first one identified and expressed in not only humans but also other mammals, such as pigs, rats and mice [2, 4, 10, 11].

Unlike other hair-bearing mammals, human sebaceous glands (SG) highly express FADS2 rather than SCD; palmitic acid 16:0 is preferentially converted to sapienic acid 16:1n-10 rather than 16:1n-7 [12]. Sapienic acid is the predominant unsaturated fatty acid in human sebum [4, 13] and is a unique feature of the SG because it is not abundant in any other human tissue [14]. Increased sapienic acid level and lower 16:0/16:1 ratio caused by FADS2 activity alteration are associated with acne development [13, 15-17].

Besides normal (n-) fatty acids like palmitic acid and sapienic acid, saturated branched chain fatty acids (SBCFA) terminating with a propan-2-yl (*iso*-) or butan-2-yl (*anteiso*-) group along with monounsaturated BCFA (MBCFA), constitute 5.4% and 6.6%, respectively, of total fatty acids in human skin lipids, followed by 9.4% of normal odd chain fatty acids (n-OCFA) [18]. They are also identified in vernix caseosa wax ester, and likely relevant to SG activity in utero [19]. Specifically, *iso*-6Z-16:1, n-6Z-17:1 and *anteiso*-6Z-17:1 represent 3.96%, 1.31% and 0.81%, respectively, of the human skin surface lipids which mostly are synthesized in SG [18]. A study reporting fatty acid composition of sebum wax esters, *iso*-16:1 comprised of 20% of total MUFA, only less than the n-16:1 at 50%, followed by *anteiso*-17:1, n-17:1, *iso*-15:1, *iso*-18:1 and *iso*-17:1 which were 0.1-10% of total MUFA [20]. Interestingly, even though *anteiso*-15:0 is the predominant SBCFA which accounts for 20% of the total SFA in sebum wax esters, no *anteiso*-15:1 was reported in this study [20]. No data on the desaturase involved and the biological pathway converting these

BCFA to monounsaturates in human SG is not investigated yet.

The biochemical pathway to monounsaturated fatty acids may be via direct conversion from the corresponding saturated fatty acid via a desaturase enzyme (e.g. 18:0 → 18:1n-9 via SCD) or by desaturation of a saturate followed by chain elongation (e.g. 16:0 → 16:1n-7 → 18:1n-7 via SCD and ELOVL5/6). Here we hypothesize that FADS2 gene product desaturates BCFA, due to its high expression in BCFA-rich human SG. We tested the function of FADS2 with several *iso* and *anteiso* BCFA prominent in skin lipids. We also characterized FADS2-coded activity towards n-17:0→n-17:1 as a hypothetical explanation for the presence of n-6Z-17:1 also found in SG, because the biosynthesis of n-17:1 has not been characterized. By establishing the role of FADS2 desaturation in human sebum, we will have better understanding on how it regulates sebum composition to support normal function of SG by modulating its unique fatty acid profile.

MCF-7 human breast cancer cells have no native FADS2 activity, but have native SCD mediated Δ9- desaturase activity like other cancer cell lines [4, 21-26]. Previously we reported FADS1 could compete with FADS2 for the same fatty acid substrate [27]. Here by using MCF-7 stably expressing FADS1 and FADS2, we investigate the role of FADS2 desaturase enzymatic activity in the metabolism of BCFA and n-OCFA.

Materials and Methods

Materials

Fatty acids standards *anteiso*-15:0, *iso*-16:0, *iso*-18:0, *iso*-17:0, *anteiso*-17:0 and n-17:0 were obtained from Larodan Fine Chemicals (Malmo, Sweden) and Sigma-Aldrich (St. Louis, MO). Cell culture reagents including media, trypsin, phosphate-buffered saline (PBS), fetal bovine serum (FBS) and others were obtained from Life Technologies (NY) and Thermo Fisher Scientific (MA). Cell dishes, serological pipette and pipette tips are from Corning (MA). Chemical analysis solvents for fatty acid analysis and GC-MS system were at HPLC grade and purchased from Sigma-Aldrich (St. Louis, MO) and Burdick & Jackson (Muskegon, MI).

Cell culture and treatment

MCF-7 cells stably expressing FADS1 and FADS2 were generated using the pcDNA3.1 expression vector system and empty vector cells were used as control; as described previously [5]. As antibiotic-resistant transformants were selected for creating pure stably transformed FADS1 and FADS2 cells, we expect all cells will express FADS1 and FADS2. The stable cells were grown at 37°C with 5% $CO_{2,}$ on minimum essential medium alpha (MEM-α) with 10% (v/v) FBS and 10mM buffer [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEPES] and 0.5 mg/ml geneticin. 1×10^6 cells were seeded in 60 × 15 mm cell culture dishes and were grown for 48h until reach 80% confluence. Cells were incubated with 50 μM of bovine serum albumin (BSA) bound FA

substrates individually (*anteiso*-15:0, *iso*-16:0, *iso*-18:0, *iso*-17:0, *anteiso*-17:0 or n-17:0). BSA bound substrates were prepared by the following procedures: 100mM FA stock in ethanol was made by dissolving saturated branch chain fatty acids (SBCFA) and n-SFA into absolute ethanol. Then 2mM BSA bound FA stock was made by mixing 200μl of 100mM FA stock in ethanol with FA free BSA at the concentration of 0.66mM in 10ml of PBS. So the final concentration ratio of FA: BSA is 3:1. Once incubated overnight at 37^0C, BSA bound FA were diluted to 50 μM with FBS free media after filtration using 0.22 μm syringe, and then added to cells. After 24 h of additional incubation, cell pellets for fatty acid analysis were harvested using trypsin followed by centrifuging.

Fatty acid extraction and analysis

Fatty acid methyl esters (FAME) were extracted and derivatized from the harvested MCF-7 cell pellets based on the modified one-step method of Garces and Mancha [28]. They were dissolved in heptane and stored at -20^0C until analysis.

FAME were structurally identified by gas chromatography (GC) - covalent adduct chemical ionization (CACI) mass spectrometry (MS) and CACI-MS/MS using Shimadzu (Columbia, MD) GCMS-TQ8040. GCMS is equipped with a BPX70 capillary column (25 m × 0.22 mm × 0.25 μm; SGE Inc., Austin, TX) to separate FAME and using an acetonitrile-derived reagent ion to interact with

the DB of FAME for localizing DB positions. GC setting were as follows: injection temperature was 250^{0}C in splitless mode; sample was purged at 1 min after injection; the initial column oven temperature was 80^{0}C; it ramped to 170^{0}C at the rate of 15^{0}C/min and held for 4 min, then ramped to 240^{0}C at the rate of 7^{0}C/min and held for 10 min; the total program time was 30 min. MS parameters were as follows: ion source temperature was 230^{0}C; interface temperature was 240^{0}C; solvent cut time was 1.5 min; detector voltage was 1.5kV. [M+54]+ ions of FAME were isolated for fragmentation in CACI-MS/MS mode with 6V of collision energy. The GC elution of FAME under our condition are in the order of *iso*-, *anteiso*- and n-, when carbon numbers are the same [29].

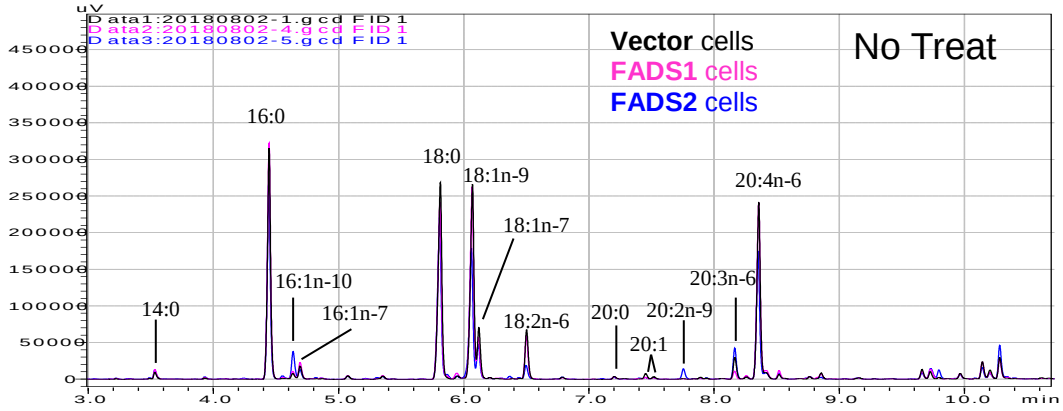
FAME were quantified by Shimadzu (Austin, TX) QS2010 Plus GC-flame ionization detection (GC-FID). GLC462 (Nu-Check Prep, Inc.) containing common FAME at equal weight was used to calculate response factors of all fatty acids [30]. Percentage conversion of substrate (S) to product (P) was calculated as P / (S + P), which expressed as %. Four biological replicates were used and values are expressed as mean ± SD.

Results

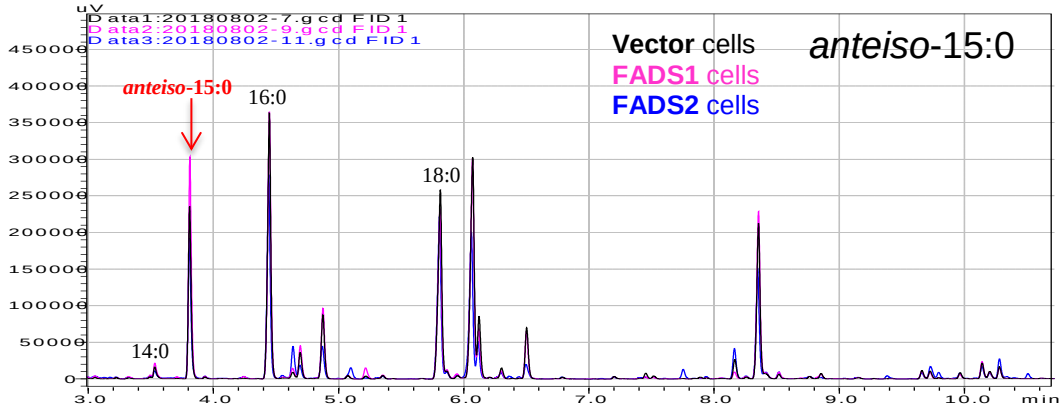
Figure 4.1 shows GC-FID chromatograms of control, FADS1 and FADS2 stable MCF7 cells treated with zero or 50 μM of individual fatty acid substrate

anteiso-15:0, *iso*-16:0, *iso*-18:0, *iso*-17:0, *anteiso*-17:0 or n-17:0. MCF7 stable cells have no native BCFA and negligible amount of n-17:0 (**Figure 4.1A**), but readily take up these substrates when cells ae treated with exogenous fatty acids (**Figures 4.1B-4.1G**). When treated with *anteiso*-15:0, no novel desaturation product was generated in either empty vector control, FADS1 or FADS2 cells (**Figure 4.1B**). However, when treated with *iso*-16:0, *iso*-18:0, *iso*-17:0, *anteiso*-17:0 or n-17:0, novel desaturation products were produced in FADS2 cells but not in control and FADS1 cells, respectively (**Figures 4.1C-4.1G**). Additionally, in **Figures 4.1D-4.1G**, direct desaturation products *iso*-18:1, *iso*-17:1, *anteiso*-17:1 and n-17:1 are elongated to *iso*-20:1, *iso*-19:1, *anteiso*-19:1(coelutes with 18:2n-6) and n-19:1, respectively.

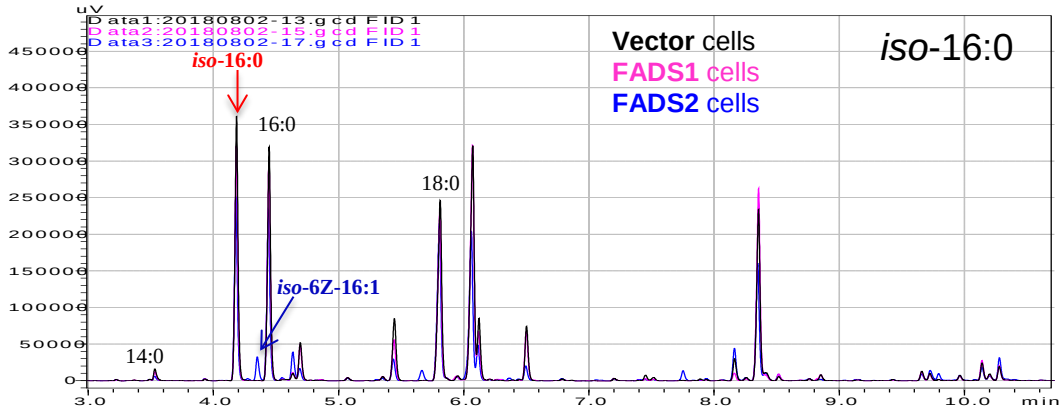
A


Vector cells
FADS1 cells
FADS2 cells
No Treat
14:0
16:0
16:1n-10
16:1n-7
18:0
18:1n-9
18:1n-7
18:2n-6
20:0
20:1
20:2n-9
20:3n-6
20:4n-6
min

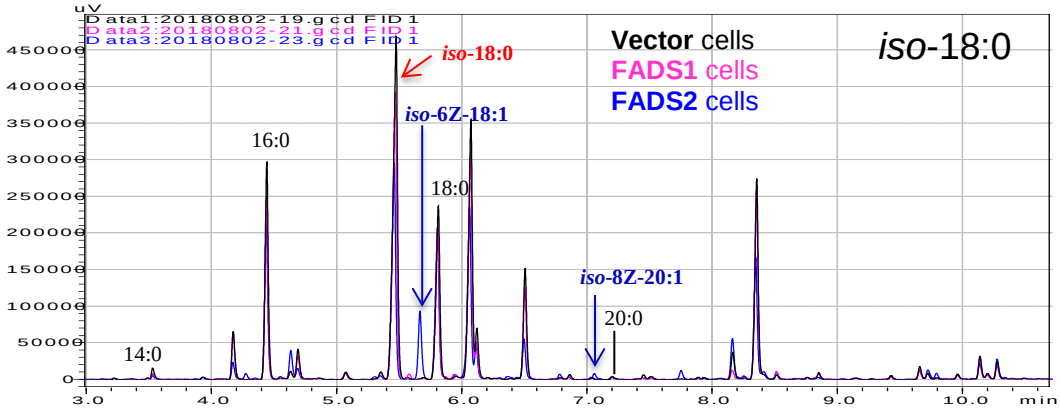
B


Vector cells
FADS1 cells
FADS2 cells
anteiso-15:0
anteiso-15:0
14:0
16:0
18:0
min

C


Vector cells
FADS1 cells
FADS2 cells
iso-16:0
iso-16:0
14:0
16:0
18:0
iso-6Z-16:1
min

D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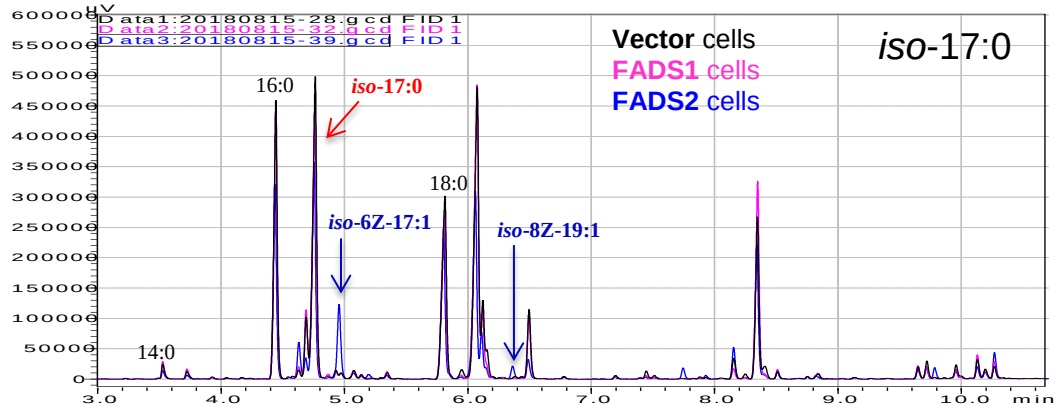
Data2:20180802-21.gcd FID1
Data3:20180802-23.gcd FID1
uV
Vector cells
FADS1 cells
FADS2 cells
iso-18:0
iso-6Z-18:1
16:0
18:0
iso-8Z-20:1
20:0
14:0
min

E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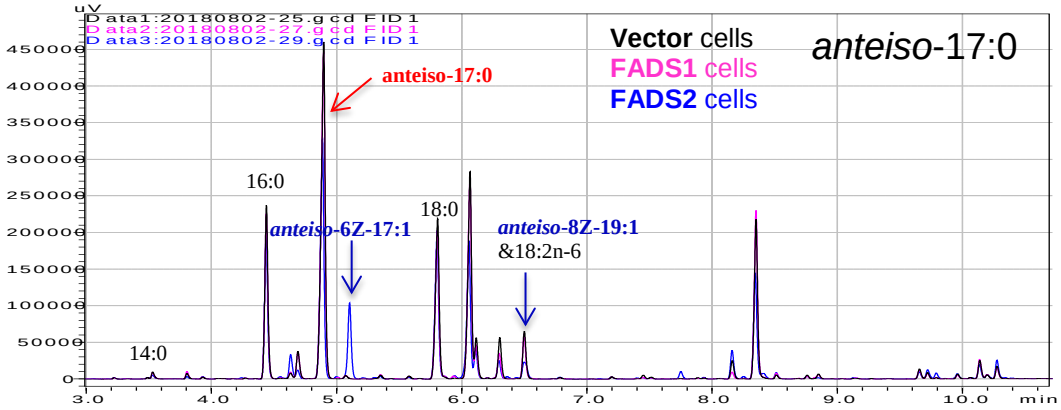
Data3:20180815-39.gcd FID1
uV
Vector cells
FADS1 cells
FADS2 cells
iso-17:0
16:0
iso-6Z-17:1
18:0
iso-8Z-19:1
14:0
min

F


Data1:20180802-25.gcd FID1
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Data3:20180802-29.gcd FID1
uV
Vector cells
FADS1 cells
FADS2 cells
anteiso-17:0
16:0
anteiso-6Z-17:1
18:0
anteiso-8Z-19:1
&18:2n-6
14:0
min

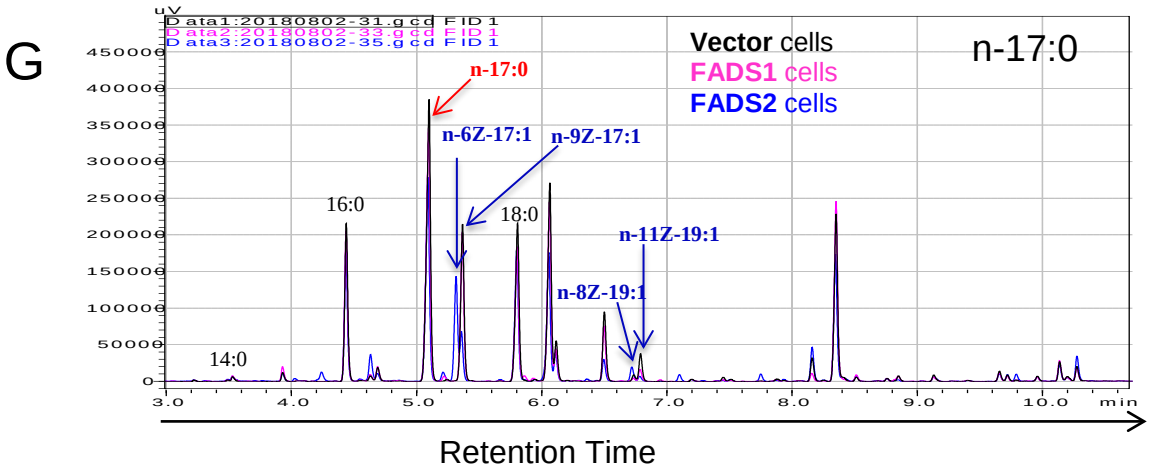
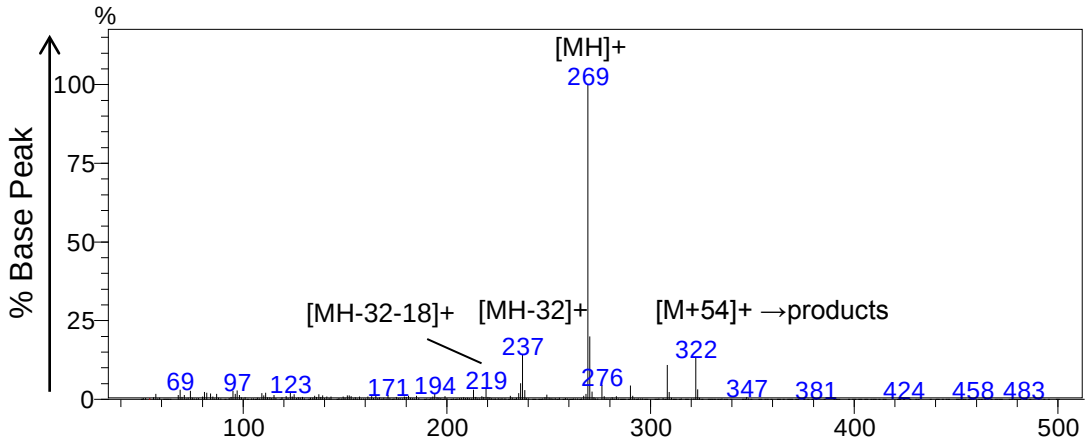


Figure 4.1 GC-FID chromatograms of control (black), FADS1 (pink) and FADS2 (blue) stable MCF7 cells treated with no substrate and 50 μM of fatty acid substrates *anteiso*-15:0, *iso*-16:0, *iso*-18:0, *iso*-17:0, *anteiso*-17:0 or n-17:0.

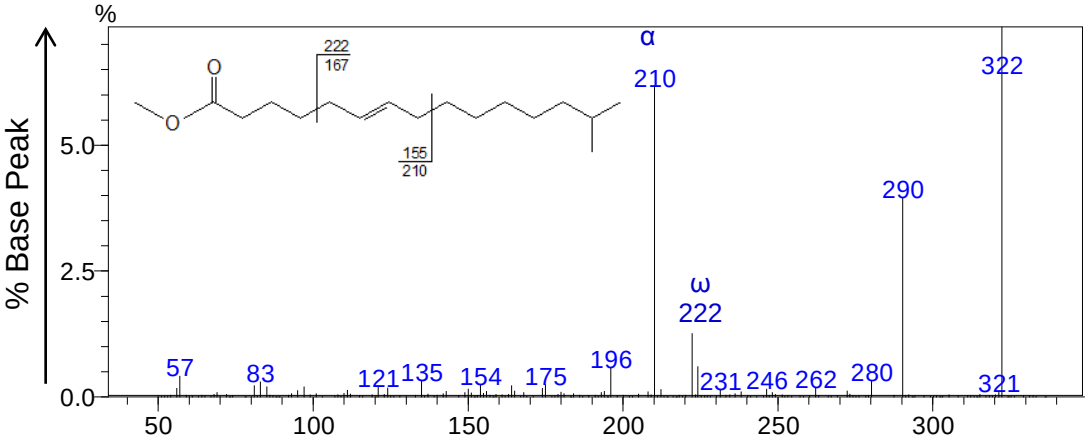
Native cell fatty acid peaks are marked in black; the treated saturated fatty acid substrates are marked out by red arrow; novel direct desaturation products and secondary elongation products generated in FADS2 cells are marked out by blue arrow. Horizontal axis, retention time; vertical axis, FID signal response.

GC-CACI-MS and CACI-MS/MS analyses were performed to identify the direct novel desaturation products in FADS2 cells including *iso*-6Z-16:1, *iso*-6Z-18:1, *iso*-6Z-17:1, *anteiso*-6Z-17:1 or n-6Z-17:1 FAME, and spectra were shown in **Figure 4.2**. **Figures 4.2A1-4.2F1** show the characterized ions [M+54]+, [MH]+, [MH-32]+ and [MH-32-18]+ of the corresponding FAME; and **Figures 4.2A2-4.2F2** are collisional induced activation spectra (MS/MS) of [M+54]+ with diagnostic α and ω ions locating the single DB. Each FA product is identified by both CACI-MS and CACI-MS/MS information. Specifically, *iso*-6Z-16:1 FAME was characterized by CACI-MS with characteristic ions (m/z 219, 237, 269, 322) and by CACI-MS/MS with diagnostic ions (m/z 210, 222) (**Figure 4.2A**); *iso*-6Z-18:1 FAME was identified with characteristic ions (m/z 247, 265, 297, 350) and diagnostic ions (m/z 210, 250) (**Figure 4.2B**); *iso*-6Z-17:1 FAME was identified with characteristic ions (m/z 233, 251, 283, 336) and diagnostic ions (m/z 210, 236) (**Figure 4.2C**); D. *anteiso*-6Z-17:1 FAME was identified with characteristic ions (m/z 233, 251, 283, 336) and diagnostic ions (m/z 210, 236) (**Figure 4.2D**); n-17:1Δ6 FAME was identified with characteristic ions (m/z 233, 251, 283, 336) and diagnostic ions (m/z 210, 236) (**Figure 4.2E**); n-6Z-17:1 FAME was identified with characteristic ions (m/z 233, 251, 283, 336) and diagnostic ions (m/z 252, 194) (**Figure 4.2F**).

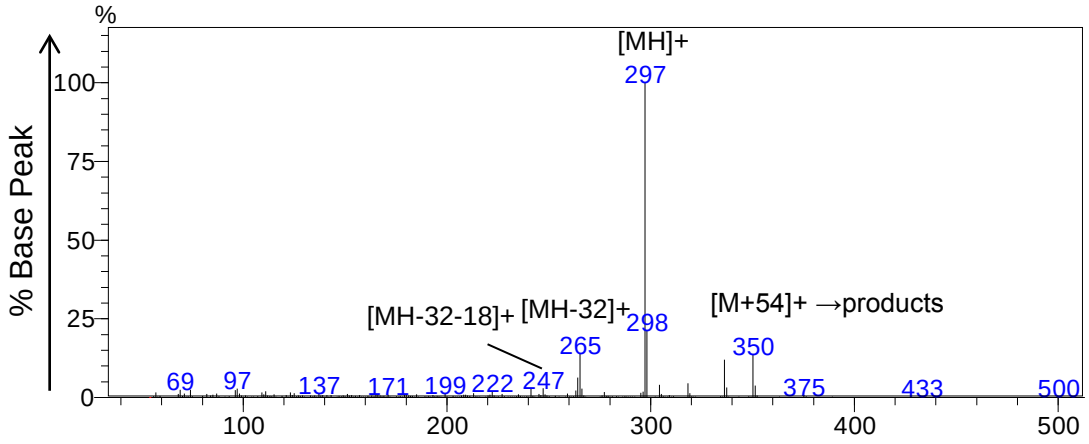
A1. CIMS *iso*-6Z-16:1



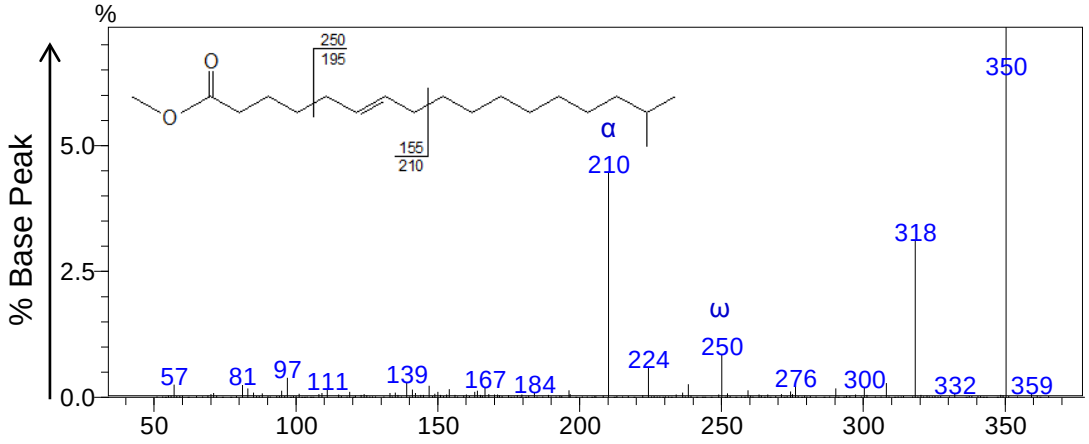
A2. CIMS2 *iso*-6Z-16:1



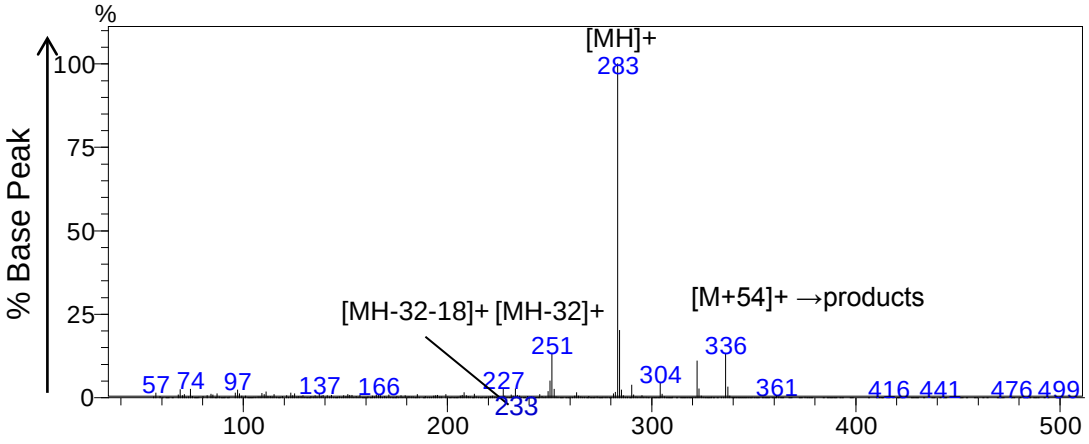
B1. CIMS *iso*-6Z-18:1



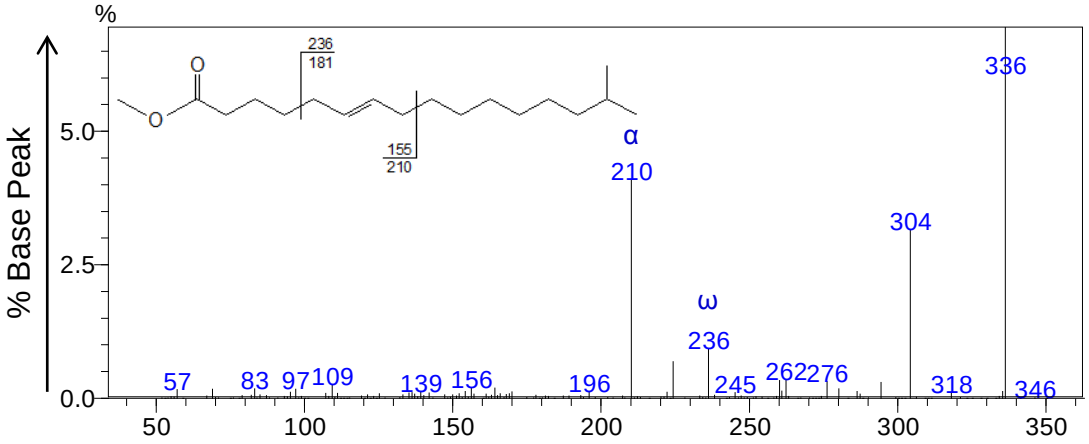
B2. CIMS2 *iso*-6Z-18:1



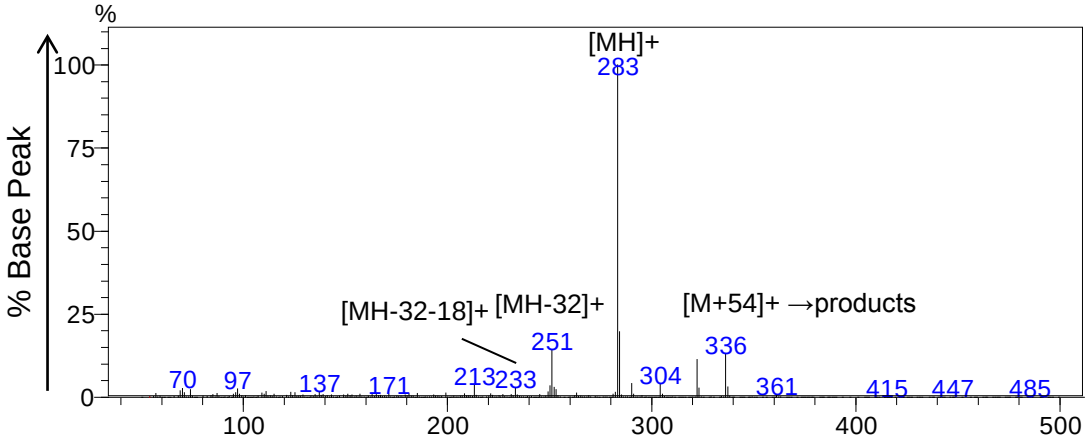
C1. CIMS *iso*-6Z-17:1



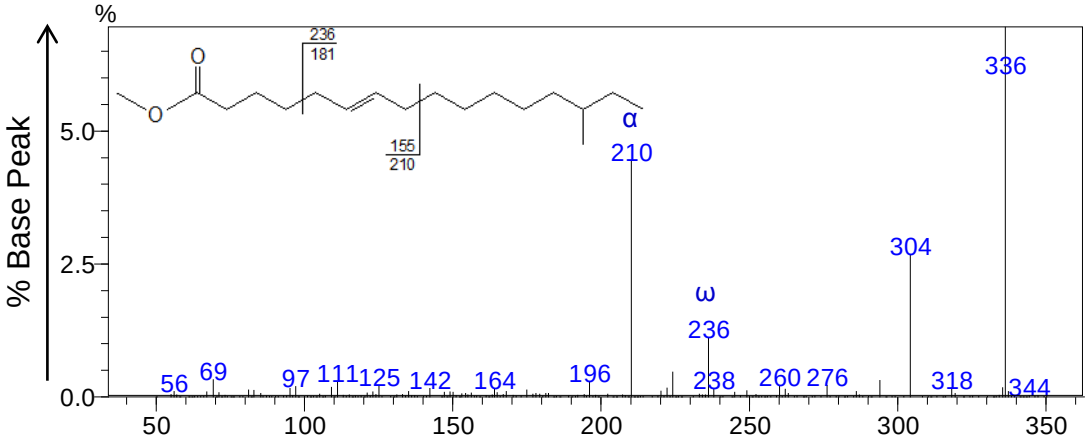
C2. CIMS2 *iso*-6Z-17:1



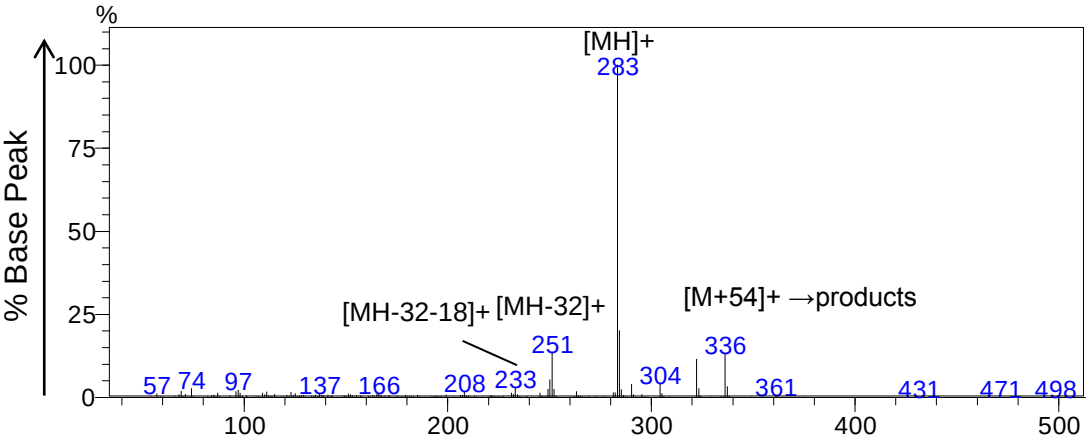
D1. CIMS *anteiso*-6Z-17:1



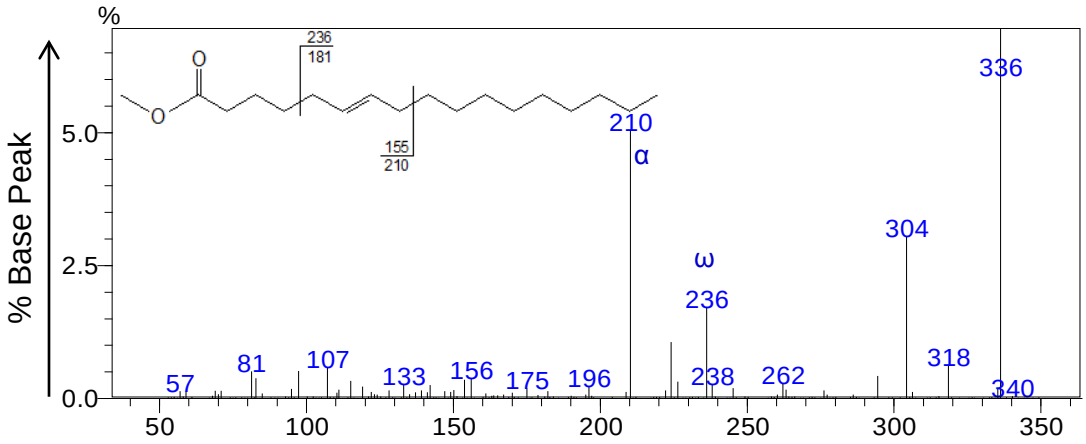
D2. CIMS2 *anteiso*-6Z-17:1



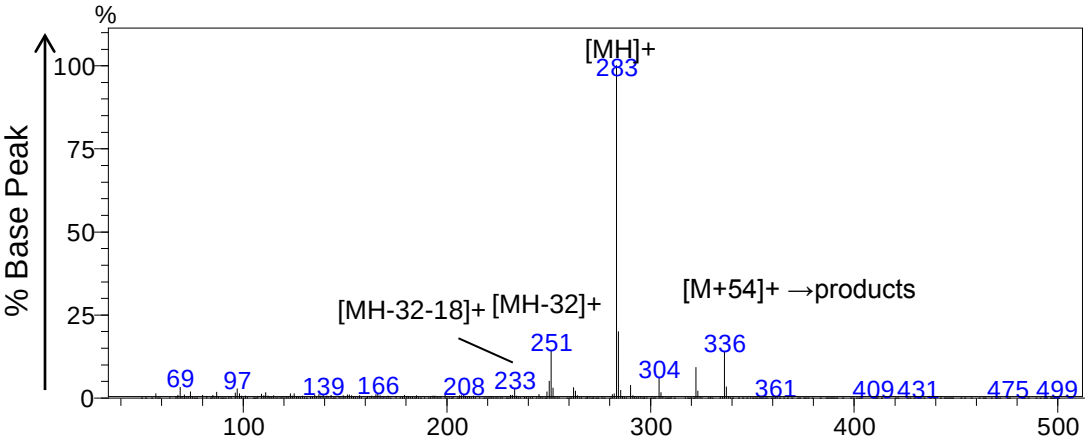
E1. CIMS n-6Z-17:1



E2. CIMS2 n-6Z-17:1



F1. CIMS n-9Z-17:1



F2. CIMS2 n-9Z-17:1

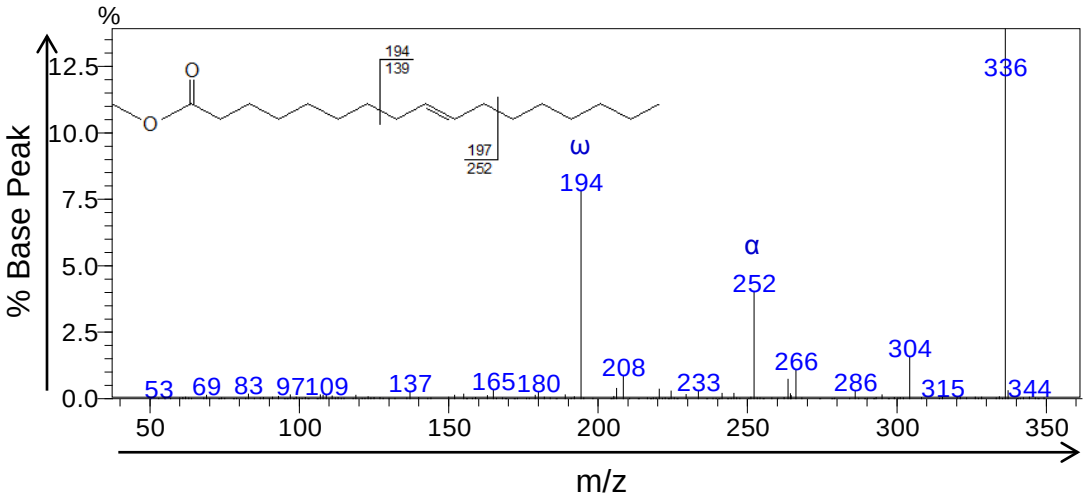


Figure 4.2 CACI-MS and CACI-MS/MS spectra of desaturation products *iso*-6Z-16:1, *iso*-6Z-18:1, *iso*-6Z-17:1, *anteiso*-6Z-17:1, n-6Z-17:1 and n-9Z-17:1 FAME.

A. CACI-MS and CACI-MS/MS spectrum of *iso*-6Z-16:1 FAME showing the characteristic ions (m/z 219, 237, 269, 322) and diagnostic ions (m/z 210, 222); B. CACI-MS and CACI-MS/MS of *iso*-6Z-18:1 FAME showing the characteristic ions (m/z 247, 265, 297, 350) and diagnostic ions (m/z 210, 250); C. CACI-MS and CACI-MS/MS of *iso*-6Z-17:1 FAME showing the characteristic ions (m/z 233, 251, 283, 336) and diagnostic ions (m/z 210, 236); D. CACI-MS and CACI-MS/MS of *anteiso*-6Z-17:1 FAME showing the characteristic ions (m/z 233, 251, 283, 336) and diagnostic ions (m/z 210, 236); E. CACI-MS and CACI-MS/MS of n-6Z-17:1 FAME showing the characteristic ions (m/z 233, 251, 283, 336) and diagnostic ions (m/z 210, 236); F. CACI-MS and CACI-MS/MS of n-9Z-17:1 FAME showing the characteristic ions (m/z 233, 251, 283, 336) and diagnostic ions (m/z 252, 194).

FA with the same carbon and DB numbers such as n-6Z-17:1 and n-9Z-17:1 share the same characterized ions but distinguished by diagnostic ions so that diagnostic ions would help with localizing DB positions. FA with both the same carbon/DB numbers and DB positions, such as *iso*-6Z-17:1, *anteiso*-6Z-17:1 and n-6Z-17:1, are only different in the terminal end structure of carbon chain; they share similar characteristic ions and diagnostic ions. Thus their differences in structure are distinguished by the branched terminal ends corresponding to the treated saturated fatty acids structures.

Similarly, the secondary elongation products from direct desaturation products, including *iso*-8Z-20:1, *iso*-8Z-19:1, *anteiso*-8Z-19:1, n-8Z-19:1 and n-11Z-19:1, are verified by CACI-MS and CACI-MS/MS as well, and CIMS and CIMS/MS spectra are summarized in **Figure S4.1**. Each secondary elongation product shares the same ω diagnostic ion with the corresponding direct desaturation product. Besides, even though both secondary elongation product and direct desaturation product are found in FADS2 cells, their concentrations are quite different: secondary elongation product is at quite a small amount which is less than 1% of the total fatty acids in FADS2 cells, while direct desaturation product is 5-8%. Both of the identified DB position and the difference of concentrations between direct FADS2 synthesized monoenes and secondary elongation products indicate the treated saturated fatty acids are primarily desaturated and then elongated. For instance, *iso*-8Z-19:1 shares the same ω diagnostic ion (m/z 236) with *iso*-6Z-17:1 and α fragment mass increases by

28. Moreover, *iso*-8Z-19:1 is only found in FADS2 but not control and FADS1 cells and its concentration is lower than *iso*-6Z-17:1, indicating it prefers to follow the pathway *iso*-17:0 → *iso*-6Z-17:1 → *iso*-8Z-19:1 rather than *iso*-17:0 → *iso*-19:0 → *iso*-19:1.

The percentage conversions of fatty acid substrate to desaturation product in FADS2 cells compared to control are presented in **Table 4.1**. Control MCF-7 cells cannot desaturate any tested BCFA. While FADS2 is able to Δ6-desaturate *iso*-16:0, *iso*-18:0, *iso*-17:0 and *anteiso*-17:0 to single product *iso*-6Z-16:1, *iso*-6Z-18:1, *iso*-6Z-17:1 and *anteiso*-6Z-17:1, respectively, at percentage conversions of 11.04 ± 0.37 (%), 19.87 ± 2.49 (%), 25.76 ± 0.52 (%) (coelutes with 3.52 ± 0.06 (%) of native MCF-7 cell FA) and 21.37 ± 1.20 (%) (coelutes with 0.88 ± 0.06 (%) of native n-17:0), respectively. Native cell FA coeluting with *iso*-6Z-17:1 and *anteiso*-6Z-17:1 are constantly existed in control and FADS1 cells; they are quite small and hard to be separated from FADS2 desaturation products, so we didn't exclude them when calculating percentage conversions. n-17:0 in control cells was converted to single desaturation product n-9Z-17:1 via SCD Δ9-desaturation at percentage conversion of 33.10 ± 1.62 (%); while in FADS2 cells, it was converted to both n-9Z-17:1 by SCD Δ9-desaturation and n-6Z-17:1 by FADS2 Δ6-desaturation at percentage conversion of 17.19 ± 6.14 (%) and 22.52 ± 5.40 (%), respectively.

Table 4.1 Percentage conversion of fatty acid substrate to desaturation product in FADS2 cells compared to control.

FA Substrate	**FA Product**	**Conversion (%) in FADS2 cells**	**Conversion (%) in Control cells**	**Active Desaturase**
anteiso-15:0	-	0	0	-
iso-16:0	*iso*-6Z-16:1	11.04 ± 0.37	0	FADS2 Δ6
iso-18:0	*iso*-6Z-18:1	19.87 ± 2.49	0	FADS2 Δ6
iso-17:0	*iso*-6Z-17:1	25.76 ± 0.52	3.52 ± 0.06¶	FADS2 Δ6
anteiso-17:0	*anteiso*-6Z-17:1	21.37 ± 1.20	0.88 ± 0.06§	FADS2 Δ6
n-17:0	n-6Z-17:1	22.52 ± 5.40	0	FADS2 Δ6
	n-9Z-17:1	17.19 ± 6.14	33.10 ± 1.62	SCD Δ9

Percent conversion of substrate (S) to product (P) were calculated as P / (S + P), which expressed as %.
¶ *iso*-17:1 coelutes with native FA, so the percentage conversion in control represents (native peak only) / (native peak+iso-17:0).
§ *anteiso*-17:1 coelutes with n-17:0, so the percentage conversion in control represents (n-17:0 only) / (n-17:0+*anteiso*-17:0).

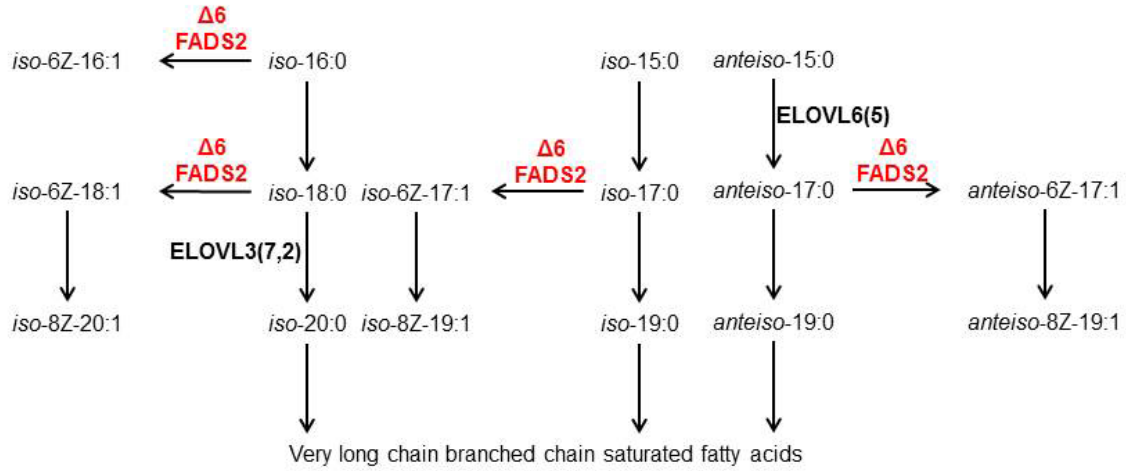
Discussion

SCD is expressed ubiquitously in human tissues [23]. The wild type MCF-7 cells have SCD and FADS1 activity but lack FADS2 activity [24-26]. In this study, vector MCF-7 cells express native SCD and FADS1 activities like wild type ones; stable FADS1 MCF-7 cells express native SCD and enhanced FADS1 activities; while stable FADS2 MCF-7 cells express native SCD, native FADS1 and stably enhanced FADS2 activities [5]. Thus, the novel desaturation products generated in FADS2 cells are due to the stably enhanced FADS2 activity. The stable FADS2 cell FA composition contains both SCD products and FADS2 products at the same time, for example 16:1n-7 and 16:1n-10 [4].

The biosynthesis pathways of saturated and monounsaturated branched/normal fatty acids in mammals are summarized in **Figure 4.3**, including enzymatic activities established in the present study (red) and reported in previous studies of our lab or others (black) (Wang, et al., 2018a, in press; Wang, et al., 2018b, under review) [4, 10]. SCD is considered the major desaturase catalyzing normal saturated fatty acids (SFA) to MUFA, while FADS2 desaturation activities are mainly found in generating normal polyunsaturated fatty acids (PUFA) [4]. In normal MUFA and mead acid biosynthesis metabolism, until now FADS2 activities are reported for three substrates, 16:0 → 16:1n-10, 18:1n-9 → 18:2n-9 and 20:1n-9 → 20:2n-9 [4, 7, 12]. Our present study extends FADS2 activities towards BCFA and normal

OCFA for MUFA biosynthesis: it reveals that FADS2 mediates Δ6-desaturation towards BCFA *iso*-16:0 → *iso*-6Z-16:1, *iso*-18:0 → *iso*-6Z-18:1, *iso*-17:0 → *iso*-6Z-17:1, *anteiso*-17:0 → *anteiso*-6Z-17:1 (**Figure 4.3A**); and promotes n-OCFA n-17:0 → 17:1n-11/n-6Z-17:1 in concurrence with n-17:0 → 17:1n-8/n-9Z-17:1 catalyzed by SCD (**Figure 4.3B**). More than half of n-17:0 is utilized to produce n-6Z-17:1 rather than n-9Z-17:1 in FADS2 cells, indicating FADS2 is likely to compete with SCD in desaturating n-17:0. n-17:0 and n-16:0 share the similarities that both of them are able to access FADS2 Δ6-desaturation and SCD Δ9-desaturation at the same time, and FADS2 activity is predominant when SCD expression is limiting [4, 31, 32].

A. branched chain fatty acids



B. normal fatty acids

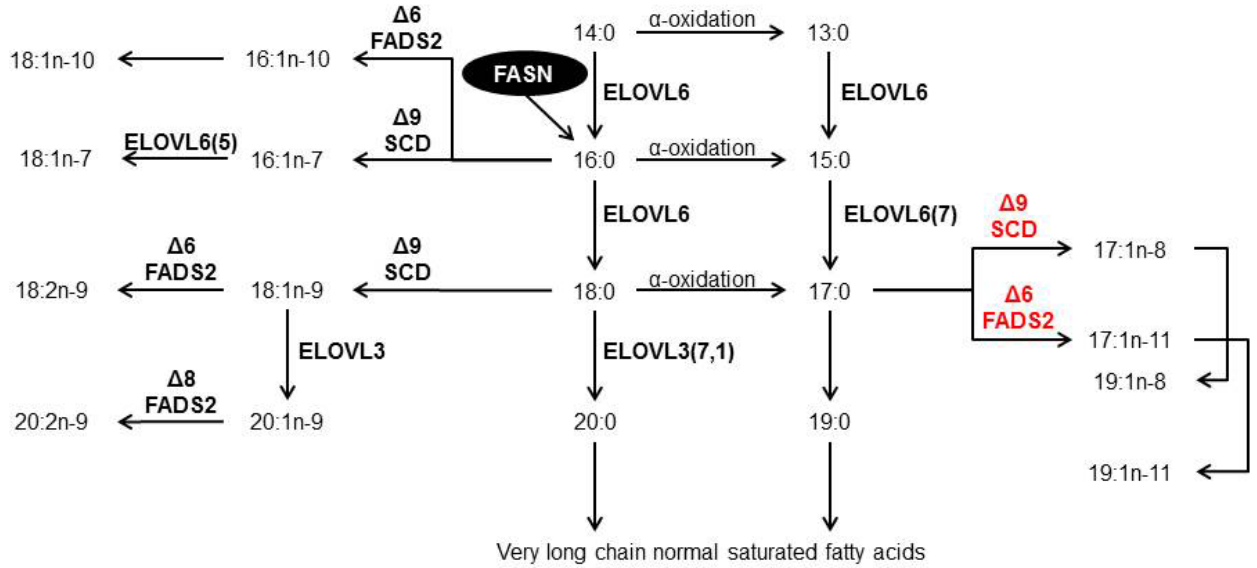


Figure 4.3 Biosynthesis of long chain saturated and monounsaturated branched chain or normal fatty acids in mammals.

A. Saturated and monounsaturated branched chain fatty acid (SBCFA and MBCFA) biosynthesis pathways; B. Saturated and monounsaturated normal/linear chain fatty acid (n-SFA and n-MUFA) biosynthesis pathways. Genes encoding elongation (ELOVL) and desaturation (SCD and FADS) enzymes are indicated in pathways. Enzymatic activities shown in red are established based on the present study; ones shown in black are from previous studies of ours or others (Wang, et al., 2018a, in press; Wang, et al., 2018b, under review) [4, 7, 10].

The SG on human skin are rich in BCFA saturates and BCFA monounsaturates [18-20], highly express FADS2 [12] and weak or no expression of SCD [12, 33]. BCFA behave quite differently from normal FA (n-FA) for MUFA biosynthesis (**Figure 4.3**). The stable FADS2 cells treated with BCFA *iso*-16:0, *iso*-18:0, *iso*-17:0 and *anteiso*-17:0 produce direct MUFA products via Δ6-desaturation, whereas SCD mediated Δ9-desaturation products are not detected. As FADS2 is highly expressed in human SG, our results may explain why MBCFA are abundant in human SG. Moreover, our study didn't found any FADS2 activity towards *anteiso*-15:0, which is in accordance with the lack of *anteiso*-15:1 when *anteiso*-15:0 is presented in the reported human SG fatty acid composition [20].

Human SG and its lipid composition are unique in several aspects when compared to other human tissues/organisms or other mammalian species. Firstly, sebaceous lipid composition of humans is quite different from that of other mammals [34-36]. Sebaceous lipids mainly consist of triglycerides, wax esters and squalene [36]. High levels of long chain BCFA and n-OCFA components in sebaceous lipids, especially in wax esters, build up the special fatty acid profile of sebum [18]. Moreover, highly expressed FADS2 is another unique feature of human SG; that is opposite to that of other mammalian SG such as pig and mouse's in which SCD predominantly expressed [4, 12, 16, 33]. The shift from SCD Δ9- to FADS2 Δ6- desaturation has been regarded as a unique feature of human sebaceous cell maturation [15, 16].

Acne is a common human skin inflammatory disease but is rare in other hair-bearing mammals [12, 16, 37]. The alteration of fatty acid composition influenced by FADS2 and SCD desaturases, as well as the elevated sebum excretion, are concurrent events associated with the acne development on skin [15, 36-38]. Among sebaceous lipids, the FA specially generated from SG are considered a feature of the pathophysiology of acne [37]. One of these FA is sapienic acid (16:1n-10). Sapienic acid is the first Δ6-monounsaturate discovered in human sebum and known to be synthesized by action of FADS2 [12]. It has been reported only in human sebaceous lipids but not in other human tissues/organisms or other animal sebum secretions, and is the predominant MUFA in human SG wax esters [4, 13-15, 36]. Lower 16:0/16:1 ratio in is detected in skin triglycerides and wax esters of acne patients, suggesting higher unsaturation degree and increased sapienic acid led by incorrect FADS2 activity are characteristics of acne formation [13, 15-17]. In this study, the new Δ6-monounsaturates generated from BCFA and n-OCFA are also unique in sebaceous lipids composition and also produced via FADS2 Δ6-desaturation, therefore they may share similar function as sapienic acid. However, it is still unknown why human SG prefer FADS2 Δ6-desaturation rather than the common SCD Δ9-desaturation for MUFA production, and what exact function of these Δ6-monounsaturates in human sebum.

The relative proportions of MUFA and saturated BCFA regulate membrane

fluidity of bacterial cells [39-42]. Bacteria increase fatty acid unsaturation and/or chain branching to keep membrane fluidity in response to a decreased growth temperature [42]. Human SG FA composition contains only 2.9% of PUFA, but 47.1% of MUFA in which BCFA and n-OCFA are presented at comparable levels [18]. Thus, the monounsaturated BCFA and n-OCFA in human SG are presumed to maintain sebum fluidity and support normal human skin function, such as the adaption to thermal change.

Besides the direct Δ6- or Δ9- desaturation products, we also observed that *iso*-8Z-20:1, *iso*-8Z-19:1, *anteiso*-8Z-19:1, n-8Z-19:1 and n-11Z-19:1 generated by secondary elongation after desaturation. Unlike their direct desaturation products *iso*-6Z-18:1, *iso*-6Z-17:1, *anteiso*-6Z-17:1, n-6Z-17:1 and n-9Z-17:1 which are 5-8% of the total fatty acids, these secondary elongation products comprise less than 1%. The low concentration of secondary elongation products may explain why they are not revealed in previous skin lipid studies [18, 20]. Regardless of their low concentration, the presence of secondary elongation products may have physiological importance such as tweaking sebum fluidity along with common monoenes, and interconverting with the direct desaturation products to perform physiological activities in different circumstances.

In conclusion, this work characterized FADS2 activities in producing predominant human sebaceous MUFA categories, identified FADS2 mediated

Δ6-desaturation towards BCFA *iso*-16:0 → *iso*-6Z-16:1, *iso*-18:0 → *iso*-6Z-18:1, *iso*-17:0 → *iso*-6Z-17:1, *anteiso*-17:0 → *anteiso*-6Z-17:1, and also detected FADS2 activity on n-OCFA n-17:0 → n-6Z-17:1. It extended FADS2 activity range to BCFA and n-OCFA, and enhanced the importance of FADS2 in MUFA metabolism. It is the first time to associate SG's high FADS2 expression with its unique BCFA and n-OCFA monounsaturates composition. Further studies are necessary to explore the correlation between acne development and sebum fatty acid compositional changes catalyzed by different desaturases.

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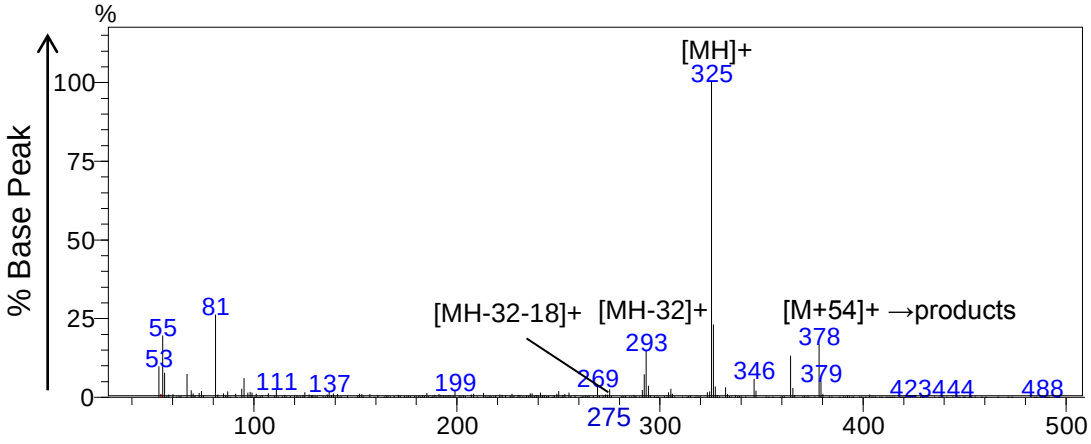
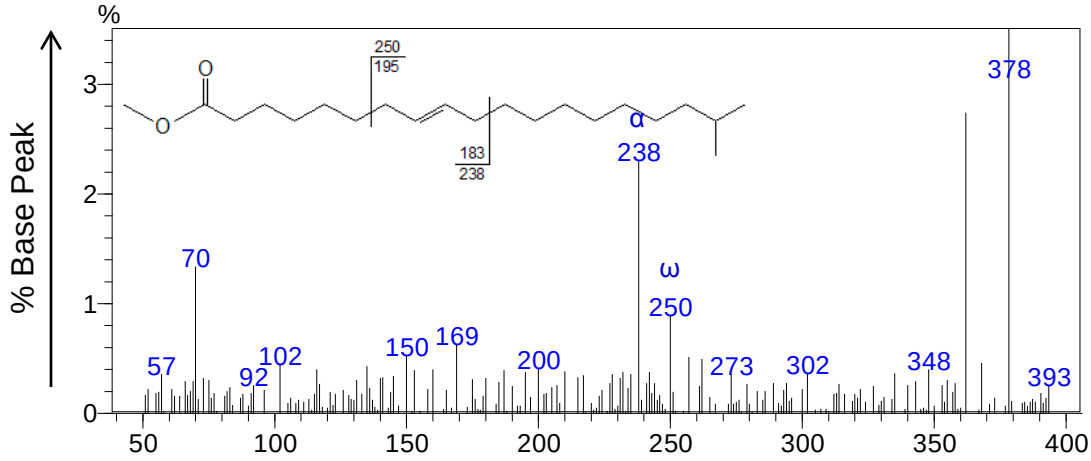
APPENDIX

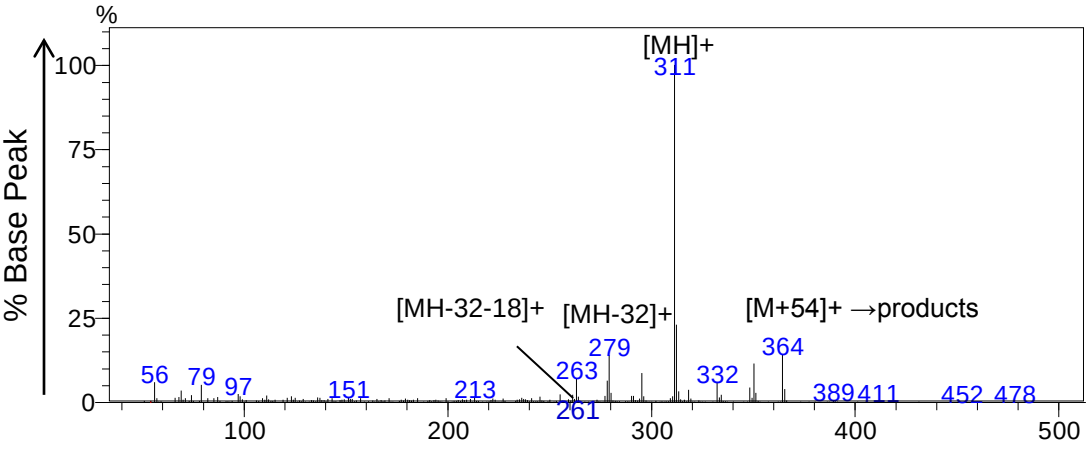
A1. CIMS *iso*-8Z-20:1



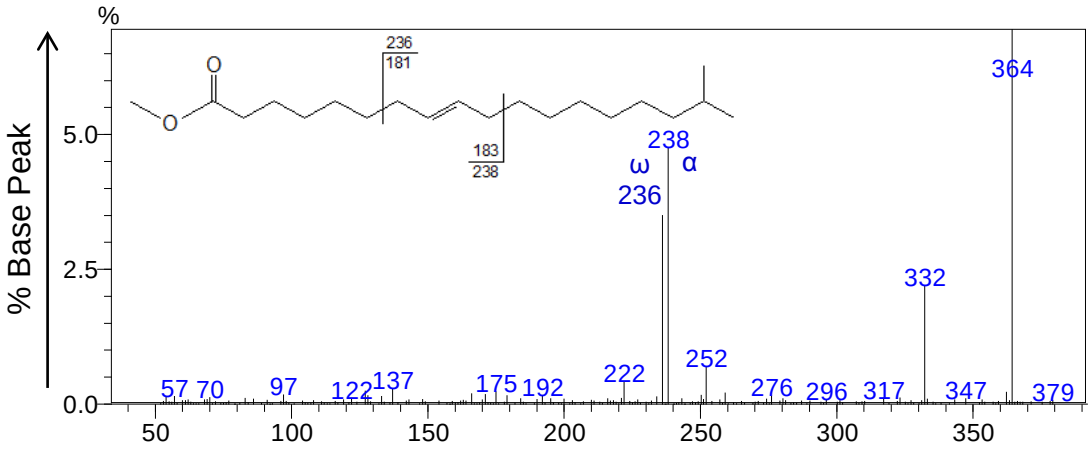
A2. CIMS2 *iso*-8Z-20:1



B1. CIMS *iso*-8Z-19:1



B2. CIMS2 *iso*-8Z-19:1



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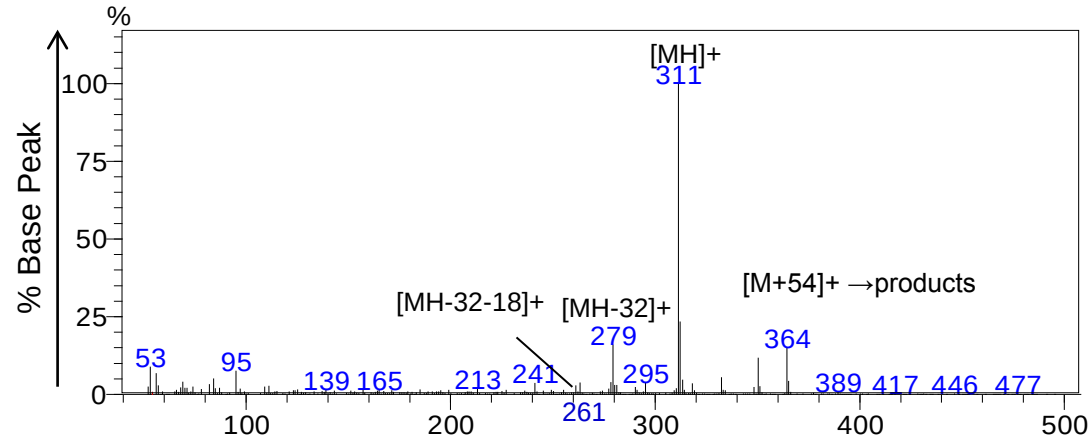

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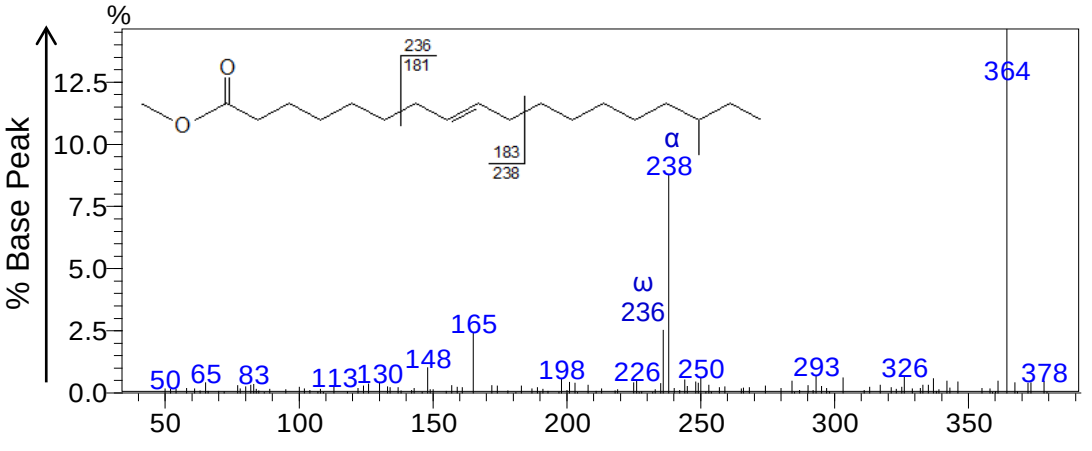

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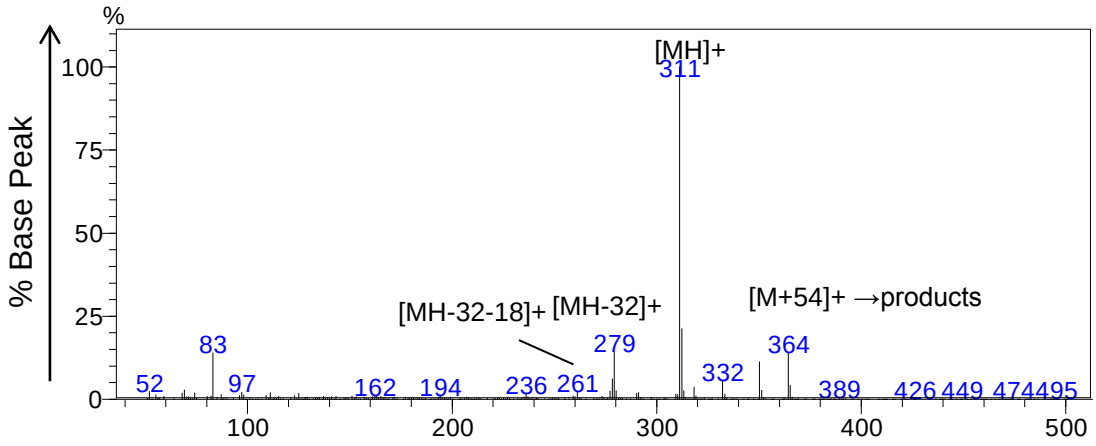

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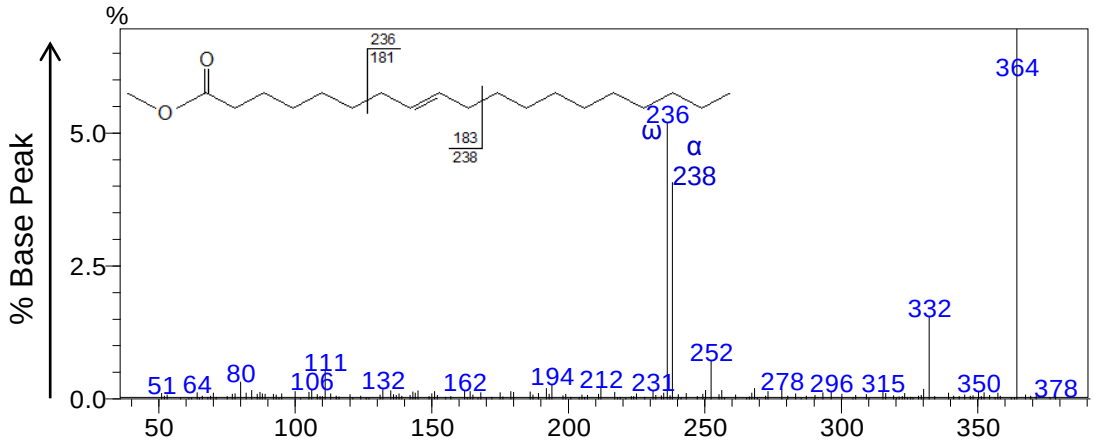
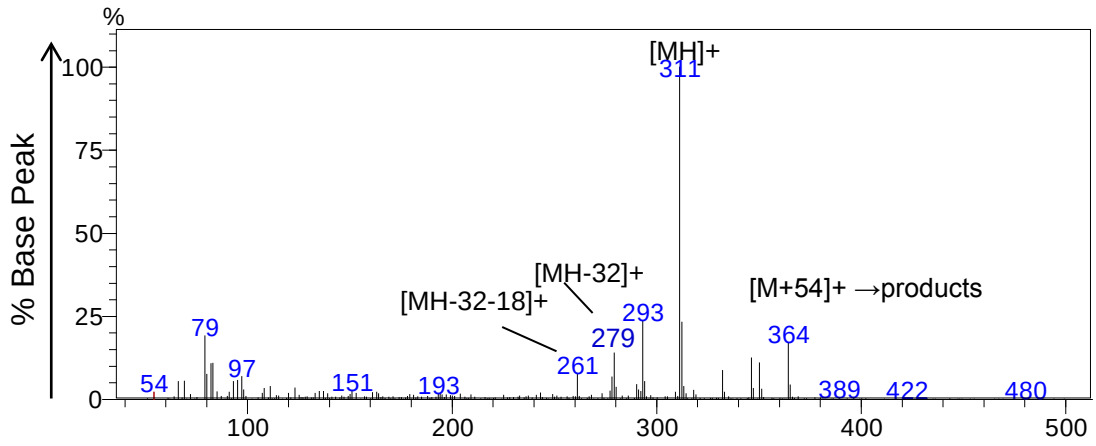


E1. CIMS n-11Z-19:1



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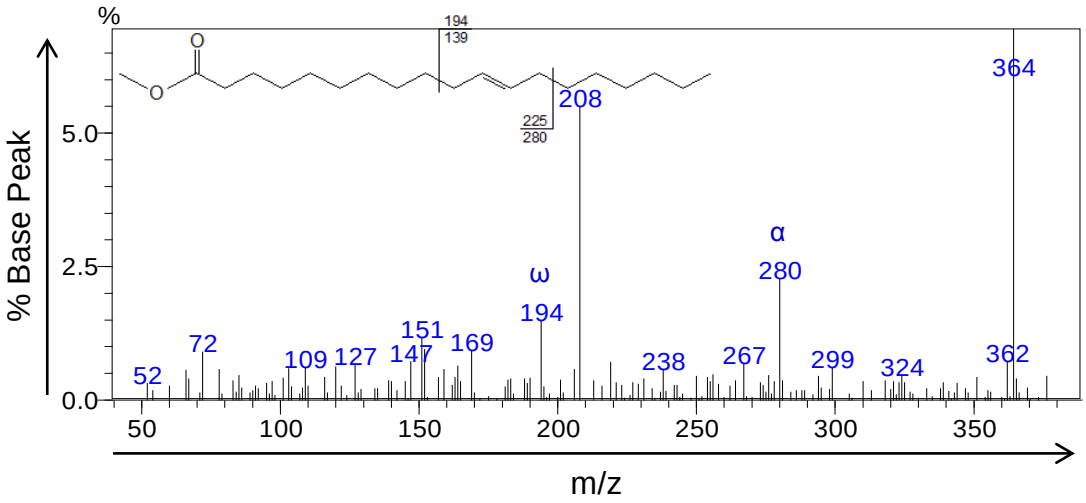

Figure S4.1 CACI-MS and CACI-MS/MS spectra of desaturation products *iso*-8Z-20:1, *iso*-8Z-19:1, *anteiso*-8Z-19:1, n-8Z-19:1 and n-11Z-19:1 FAME. A. CACI-MS and CACI-MS/MS of *iso*-8Z-20:1 FAME showing the characteristic ions (m/z 275, 293, 325, 378) and diagnostic ions (m/z 238, 250); B. CACI-MS and CACI-MS/MS of *iso*-8Z-19:1 FAME showing the characteristic ions (m/z 261, 279, 311, 364) and diagnostic ions (m/z 238, 236); C. CACI-MS and CACI-MS/MS of *anteiso*-8Z-19:1 FAME showing the characteristic ions (m/z 261, 279, 311, 364) and diagnostic ions (m/z 238, 236); D. CACI-MS and CACI-MS/MS of n-8Z-19:1 FAME showing the characteristic ions (m/z 261, 279, 311, 364) and diagnostic ions (m/z 238, 236); E. CACI-MS and CACI-MS/MS of n-11Z-19:1 FAME showing the characteristic ions (m/z 261, 279, 311, 364) and diagnostic ions (m/z 280, 194).

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

Conclusions

In human n-ECFA metabolism, ELOVL1, 3, 6, 7 are known to mediate saturated and monounsaturated n-ECFA elongation, ELOVL2, 5 are active for polyunsaturated n-ECFA elongation, while ELOVL4 accounts for elongation of VLCFA with more than 24 carbons in the chain regardless of the unsaturation degree. SCD mediated ∆9-desaturation is mainly for MUFA production, while FADS1 ∆5 and ∆7 -desaturation as well as FADS2 ∆4, ∆6 and ∆8 – desaturation participate more in PUFA production than MUFA production. Complementarily, our studies characterized the major elongases and desaturase enzymatic activities in human n-OCFA and BCFA metabolism *in vitro*. Studies were conducted using ELOVLx (ELOVL1-7) transient transfected or FADSx (FADS1-2) stably expressed MCF-7 human breast cancer cells, to characterize the enzymatic activities in n-OCFA and BCFA metabolism.

We found ELOVL6 is well conserved across evolutionarily distant related genomes (human to zebrafish), and of importance in n-OCFA elongation, primarily catalyzing n-13:0 → n-15:0 and n-15:0 → n-17:0. ELOVL7 has moderate activity towards n-15:0 → n-17:0. No ELOVL1, 3, 6, 7 elongation activities detected for n-17:0 → n-19:0. This indicates nutrition studies regarding n-15:0 and n-17:0 as biomarkers of specific food intake should

consider the n-OCFA endogenous metabolism in light of these findings.

ELOVLx also showed significant activities in BCFA elongation: among ELOVL1-7, ELOVL6 is mainly responsible for catalyzing *anteiso*-15:0 → *anteiso*-17:0, while ELOVL3 is active toward *iso*-18:0 → *iso*-20:0. Additional competition studies between BCFA and n-SFA for ELOVLx mediated elongations revealed ELOVL6 prefers to catalyze elongations of n-16:0 than *anteiso*-15:0, and ELOVL3 prefers n-18:0 to *iso*-18:0, providing clues to metabolic conditions that are related to local excess of n-SFA.

Our data revealed FADS2 plays a vital role in producing MUFA in n-OCFA and BCFA metabolism. FADS2 is able to Δ6-desaturate BCFA *iso*-16:0 → *iso*-6Z-16:1, *iso*-18:0 → *iso*-6Z-18:1, *iso*-17:0 → *iso*-6Z-17:1 and *anteiso*-17:0 → *anteiso*-6Z-17:1, and also n-OCFA n-17:0 → *anteiso*-6Z-17:1. FADS2 is uniquely highly expressed in human skin sebaceous glands (SG), of which FA composition comprises comparable levels of saturated and monounsaturated BCFA and n-OCFA. Our findings emphasize the role of FADS2 in creating the unique monounsaturated BCFA and n-OCFA composition of human SG.

In summary, our studies extended the range of ELOVLx and FADS2 activities to n-OCFA and BCFA, and verified that both n-OCFA and BCFA could be metabolized in humans via elongation and desaturation. The elongations of saturated n-OCFA and BCFA are operated by ELOVLx which were found to

have a higher affinity towards saturated n-ECFA, if they co-exist in the cell environment. The desaturation of n-17:0 is similar to n-16:0, which could be converted to monoenes by both SCD and FADS2 activities. While the desaturation of saturated BCFA are special, because only FADS2 but not SCD products were detected when BCFA are available. The similarity and difference between n-OCFA, BCFA and n-ECFA metabolism will have implications in the metabolic conditions relevant to FA composition shift, especially in investigating the dysfunctions of skin-like tissues that are rich in n-OCFA and BCFA.

Future Work

n-OCFA and BCFA have been mainly investigated in bacteria, but neglected in food and nutrition. Despite of their relatively low levels, their appearance in dairy, beef and seafood as well as in human GI tract and skin shows they are digested and utilized for key functions in humans. Studies in this dissertation are the first work exploring the metabolism of n-OCFA and BCFA in human cell model, and provide clues to the major rate-limiting enzymes catalyzing their elongation and desaturation pathways. Compared to n-ECFA metabolism, the whole systems of n-OCFA and BCFA are far from established. Additional studies are necessary to characterize the elongase/desaturase substrate specificities to shorter chain and longer chain n-OCFA and BCFA.

In current studies, we found overlapping function of ELOVLx: both ELOVL6 and ELOVL7 could mediate n-15:0 elongation; and ELOVL2, ELOVL3 and ELOVL7 catalyze *iso*-18:0 elongation. Also, we showed human elongase/desaturase activity towards multiple substrates simultaneously: ELOVL3 acts on both n-18:0 and *iso*-18:0; ELOVL6 acts on both n-16:0 and *anteiso*-15:0; FADS2 not only catalyzes desaturation of n-16:0 but also *iso*-16:0, *iso*-18:0, *iso*-17:0, *anteiso*-17:0 and n-17:0. The competition studies between BCFA and n-SFA for ELOVL6/3 mediated elongations shown in Chapter 3 revealed that BCFA compete with n-SFA for ELOVL6/3 mediated elongation. Thus, in metabolic diseases that are related to local excess of n-SFA, such as diabetes, increasing BCFA intake may displace n-SFA and influence the disease phenotype. Our findings also indicate BCFA and n-FA may have potential competition for FADS2 mediated desaturation. Since human skin-like tissues are rich in both BCFA and n-FA, and featured by high FADS2 expression, further exploration of the potential competition between BCFA and n-FA for FADS2 activity, and illustrate the implication of this competition in skin function.

The FA compositions are shifted in several skin diseases due to gene regulation. For instance, mice with ELOVL3-ablation showed skin dysfunction and no obvious FA with more than 20 carbons [1]; atopic dermatitis (AD) patients have decreased ELOVL3 and ELOVL6 expression in stratum corneum compared to healthy subjects, leading to a decreased proportion of

VLCFA in skin lipids [2]; and acne development is associated with lower 16:0/16:1 ratio and higher unsaturation degree of lipids resulted from incorrect FADS2 activity [3, 4]. However, no causation is established between gene-regulated FA composition shift and skin dysfunction yet. Therefore, it would be interesting to further study the gene regulation of multiple FA biosynthesis pathways in skin tissues, and to investigate the gene-modulated FA difference between patients and normal population. Moreover, the role of BCFA in skin is still unknown. Inspired by BCFA biophysical function in bacteria [5, 6], BCFA in skin are presumed to maintain sebum fluidity when PUFA are low in skin lipids [7], and support normal human skin function such as the thermal adaptation, though more evidence is needed to support this claim.

In this dissertation, novel biosynthesis pathways of food-based n-OCFA and BCFA are established in human cell model. Further animal studies and clinical trials are warranted in this direction to further reveal the complex metabolism of these FA after digestion.

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