A NOVEL ALTERNATE PATHWAY FOR LONG CHAIN POLYUNSATURATED FATTY ACID BIOSYNTHESIS AND ALTERNATIVE TRANSCRIPTS OF FATTY ACID DESATURASE GENES

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Long chain polyunsaturated fatty acids (LCPUFA), especially arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3) are essential for human health and cognitive development, are the major structural components of the cell membranes, are precursors for cellular signaling molecules and are also known to regulate transcription of genes. It is widely believed that the biosynthesis of LCPUFA from linoleic acid (18:2n-6) and linolenic acid (18:3n-3) takes place by alternating series of position-specific desaturases, chain-elongation reactions and a peroxisomal beta-oxidation for DHA synthesis. The existence of an alternative biosynthetic pathway was first observed in a protozoan (*Euglena gracilis*), in which 18:2n-6 and 18:3n-3 are first elongated to 20:2n-6 and 20:3n-3, followed by Δ8- and Δ5-desaturation, respectively to yield 20:4n-6 and 20:5n-3. We hypothesized that the primate *FADS2* gene product would have Δ8-desaturase activity and cloned baboon *FADS2* into *Saccharomyces cerevisiae*, to test for gain of Δ8-desaturation activity. The novel data that we have published (park et al, 2009, JLR) show evidence that the *FADS2* protein product **is a Δ8-desaturase** (as well as a Δ6-desaturase).

FADS1, *FADS2,* and *FADS3* are localized within a 100 kb region on human chromosome 11q12-13.1. *FADS1* and *FADS2* genes encode for enzymes catalyzing Δ5- and Δ6- & Δ8-desaturation, respectively and only a single transcript has been identified for both genes. Function(s) of *FADS3* showing high homology to *FADS1* and *FADS2* are unknown. We reported the results for the existence of alternative transcripts (AT) for *FADS2* and *FADS3* genes. Investigating the role of *FADS* genes, we performed RT-PCR analysis using baboon cDNA and detected an AT for *FADS2* and multiple AT for *FADS3* generated by alternative splicing (Park et al, 2009, Mol Biol Reports; Park et al, 2009, Gene). We also found that these AT are well conserved across several vertebrate species. The *FADS2* AT and *FADS3* AT are expressed in many neonate baboon tissues. *FADS3* AT also showed changes in abundance in response to human neuronal cell differentiation. Determining the functions of these AT, conserved among species, provides a putative mechanism for understanding of LCPUFA biosynthetic regulation, and how it differs among individuals.

BIOGRAPHICAL SKETCH

WOO JUNG PARK was born in Seoul, South Korea in 1975. He has two sisters and lived with his family, including his grandfather in law for about 20 years. He entered Kyung Hee University in 1994 and received his Bachelor degree in 2000 and his Masters degree in 2002 from Kyung Hee University in South Korea. He met his wife, Jiyoung in 2002, June and got married with his wife on the next day of 2004 Christmas. After thirty years in Korea, he moved to Ithaca, U.S. and started his Ph.D. studies in the field of Food Science and Technology in fall, 2005. He received his Ph.D. degree from Cornell University in February, 2010. His research interest is fatty acid desaturase genes and their function in long chain polyunsaturated fatty acid synthesis.

For

my Father,

my Mother,

and

my wife Jiyoung

ACKNOWLEDGMENTS

Completing this dissertation, I am reminded about my last few years in Ithaca and in my PhD lab when it occasionally provided me with difficulties that were hard to tolerate and at the same time, gave me happiness which may never come again in my life.

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LIST OF ABBREVIATIONS

A. castellanii: Acanthamoeba castellanii

ALA: α-Linolenic acid (18:3n-3)

ARA: Arachidonic acid (20:4n-6)

AS: Alternative splicing

AT: Alternative transcript

CACI: Covalent adduct chemical ionization

DHA: Docosahexaenoic acid (22:6n-3)

DGLA: Dihomo-gamma-linolenic acid (18:3n-6)

EPA: Eicosapentaenoic acid (20:5n-3)

E. gracilis: Euglena gracilis

FADS1: Fatty acid desaturase 1

FADS2: Fatty acid desaturase 2

FADS3: Fatty acid desaturase 3

FAME: Fatty acid methyl ester

FID: Flame ionization detector

GC: Gas chromatography

MS: Mass spectrometry

LA: Linoleic acid (18:2n-6)

LCPUFA: Long chain polyunsaturated fatty acid

PUFA: Polyunsaturated fatty acid

P. marinus: Perkinsus marinus

S. cerevisiae: *Saccharomyces cerevisiae*

SNP: Single nucleotide polymorphism

CHAPTER 1

INTRODUCTION

6.1. Food and LCPUFA

With the growing concerns over optimal nutrition for human health, interests in functional food and dietary nutrients have significantly increased. As an essential requirement for the human diet, dietary fat is important as it is a source of energy as well as as a source of essential fatty acids and fat-soluble vitamins. However, regardless of the sufficient supply of fat, the nutritional composition of western diet is widely believed to have adversely affected human health and contributed to the prevalence of chronic diseases (Roche, 1999). Nutritional imbalance between n-6 and n-3 fatty acids has been a serious concern in the western diet and in developing countries. High intakes of n-6 fatty acids are thought to induce deleterious shifts in blood clotting propensity, vasospasm, and vasoconstriction, and decrease in bleeding time (Roche, 1999; Simopoulos, 1999).

Mammalian fatty acids consist of hydrocarbon chains with diverse lengths and with single or double bonds. The n-6 and n-3 fatty acids are classified according to the place of the first double bond close to the methyl end of the fatty acid. Long chain polyunsaturated fatty acid (LCPUFA), of the n-6 and n-3 families, especially arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-6) are the most physiologically potent and contribute significantly to human health, especially

to vision and cognition during various stages of development and aging. However, mammals cannot synthesize these fatty acids from acetyl CoA because they lack Δ12- and Δ15- desaturase enzymes, hence, these LCPUFA must be directly consumed from diet such as fish, or meat. They also can be synthesized from dietary essential fatty acids (EFA) (Burr and Burr, 1973) linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) by a series of desaturation and elongation reactions. The main sources for LA are corn, sunflower seeds, safflower seeds, cotton seeds, and soybeans and those for ALA are green leafy vegetables, flaxseed, canola, soybean, and walnuts (Roche, 1999).

1.2. LCPUFA and human health

LCPUFA generate various bioactive metabolic compounds to regulate inflammatory responses, play role as secondary messengers, and mediate functions for vascular tone and platelet aggregation, contributing to membrane biophysical properties and integrity and hence are indirectly associated with diverse chronic disease such as cardiovascular disease, diabetes, arthritis, and cancer (Simopoulos, 1999; Le et al., 2009). Insulin sensitivity is associated with phospholipid fatty acid composition (Benatti et al., 2004). Ratio of n-6/n-3 fatty acids affects cell adhesion and invasion-related gene expression in human lung cancer cells (Xia et al., 2005). Many epidemiological and clinical studies imply that n-3 fatty acids affect on the arrhythmic action of patients with coronary heart disease (Benatti et al., 2004).

Eicosanoids generated from n-6 and n-3 fatty acids have pro-inflammatory and anti-inflammatory activities, respectively (Lee et al., 1984). EPA has been reported to be a precursor of resolvin E1, a mediator that reduces the inflammatory response through the regulation of leukocyte extravasation (Serhan et al., 2002; Dona et al., 2008). DHA is a precursor of neuroprotectin D1, which is a lipid mediator to protect neurons and retinal cells under oxidative stress conditions (Mukherjee et al., 2004). EPA and DHA inhibit the production of eicosanoids from ARA, producing eicosanoids from EPA and of anti-inflammatory resolvins and similar metabolic regulators from DHA. The eicosanoid syntheses are regulated by COX and 5-lipooxygenase pathways (Le et al., 2009). In addition, n-3 LCPUFA act on other pro-inflammatory responses such as reducing leukocyte chemotaxis, adhesion molecule expression and inflammatory cytokine production (Calder, 2008). These anti-inflammatory effects were processed via pro-inflammatory transcription factor NF-κB and probably via activation of anti-inflammatory transcription factor PPAR-γ (peroxisome proliferator-activator receptor γ) (Calder, 2008).

In the vertebrate central nervous system (CNS), ARA and DHA are prevalent fatty acids and have critical roles for brain function, neuronal development, and cognitive function (Brenna and Diau, 2007). These fatty acids are major constituents of brain membranes, and studies indicated the importance of DHA in neurogenesis (Calderon and Kim, 2004; Cao et al., 2005), neurotransmission (Chalon, 2006), and protection against oxidative stress (Bazan, 2006). Recent genome wide expression studies have shown that dietary DHA and ARA changes expression of thousands of genes in the brain, and liver tissues (Kitajka et al., 2004; Berger et al., 2006; Kothapalli et al.,

2007). Our own recent studies have shown that CNS DHA is regulated by dietary DHA through modulation of biosynthesis, however, that of CNS ARA is mediated depending on the level of incorporation or utilization (Brenna and Diau, 2007; Hsieh and Brenna, 2009). In addition to brain, rod outer segments of retina also consist of high concentration of DHA. The function of DHA is also very important for visual capability (Benolken et al., 1973).

1.3. Synthetic pathway of LCPUFA

Figure 1.1 shows the widely accepted synthetic pathway in mammals before our recent publication. Plants are able to produce LA and ALA, dietary EFA, which are synthesized by Δ15- and Δ12- desaturation from oleic acid (OA, 18:1n-9). However, mammals have to consume EFA from plant sources and synthesize LCPUFA or have to directly eat LCPUFA from foods. In the currently accepted pathway, Δ6-desaturation is believed to be the rate-limiting step from LA to γ-linolenic acid (GLA, 18:3n-6). After elongation from GLA, dihomo-gamma-linolenic acid (DGLA, 20:3n-6) is Δ5-desaturated to ARA. With two consecutive elongations, another Δ6-desaturation is followed and finally β-oxidized to docosapentaenoic acid (DPA n-6, 22:5n-6). Like the n-6 pathway, common enzymes catalyze desaturation and elongation for n-3 LCPUFA synthesis. Though there is no clear evidence in mammals regarding a Δ4-desaturation step to form DHA, this step has been demonstrated in marine microorganism (Qiu et al., 2001).

An alternative LCPUFA synthetic pathway via Δ8-desaturation has been

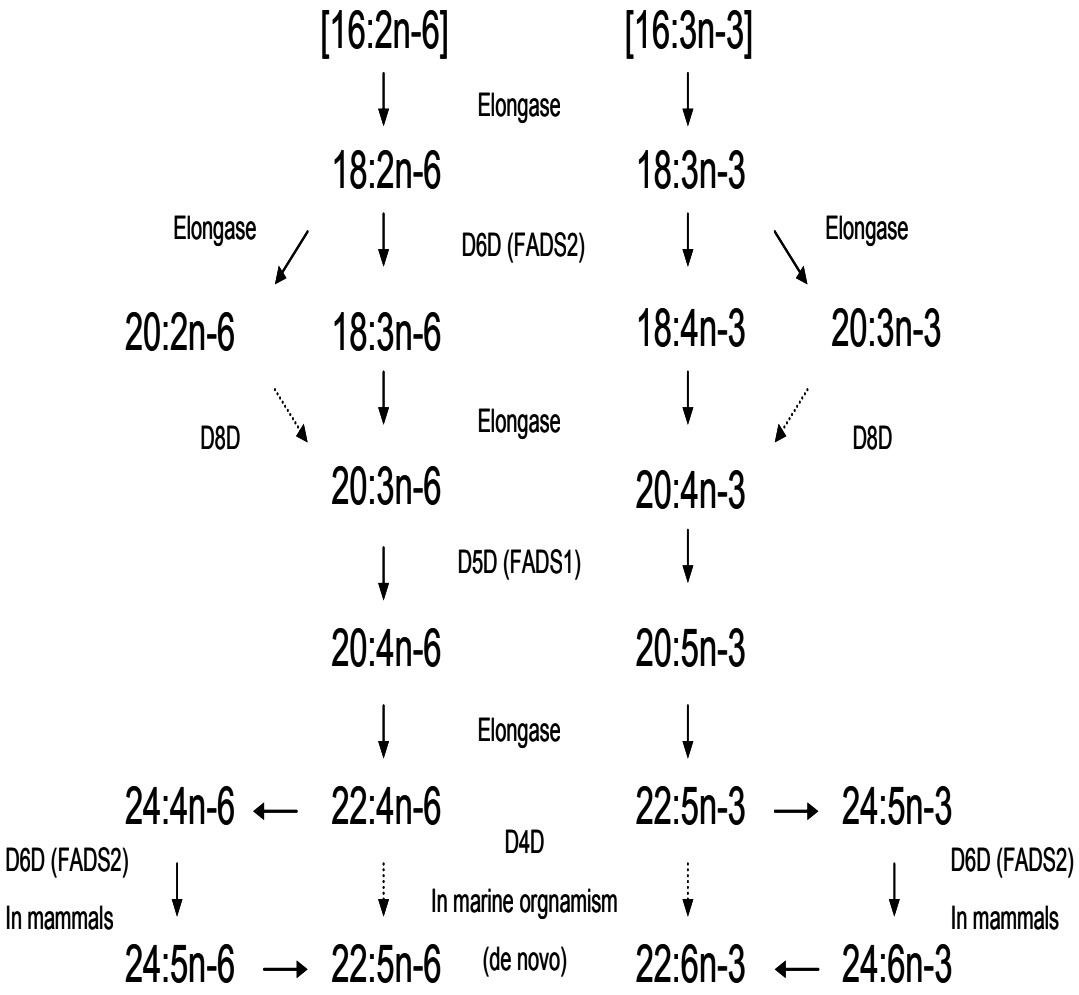

Figure 1.1. LCPUFA synthesis, as previously accepted. Two carbons of 24 fatty acids are shortened by peroxisomal β-oxidation. D4D, D5D, D6D, and D8D indicate Δ4-, Δ5-, Δ6-, and Δ8-desaturases, respectively.

demonstrated by molecular cloning and functional characterization in the unicellular organisms, in *Euglena gracilis*, *Acanthamoeba castellanii*, *Perkinsus marinus* (Wallis and Browse, 1999; Chu et al., 2004; Sayanova et al., 2006). However, the existence of Δ8-desaturation has been in question (Chen et al., 2000) though many studies have reported the Δ8-desaturation activity in mammals. Schaeffer et al showed higher levels of 20:2n-6, substrate of Δ8-desaturation, and significantly reduced levels of 18:3n-6, substrate of Δ6-desaturation, and higher levels of 20:3n-6, in human plasma and red cells in individuals homozygous for the minor alleles of several single nucleotide polymorphisms in *FADS2* gene (Schaeffer et al., 2006). This observation might be explained by the existence of Δ8-desaturation.

1.4. Desaturases of LCPUFA

Desaturases for LCPUFA are non-heme-iron-containing, membrane-bound enzymes that catalyze the addition of a double bond in a fatty acid chain. They have an N-terminal cytochrome b5 domain and 3 histidine motifs ($HX_{3-4}H$, $HX_{2-3}HH$, and $H/QX_{2-3}HH$) (Nakamura and Nara, 2004). On human choromosome 11 (11q12-13.1), fatty acid desaturase (*FADS*) genes consisting of *FADS1*, *FADS2*, and *FADS3* are clustered within a 100 kb region (Marquardt et al., 2000) (Figure 1.2). The mouse homologues of these genes are located on chromosome 19 as a cluster (Nakamura and Nara, 2004). All three desaturase genes contain the same 12 exons and 11 introns; FADS1 and 2 putative proteins consist of 444 amino acids which is different from FADS3 that is comprised of a 445 amino acid protein. The location of desaturase genes

suggested that the genes are thought to have arisen by gene duplication during the evolution (Marquardt et al., 2000).

The *FADS1* gene product is known as the Δ5-desaturase. It introduces a double bond in last step of the production of ARA and EPA from DGLA and 20:4n-3, respectively (Cho et al., 1999a). The *FADS2* gene product catalyzes the introduction of a Δ6 double bond from LA to GLA and ALA to stearidonic acid (18:4n-3) (Cho et al., 1999b). In addition to the desaturation of palmitic acid (16:0) (Ge et al., 2003; Guillou et al., 2003), *FADS2* gene product catalyzes Δ6-desaturation from 24:4n-6 to 24:5n-6 and from 24:5n-3 to 24:6n-3 (D'Andrea et al., 2002). The activity of FADS3 gene product remains still unknown, though the first report of the gene was in 2000 (Marquardt et al., 2000). Apart from mammals, the zebrafish desaturase contain both Δ5- and Δ6- desaturation activities (Hastings et al., 2001) and *Caenorhabditis elegans* omega-3 desaturase (FAT-1) catalyzes a change from n-6 fatty acids to n-3 fatty acids by introducing a double bond between 3rd and 4th carbons from the methyl end of a fatty acid chain (Spychalla et al., 1997).

Recently, reports of FADS2 deficient (*FADS2* -/-) mice suggested the physiological roles *in vivo*. FADS2 deficiency did not affect the viability and development of mice, independent of the support of LCPUFA, but the deficiency induced the reproductive abnormality and sterility of mice (Stoffel et al., 2008; Stroud et al., 2009). Stroud et al. also reported that *FADS2* -/- mice displayed dermatitis and the symptoms disappeared after the supplementation of ARA and that *FADS2* -/- mice created the ulceration of the small intestine (Stroud et al., 2009). Interestingly, Stroud et al. showed that

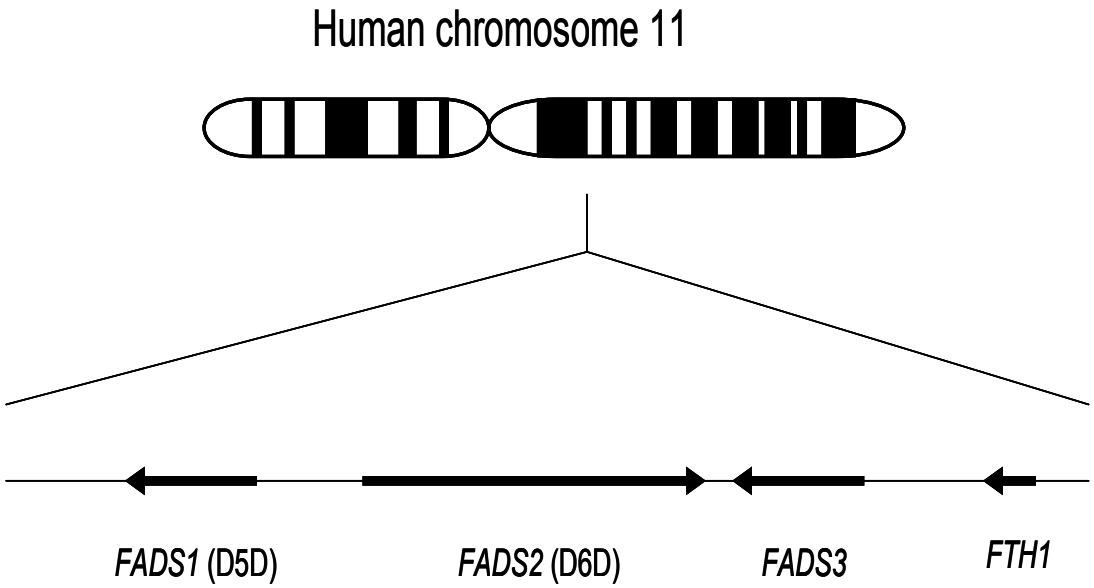

Figure 1.2. The localization of *FADS1*, *FADS2*, and *FADS3* on human chromosome 11. *FADS1*; fatty acid desaturase 1, *FADS2*; fatty acid desaturase 2, *FADS3*; fatty acid desaturase 3, FTH1; ferritin-heavy chain 1.

FADS2 -/- mice caused the high expression of *FADS3* compared to *FADS2* +/+ mice, implying a role for FADS3 in LCPUFA synthesis (Stroud et al., 2009).

1.5. FADS gene clusters and human health

Recent studies have suggested the importance of *FADS* genes for human health and cognitive development based on genome wide analyses. They show associations between fatty acid genes and various physiological conditions. An early first report about the relationship between fatty acid composition in phospholipid and *FADS* gene clusters, Schaeffer et al. 2006 showed that PUFA compositional changes in serum phospholipids are associated with FADS1 FADS2 gene clusters and ARA is most significantly associated with the genetic variants (Schaeffer et al., 2006). Another study showed that genetic variants in *FADS* gene cluster of patients with cardiovascular disease are associated with ARA, the precursor for proinflammatory eicosanoids (Malerba et al., 2008). These studies suggest that the genetic variation within *FADS* genes may have important roles for inflammatory related diseases and that the genotypes may be critical modulators of desaturase activities (Lattka et al., 2009). A recent genome wide study also suggests that enzymes for LCPUFA synthesis, specifically desaturases and elongases, influence concentrations of plasma fatty acid (Tanaka et al., 2009).

As physiological and cognitive studies, *FADS* gene clusters were investigated.

FADS1 and *FADS2* SNP (single nucleotide polymorphism) affects plasma and erythrocyte phospholipids and breast milk fatty acid compositions in pregnancy and lactation. Those SNP may have effects on the DHA, in maternal breast milk, essential for infant development (Xie and Innis, 2008). Caspi et al showed that a genetic variant of *FADS2* is associated with IQ and breastfeeding (Caspi et al., 2007), though the SNP in question was within an intron. Additionally, ADHD (Attention Deficit/Hyperactivity Disorder), one of the most usual and heritable behavioral disorders in childhood, was analyzed with genetic variants within *FADS* genes showing that a single SNP within *FADS2* is highly associated with ADHA (Brookes et al., 2006).

1.6. Regulation of desaturases in LCPUFA

Dietary LCPUFA inhibits the expression of Δ5- and Δ6-desaturases by feedback regulation (Nakamura and Nara, 2004). Sterol regulatory element binding protein-1c (SREBP-1c) and peroxisome proliferator activated receptor-α (PPARα) have been reported as regulators of the desaturases. SREBP-1c and PPARα are transcription factors for fatty acid synthesis and for fatty acid oxidation, respectively (Nakamura and Nara, 2004), but both of them were known to induce the expression of mice Δ5- and Δ6-desaturase genes in liver (Matsuzaka et al., 2002; Nakamura and Nara, 2004). Nara et al also identified the 90-bp promoter region of *FADS2* gene which is not only required for the activation of the gene by SREBP-1c but for the suppression of *FADS2* gene by LCPUFA (Nara et al., 2002).

In addition, both Δ5- and Δ6-desaturses are regulated by various hormones. Insulin activates both enzymes, but glucagon, adrenaline, glucocorticoids, mineralocorticoids, oestriol, oestradiol, testosterone, and ACTH suppress both enzymes (Brenner, 2003). Previous studies showed that proportions of ARA and EPA in liver were reduced by insulin-dependent diabetes, however insulin increased these fatty acids. Interestingly, DHA displayed the opposite; an increase in liver DHA is associated in insulin-dependent diabetes (Hu et al., 1994; Chanussot et al., 1997; Brenner et al., 2000; Brenner, 2003). These results imply that the enzymes for synthesis for DHA are not affected by insulin or the biosynthesis for DHA could be different from ARA and EPA.

1.7. Elongases of LCPUFA

Elongases, or fatty acid chain elongation system (FACES), are the enzymes which catalyze an increase in chain length by adding two carbons to the carboxyl end group of a fatty acid chain. FACES consists of four enzyme units, condensing enzyme, β-ketoacyl CoA reductase, β-hydroxyacyl CoA dehydrase, and *trans*-2-enoyl CoA reductase (Leonard et al., 2004; Jakobsson et al., 2006), whereas the elongase is the first condensing enzyme. The elongases are generally able to be classified depending on the degree of unsaturation and the chain length. Human *ELOVL2*, *4*, and *5* genes encode for enzymes mediating elongation of PUFA. The *ELOVL2* gene product, consisting of 296 amino acids, has elongation activity for C_{20} and C_{22} PUFA (Leonard et al., 2004). The *ELOVL4* gene product, encoding for 314 amino acids, is highly expressed in retina, brain, and testis (Leonard et al., 2002),

which suggests that it might be related to the synthesis of DHA. The *ELOVL5* gene product encodes for 299 amino acids and elongates C_{18}-C_{20} PUFA (Leonard et al., 2004; Jakobsson et al., 2006). Additionally, mouse *Elovl2* showed activity against DGLA to some degree, however, human *ELOVL2* was not able to elongate the fatty acid, suggesting that there is a functional divergence between species. *ELOVL* genes in the ER are also thought to have a function in the biosynthesis of PPARα ligands for the synthesis of 22-carbon fatty acids (Jakobsson et al., 2006), but further study is still required on this point.

1.8. Alternative splicing

Alternative splicing (AS) has been identified to be critical in molecular and cellular mechanisms. Recent studies show that AS occurs in more than 95% of human multiexon genes (Calarco et al., 2009). AS events can be divided into several splicing patterns such as exon skipping, alternative 5' splice sites, alternative 3' splice sites, and retained introns (Stamm et al., 2005). By diverse combination of splicing patterns, many transcripts and isoforms can be generated. For instance, the human neurexin3 gene is able to generate more than 1700 transcripts, and *Drosophila* Down syndrome cell adhesion molecule (DSCAM) is able to form over 38,000 proteins by alternative splicing (Celotto and Graveley, 2001).

AS mediates gene expression and contributes to protein diversity by generating a number of mRNA and protein isoforms from the low number of

eukaryotic genes (Black, 2000; Graveley, 2001). It also has an effect on the structure of mRNA and their protein. These effects are capable of changing the features of protein functions: binding properties, enzyme activity, intracellular localization, protein stability, phosphorylation, and glycosylation patterns (Stamm et al., 2005). Though AS has been reported in various tissues, the most prevalent regulatory tissues for this event are in the brain and nervous system for functions such as synaptogenesis, neurite outgrowth, axon guidance, ion channel activity, and long-term potentiation (Calarco et al., 2009). For example, DSCAM is crucial in the development of neural circuitry and in dendritic self-avoidance, in which only a few different amino acids serves to identify one neuron from another. Fibroblast growth factor 8 (FGF8), regulating of vertebrate midbrain development, has two isoforms showing very different activities depending on the development (Li et al., 2007).

1.9. Summary

Long chain polyunsaturated fatty acids (LCPUFA) are critical for human health and cognition development as well as are associated with eicosanoid signaling and membrane biophysical properties. The synthesis of LCPUFA is generally believed to be regulated by Δ5- and Δ6-desaturases, elongases, and two carbon shortening β-oxidation. However, previous studies have also suggested the existence of the alternative pathway via Δ8-desaturation in LCPUFA synthesis. Herein, we hypothesized that *FADS2* gene product would show Δ8-desaturation activity and the report that we have recently published reveals that *FADS2* gene product is a ∆8-desaturase as well as ∆6-desaturase

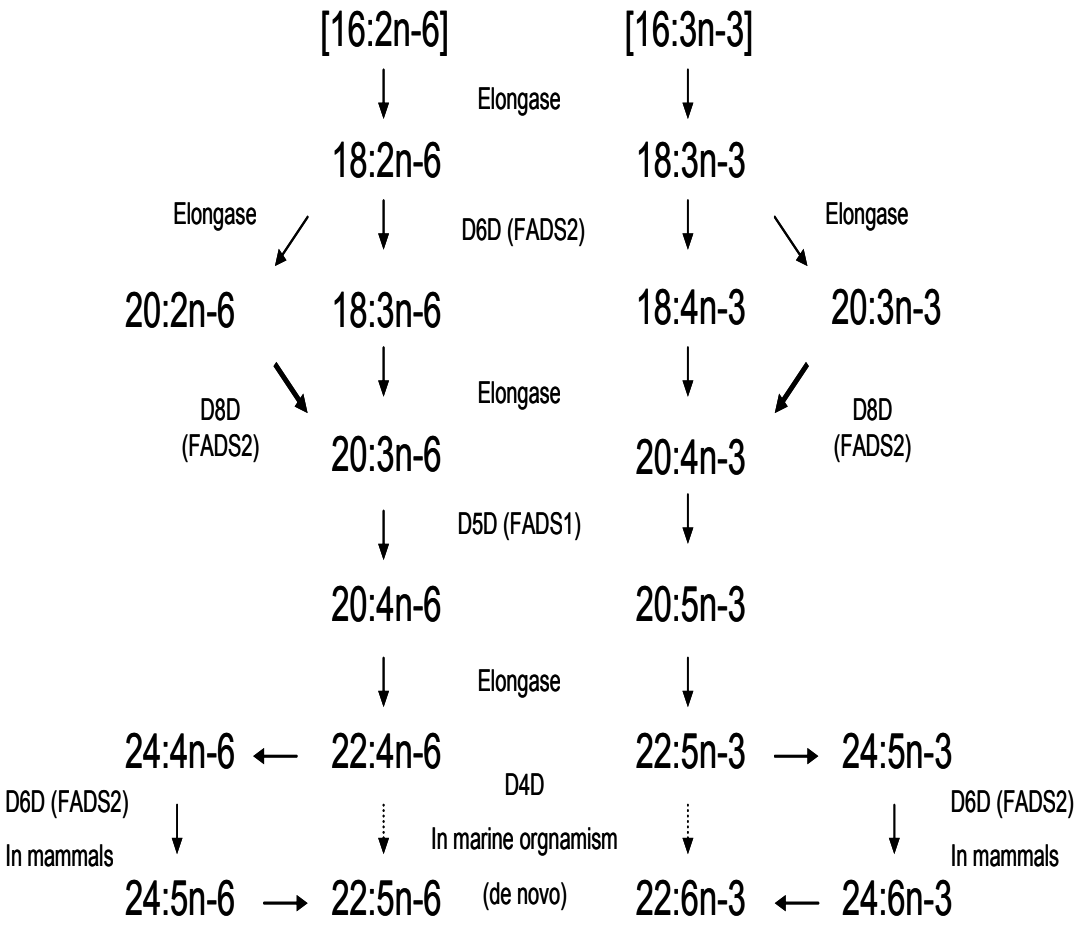

Figure 1.3. LCPUFA synthesis, as currently accepted. Two carbons of 24 fatty acids are shortened by peroxisomal β-oxidation. D4D, D5D, D6D, and D8D indicate Δ4-, Δ5-, Δ6-, and Δ8-desaturases, respectively.

(Park et al., 2009a). Figure 1.3 shows a refinement of the accepted LCPUFA pathway modified after our recent publication.

On the long arm of human chromosome 11, *FADS1*, *FADS2*, and *FADS3* are localized within a 100 kb region. The functions of these genes as well as the association between *FADS* genes and physiological conditions have been studied. In addition, the regulatory mechanisms of desaturases by transcription factors and various hormones have been reported. However, there was no clear report about AT of fatty acid desaturase genes involved in LCPUFA synthesis although importance of AS has been increasing in molecular mechanisms. We reported here the existence of AT for *FADS2* and *FADS3* generated by AS events. Functional characterization of these AT may provide clues to understanding LCPUFA synthetic regulation (Park et al., 2009b; Park et al., 2009c).

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CHAPTER 2

AN ALTERNATE PATHWAY TO LONG CHAIN POLYUNSATURATES : THE *FADS2* GENE PRODUCT Δ8-DESATURATES 20:2N-6 AND 20:3N-3[1]

2.1. Introduction

Long chain polyunsaturated fatty acids (LCPUFA) are ubiquitous in mammalian tissue, achieving highest concentrations in the membranes of neural and other excitable tissue (Brenna and Diau, 2007). LCPUFA of the n-3 and n-6 families, especially eicosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3) and arachidonic acid (ARA; 20:4n-6) are bioactive components of membrane phospholipids and serve as substrates for signaling molecules (Kinsella et al., 1990). The degree of unsaturation of the membranes is determined by the action of enzymes involved in fatty acid biosynthesis and metabolism (Voss et al., 1991). Most organisms synthesize unsaturated fatty acids, but the pathways are specific to cell types and species.

Fatty acid desaturases are enzymes that catalyze the introduction of *cis* double bonds at specific positions in a fatty acid chain (Los and Murata, 1998). Desaturases in plants and lower animal species can introduce double bonds near the methyl end. Eukaryotic cells of higher animals, fungi, and dinoflagellates express membrane bound acyl-CoA front end desaturases (Tocher et al., 1998; Nakamura and Nara, 2004) catalyzing double bond

[1] Based on the reference of J. Lipid Res. 2009 Jun;50(6):1195-202.

introduction into the ∆6, ∆5, ∆8, and ∆4 positions. Mammalian front end desaturases operate on diet-derived PUFA to synthesize LCPUFA, which can also be derived from the diet but possibly not in sufficient quantities to optimize health (Salem et al., 1996).

The front end desaturases are remarkable for their structural similarity and functional diversity. They all contain the N-terminal cytochrome b5 domain (HPGG) as electron donor and three histidine motifs "HXXXH, HXXHH and QXXHH" conserved from human to microalgae (Sperling et al., 2003). Molecular cloning and isolation of a Δ5-desaturase from *Caenorhabditis elegans* (Michaelson et al., 1998b), *Mortierella alpina* (Michaelson et al., 1998a) and a Δ6-desaturase from *C. elegans* (Napier et al., 1998), *M. alpina* (Huang et al., 1999), rat (Aki et al., 1999), and mouse (Cho et al., 1999b) have all been reported. The human *FADS* gene cluster at 11q12-q13.1 encodes two desaturases with known function, Δ5-desaturase (*FADS1*) and Δ6-desaturase (*FADS2*) (Cho et al., 1999a; Cho et al., 1999b), as well as a third putative desaturase gene (*FADS3*) (Marquardt et al., 2000) which thus far has no known substrate despite high homology to *FADS1* and *FADS2*.

Figure 2.1 shows the common n-3 and n-6 LCPUFA pathways mediated by ∆6 and ∆5 desaturases. The ∆6-desaturase (*FADS2*) is known to operate on both 18:3n-3 and 18:2n-6, resulting in the synthesis of 6,9,12,15-18:4 and 6,9,12-18:3 (gamma-linolenic acid), respectively. This step is rate-limiting, and is followed by elongation to 8,11,14,17-20:4 and 8,11,14-20:3 (dihomo-gamma-linolenic acid). A rapid ∆5-desaturation (*FADS1*) on these PUFA produces EPA and ARA. EPA can be further elongated and desaturated to yield DHA

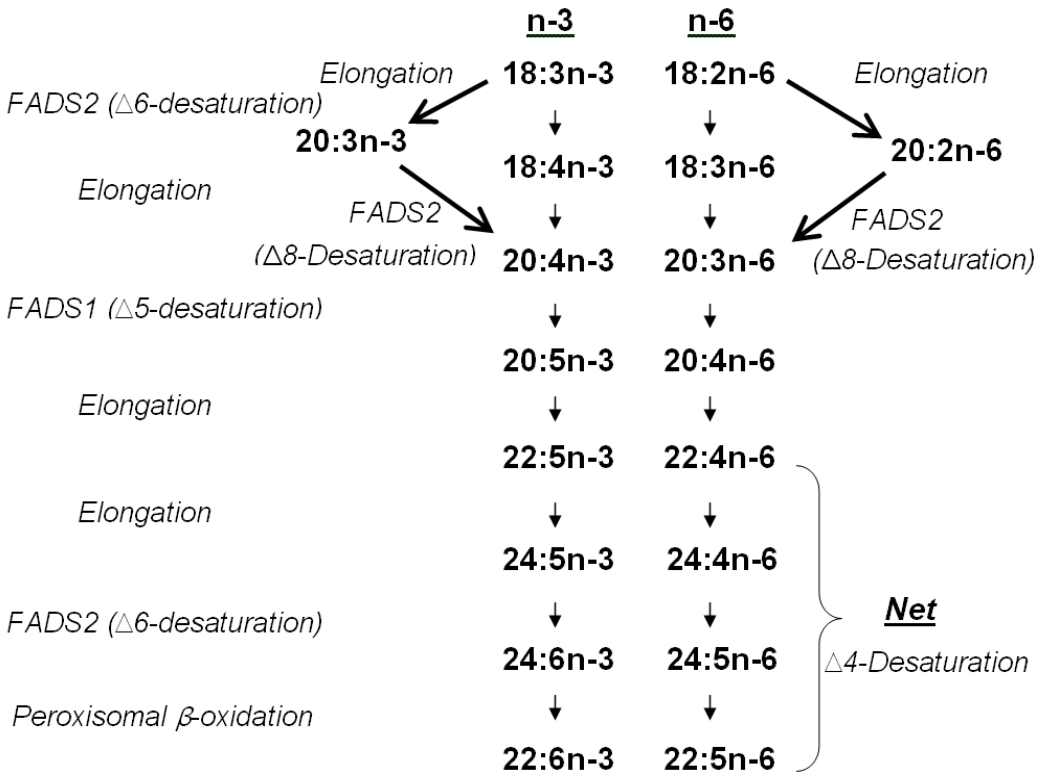


Figure 2.1. Pathways for LCPUFA Biosynthesis.
Conventional pathway consists of alternating desaturation and elongation leading to LCPUFA. Δ8-Desaturation of 20:2n-6 and 20:3n-3 would yield 20:3n-6 and 20:4n-3, intermediates in the conventional pathway to 20:4n-6 and 20:5n-3 as well as immediate eicosanoid precursors.

by the pathway shown, which is accepted as mammalian pathway, or via a ∆4-desaturase as demonstrated in *Thraustochytrium* (Qiu et al., 2001).

The operation of an alternative pathway via C20 fatty acids using a Δ8-desaturase reported in unicellular organisms (Korn, 1964b; Korn, 1964a; Lees and Korn, 1966; Qi et al., 2002), has been verified by molecular cloning and functional characterization studies in *Euglena gracilis* (Wallis and Browse, 1999), *Acanthamoeba castellanii* (Sayanova et al., 2006), and *Perkinsus marinus* (Chu et al., 2004). There are many reports of ∆8-desaturation activity in mammalian cells (Nakazawa et al., 1976; Bardon et al., 1996), rat and human testes (Albert and Coniglio, 1977; Albert et al., 1979), and in mouse liver (Schenck et al., 1996), though it has not been verified by molecular cloning and the existence of Δ8-desaturation in rat microsomes has been questioned (Chen et al., 2000). The putative substrate of the ∆8-desaturase, 11,14-eicosadienoic acid (20:2 n-6), is found in human plasma and red cells as well as other tissues, and its concentration has recently been associated with human genetic variation in the *FADS* gene cluster (Schaeffer et al., 2006; Malerba et al., 2008).

The mammalian Δ6-desaturase coded by *FADS2* uses at least 5 substrates, 18:2n-6, 18:3n-3, 24:6n-3, 24:5n-3, (D'Andrea et al., 2002; Guillou et al., 2003) and 16:0. ∆6-desaturase in the sebaceous glands catalyzes desaturation of 16:0 to 16:1n-10 (sapienate), the most abundant fatty acid in human sebum, showing that substrate specificity is influenced by the cellular environment in which it is expressed (Ge et al., 2003). We hypothesized that the primate

FADS2 gene product would have ∆8-desaturase activity and cloned baboon *FADS2* into *Saccharomyces cerevisiae*, an organism with no native PUFA biosynthetic capability, to test for gain of Δ8-desaturation activity. Here we report unambiguous evidence of the existence of Δ8-desaturation in primates, suggesting alternative pathway for LCPUFA biosynthesis.

2.2. Materials and methods

RNA isolation and cDNA synthesis

Total RNA from 30 mg neonate baboon liver tissue homogenate was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA). The yield of total RNA was assessed by 260 nm UV absorption. The quality of RNA was analyzed by 260/280 nm ratios of the samples and by agarose gel electrophoresis to verify RNA integrity. One microgram total RNA was reverse-transcribed into first strand cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The resulting cDNA was used as template for RT-PCR reactions.

Cloning of baboon *FADS2* and sequence analysis

In order to identify baboon *FADS2* cDNA sequence, primers were generated using human cDNA sequences for *FADS2* (Gen Bank Acc# NM_004265). PCR primers, *FADS2*- Forward (5'- ATG GGG AAG GGA GGG AAC CAG GGC GA -3') and *FADS2*- Reverse (5'- TCA TTT GTG AAG GTA GGC GTC CAG CCA -3') were ordered from Integrated DNA Technologies (IDT, Coralville, IA) and were amplified with baboon liver cDNA as template and high-fidelity Taq polymerase (Roche Diagnostics Ltd) using Eppendorf

gradient thermal cycler. Cycling conditions were: initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 72°C for 45 s and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. PCR product was separated by electrophoresis on 2% agarose gel stained with ethidium bromide and band of appropriate size was obtained. The PCR product was gel purified and cloned in pGEM T-Easy vector (Promega, USA) and sequenced using T7 forward and SP6 reverse universal primers at Cornell University Life Sciences Core Laboratories Center using the Applied Biosystems automated 3730 DNA analyzer. We have successfully cloned baboon *FADS2* protein coding region (GenBank Acc#EU780003). The pGEM T- Easy vector with *FADS2* was named pT*FADS2*.

Transformation into yeast (*Saccharomyces cerevisiae*)

The entire coding regions of baboon *FADS2* was amplified from pT*FADS2* with primers *FADS2*-KOZAK-Forward (5'- CCC AAG CTT ACC ATG GGG AAG GGA GGG AAC CAG GGC GA -3') including *Hin*dIII site and *FADS2*-KOZAK-Reverse (5'- CCG CTC GAG TCA TTT GTG AAG GTA GGC GTC CAG CCA -3') including *Xho*I site. The high fidelity Taq polymerase (Roche Diagnostics Ltd.) was used to minimize potential PCR errors. The amplified PCR product containing baboon *FADS2* was gel purified, restriction digested and inserted into *Hin*dIII and *Xho*I sites behind the GAL1 promoter of pYES2 vector (Invitrogen, USA) to yield the plasmid pY*FADS2*. The constructed plasmid of pY*FADS2*, was transformed into *S. cerevisiase* (strain INVSc1 from Invitrogen, USA) using S. c. Easy Comp™ Transformation Kit (Invitrogen), and the transformants were verified by DNA sequencing.

Expression of baboon *FADS2*

For functional expression characterization, transformed yeast strains with pYES2 (empty vector) as a negative control and pY*FADS2* were grown for 24h in *S. cerevisiae* minimal media without uracil. As another negative control, wild *S. cerevisiae* (INVSc1) was cultured in *S. cerevisiae* minimal medium with uracil. Expression of the transgene was induced when OD_{600} reached 0.4. At that time, appropriate fatty acids, 1mM linoleic acid (18:2n-6), alpha-linolenic acid (18:3n-3), eicosadienoic acid (20:2n-6), eicosatrienoic acid (20:3n-3) were added in the presence of 1% tergitol-Nonidet P-40 (Sigma, USA) to the cultures and were grown at 30^{o}C with constant shaking. The samples were collected after 48h for fatty acid analysis. All treatments were performed in duplicate.

Fatty acid analysis

The yeast cells were harvested by centrifugation at 4,000 rpm for 5 min. The cell pellets were washed twice with tergitol-Nonidet P-40 and finally twice with distilled water. Fatty acid methyl esters (FAME) were prepared by using modified one-step lipid extraction method of Garces and Mancha (Garces and Mancha, 1993). FAME were structurally identified by GC-CACI-MS/MS (Van Pelt and Brenna, 1999; Michaud et al., 2002; Lawrence and Brenna, 2006) and quantitatively analyzed by GC-FID. An equal weight FAME mixture was used to verify response factors on a daily basis (Diau et al., 2005). For competition experiments, GC analyses were performed in triplicate.

Materials (Chemicals)

Fatty acids (18:2n-6, 18:3n-3, 20:2n-6 and 20:3n-3) were purchased from Nu-Chek Prep, Inc, MN, USA. Uracil drop out SD-U medium and supplement contents including amino acids were obtained from Clontech, TaKaRa Bio, USA. Uracil and tergitol NP-40 was from Sigma-Aldrich, USA. pGEM-T Easy Vector II system was purchased from Promega Corporation, WI, USA. The pYES2 vector, INVSc1 strain, S. c. Easy Comp^{TM} Transformation Kit, and restriction enzymes (*Hin*dIII and *Xho*I) were obtained from Invitrogen, USA. Total RNA was isolated by using RNeasy Mini kit from Qiagen, CA, USA. cDNA synthesis kit was purchased from Bio-Rad, USA.

2.3. Results

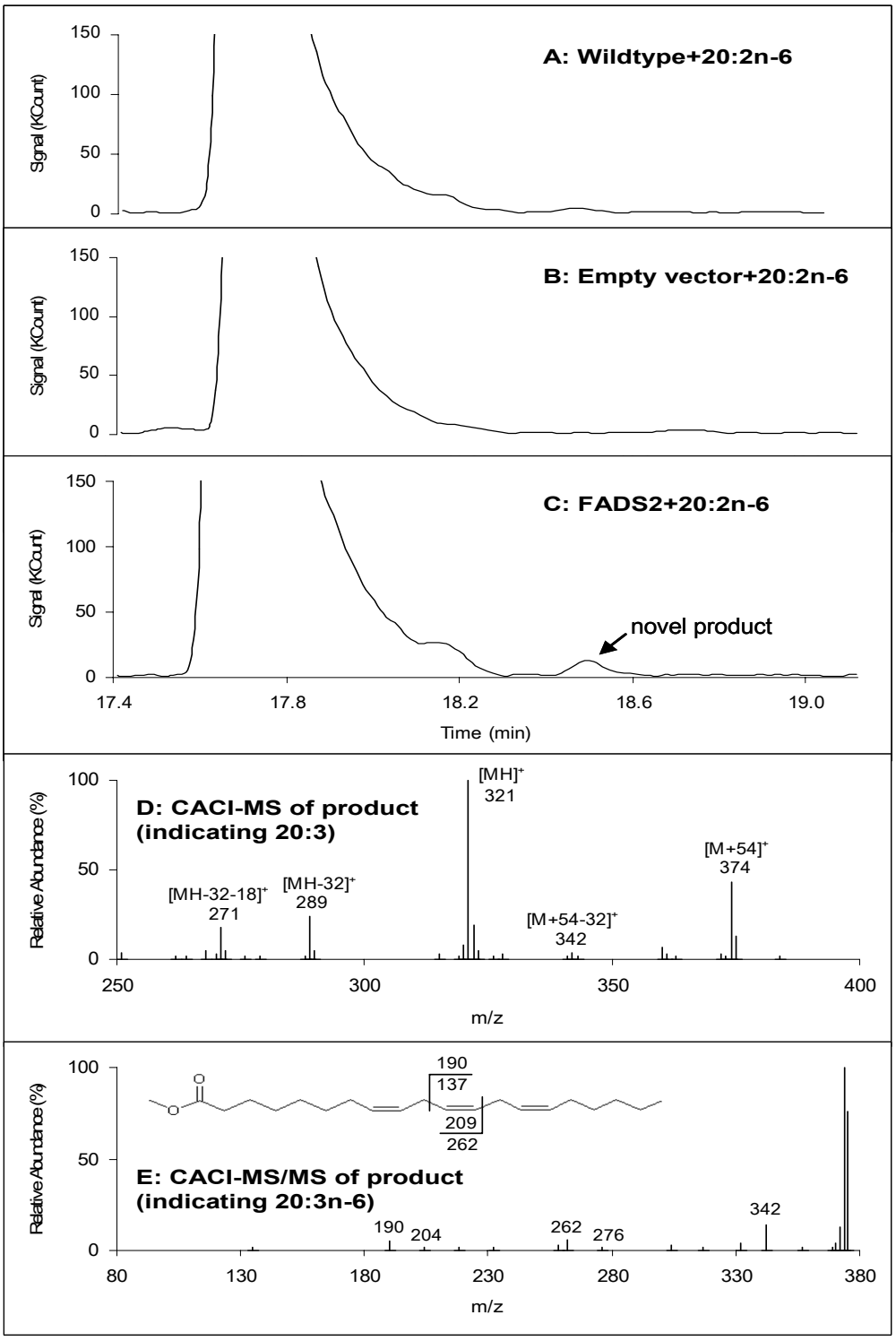
Baboon fatty acid desaturase 2 (*FADS2*):

The sequenced PCR product (Baboon *FADS2*; GenBank EU780003) revealed an open reading frame of 1,335 base pairs, encoding a protein of 444 amino acids and a stop codon. It shares a 60% homology with baboon *FADS1* (EF531577) and 62% homology with the putative baboon *FADS3* (EU780002), including "HPGG" characteristic of a cytochrome b5 domain and three conserved histidine motifs "HXXXH, HXXHH and QXXHH". Analysis and comparison of amino acid sequence of baboon *FADS2* showed 97% identity and 99% similarity with human *FADS2* (AAH09011), and 64% identity and 79% similarity with the bifunctional zebrafish desaturase (AAG25710). Baboon *FADS2* also shares homology with Δ8-desaturases from unicellular organisms

(27% identity and 43% similarity with *E. gracilis* (AAD45877), 27% identity and 40% similarity with *P. marinus* (ABF58684), and 23% identity and 38% similarity with *A. castellanii* (CAO00489)). Analysis of the baboon *FADS2* secondary structure by SOSUI software <http://bp.nuap.nagoya-u.ac.jp/sosui/> (Hirokawa et al., 1998) predicted three transmembrane regions, whereas baboon *FADS1* and the putative baboon *FADS3* had four transmembrane regions (data not shown).

The transformed yeast grown on minimal media were supplemented with various fatty acids, incubated at 30°C and harvested after 48 h. Wild type (wt) *S. cerevisiae,* and *S. cerevisiae* containing empty pYES2 vector were used as controls for every replicate. Gas chromatography-covalent adduct chemical ionization tandem mass spectrometry (GC-CACI-MS) chromatograms of fatty acid methyl ester (FAME) are presented in Figures 2.2 and 2.3 for 20:2n-6 and 20:3n-3 incubations, respectively. Panel A, B and C of both figures correspond to wt *S. cerevisiae*, *S. cerevisiae* containing empty pYES2 vector and *S. cerevisiae* containing *FADS2*, respectively. *S. cerevisiae* wt and *S. cerevisiae* with empty pYES2 vector have no activity toward 20:3n-6 and 20:4n-3, as expected. Figure 2.2, Panel C shows a new product appearing upon incubation with 20:2n-6. Panel D is the MS-1 spectrum showing peaks at *m/z* 374, 321, 289, and 271, corresponding to the $[M+54]^+$, $[MH]^+$, $[MH-32]^+$, and $[MH-32-18]^+$ ions, respectively, characteristic of a 20:3 FAME. Panel E is the collisional dissociation spectrum of $[M+54]^+$, yielding ions at *m/z* 262 and *m/z* 190 corresponding, respectively, to the α and ω diagnostic ions for 20:3n-6 and positively identifying this product. Similarly, Figure 2.3, panel D, displays MS1 ions characteristic of a 20:4 (*m/z* 372, 319, 287, 269), and

Figure 2.2. Data Showing *FADS2* Action On 20:2n-6.
(A) Reconstructed ion chromatograms (RIC) of FAME derived from 48h incubation of 20:2n-6 with wildtype *S. cerevisiae*,
(B) *S. cerevisiae* transformed with empty vector and,
(C) *S. cerevisiae* transformed with *FADS2* showing novel product.
(D) CACI-MS1 spectrum of novel product in C) showing diagnostic ions for a 20:3 FAME.


A: Wildtype+20:2n-6
B: Empty vector+20:2n-6
C: FADS2+20:2n-6
novel product
Signal (KCount)
Time (min)
D: CACI-MS of product
(indicating 20:3)
[MH]+
321
[M+54]+
374
[MH-32-18]+
271
[MH-32]+
289
[M+54-32]+
342
Relative Abundance (%)
m/z
E: CACI-MS/MS of product
(indicating 20:3n-6)
190
137
209
262
190
204
262
276
342

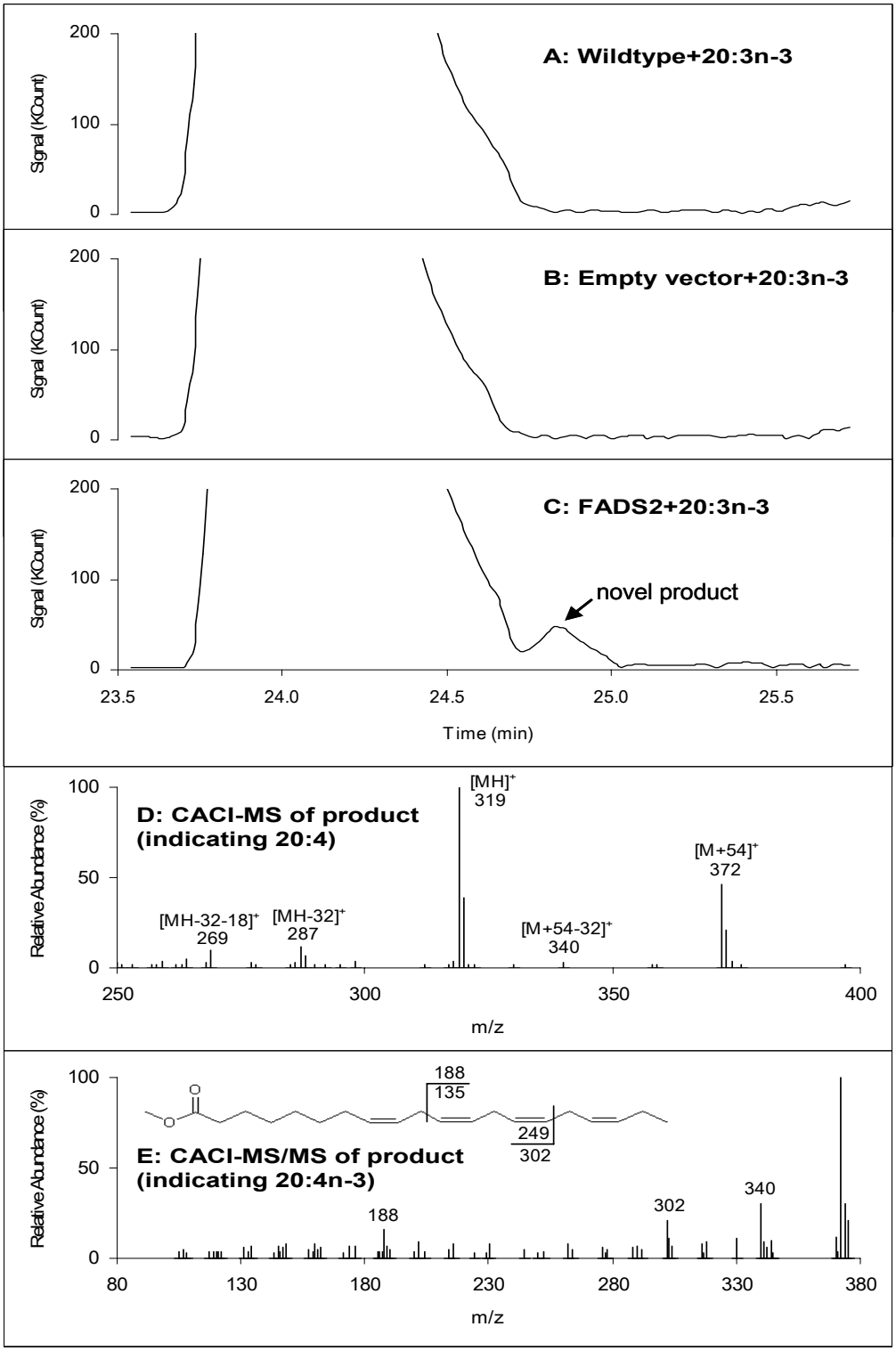


Figure 2.3 . Data Showing *FADS2* Action On 20:3n-3.
Figure Panels are analogous to those in Figure 2, and demonstrate synthesis of 20:4n-3.

collisional dissociation yields diagnostic ions *m/z* 302 and 188 in MS/MS, positively identifying 20:4n-3.

An alternative hypothesis to Δ8-desaturation of 20:2n-6 (20:2n-6 → 20:3n-6) is β-oxidation followed by entry into the normal pathway (20:2n-6 → 18:2n-6 → 18:3n-6 → 20:3n-6). Wt *S. cerevisiae* contain peroxisomes and mitochondria and thus have native β-oxidation activity, so this pathway is plausible. Figure 1.4a presents the relative product distribution with 18:2 as a substrate. No Δ6-desaturation is observed for the controls (*wt* or *vector only*), while the cells transformed with *FADS2* accumulate 18:3n-6 and about 8% of this product is further elongated to 20:3n-6 (18:2n-6 → 18:3n-6 → 20:3n-6). There is also some elongation of 18:2n-6 to 20:2n-6.

The putative intermediate of a β-oxidation-mediated alternative pathway, 18:3n-6, was detected in only one of two trials with 20:2n-6 used as a substrate. Figure 1.4b presents relative product distribution with 20:2n-6 for that trial. There is indeed some β-oxidation to 18:2n-6 in all treatments. However, Δ6-desaturation of this product to 18:3n-6 is detected in only the *FADS2* cells, and is about 7% of the 18:2n-6 product. The Δ8-desaturation product, 20:3n-6, is more than 10-fold greater in abundance than 18:3n-6, its putative intermediate in the alternative pathway. Considering the data of Figure 2.4a, the conversion of 18:3n-6 → 20:3n-6 can only account for a negligible fraction of the 20:3n-6. We conclude that Δ8-desaturation by the *FADS2* product mediates direct conversion of 20:2n-6 to 20:3n-6.

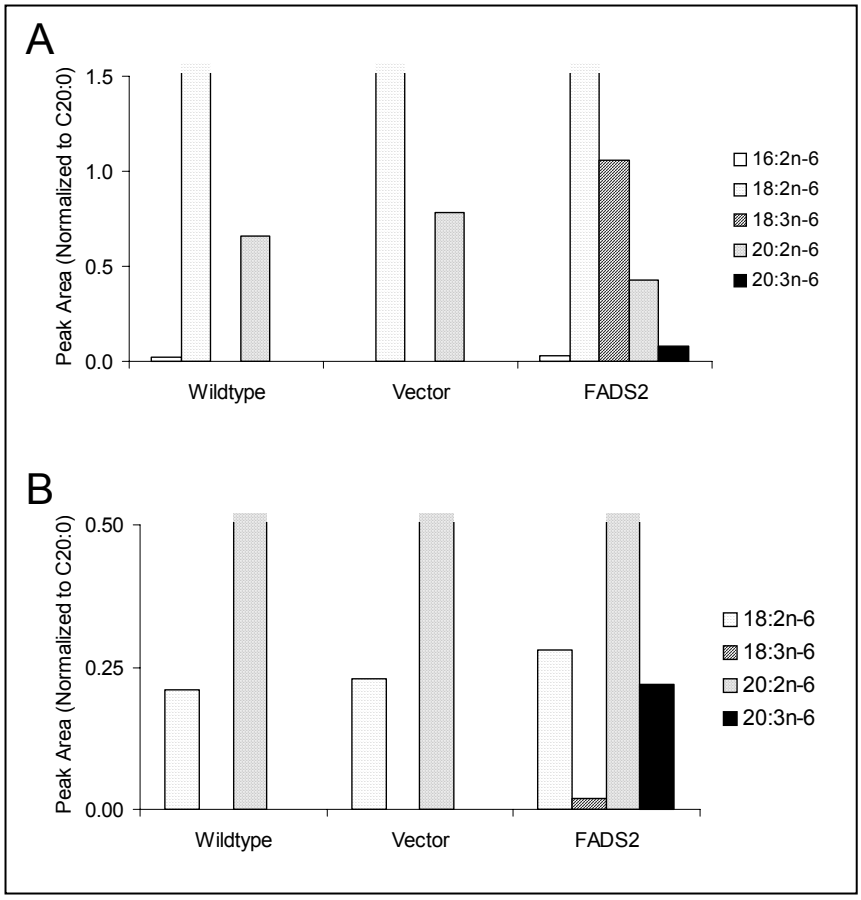


Figure 2.4. Analysis of Putative Intermediates.
(A) Peak areas of n-6 PUFA, normalized to 20:0, for wt, vector-only, and FADS2 transformed cells treated with 18:2n-6. A small amount of chain-shortening is detected (16:2n-6) in wt and FADS2 cells. The FADS2 cells uniquely show accumulation of the ∆6-desaturated product 18:3n-6, the elongated product 20:2n-6, and a small amount of 20:3n-6, the chain elongated product of 18:3n-6.
(B) All 20:2n-6 treated cells show the chain shortened product 18:2n-6, and only the FADS2 cells show a small amount of the elongated product 18:3n-6. The product of ∆8-desaturase activity on 20:2n-6, 20:3n-6, is present only in the FADS2 cells at about 10-fold higher concentration than 18:3n-6.

Once establishing that *FADS2*-transformed *S. cerevisiae* gained the ability to ∆8-desaturate 20-carbon PUFA, the relative activity was tested in competition experiments. Δ8-desaturase activity toward n-3 and n-6 fatty acids was investigated by supplementing media with a 1:1 mixture of 20:2n-6 and 20:3n-3 as substrates. Substrates and products were analyzed quantitatively by gas chromatography-flame ionization detector (GC-FID) after confirmation of structure by GC-CACI-MS and GC-CACI-MS/MS. Table 1.1 shows that 20:2n-6 → 20:3n-6 was 0.9±0.16% and 20:3n-3 → 20:4n-3 was 2.8±0.7% over a 48 h incubation, yielding a relative conversion efficiency of about 3.1 fold, favoring the n-3 PUFA.

In a second competition experiment, 1:1 mixtures of 18:2n-6+20:2n-6 or 18:3n-3+20:3n-3 were added to media to test the relative ∆6-desaturase and ∆8-desaturase activities. Table 1.2 shows that conversions 18:2n-6 → 18:3n-6 and 20:2 n-6 → 20:3n-6 were 12.2±0.16% and 1.7±0.54% respectively, yielding a relative activity of 7.2-fold favoring ∆6-desaturase. The relative conversions 18:3n-3 →18:4n-3 and 20:3n-3 → 20:4n-3 were 24.1±2.4% and 1.03±0.31% respectively, yielding the substantially greater conversion ratio of 23.

2.4. Discussion

More than 50 years ago, Thomasson showed that the n-6 PUFA were active in supporting growth of water deprived young rats raised on a diet with saturates as their only source of fat. The order of relative "vitamin F" activity was

Table 2.1. Competition between n-6 and n-3 fatty acids

Substrate Mixture	Reactions	Conversion of substrate (%) Mean ± SD	Ratio
20:2n-6+ 20:3n-3	∆8-desaturase 20:2n-6 → 20:3n-6	0.90±0.16	3.1
	∆8-desaturase 20:3n-3 → 20:4n-3	2.8±0.7	

Table 2.2. Competition between Δ6- and Δ8-desaturase activities

Substrate Mixture	Reactions	Conversion of substrate (%) Mean ± SD	Ratio
18:2n-6+ 20:2n-6	Δ6-desaturase 18:2n-6 →18:3n-6	12.2±0.16	7.2
	Δ8-desaturase 20:2n-6 → 20:3n-6	1.7±0.54	
18:3n-3+ 20:3n-3	Δ6-desaturase 18:3n-3 → 18:4n-3	24.1±2.4	23
	Δ8-desaturase 20:3n-3 → 20:4n-3	1.03±0.31	

20:4>18:2=20:3>20:2 (131:100:100:43), with relative activity of less than 10 for all other fatty acids tested, including n-3s (Thomasson, 1953). Within two decades, the pathway from 18:2 to 20:4 by sequential ∆6-desaturation-elongation-∆5-desaturation (18:2 → 18:3 → 20:3 → 20:4) emerged as the major route of biosynthesis. The alternative, elongation-∆8-desaturation-∆5-desaturation (18:2 → 20:2 → 20:3 → 20:4), has been of interest over the years because of the appearance of intermediates in mammalian tissue, as has a third alternative, elongation-∆5-desaturation-∆8-desaturation (18:2 → 20:2 → 5,11,14-20:3 → 20:4) because 20:2 is converted to 5,11,14-20:3 (sciadonic acid) by the action of ∆5-desaturase. Experiments involving only activity measurements in mammalian tissue or cells that definitively establish or rule out participation of a ∆8-desaturase are difficult to design because of the low concentration of the intermediates, indicating that molecular techniques capable of isolating a particular biochemical activity are required (Chen et al., 2000). Indeed, the existence of Δ8-desaturation as an alternative pathway to LCPUFA has been reported periodically, and ∆8-desaturase activity has been found by some and not by others (Chen et al., 2000). Presently, 20:2n-6 and 20:3n-3 are widely considered dead-end products, in part because their conversion to LCPUFA has not been unequivocally established (Stubhaug et al., 2005). However, recent studies have associated 20:2n-2 with *FADS2* polymorphisms and/or fatty acid compositions in humans (Schaeffer et al., 2006; Rzehak et al., 2008), including patients with cardiovascular disease (Malerba et al., 2008) and type 2 diabetes mellitus (Kusunoki et al., 2007). In addition, apoD knockout mice, a model for psychiatric disorders, show increased CNS 20:2n-6 and 18:2n-6 compared to wild type mice (Thomas and Yao, 2007).

Several fatty acids of chain length 16, 18, and 24 carbons are substrates for the *FADS2* gene product. Apart from 18:2n-6 and 18:3n-3, it is also known to ∆6-desaturate 24:5n-3 and 24:4n-6, as required for the coupled microsomal-peroxisomal pathway for 22:6n-3 and 22:5n-6 biosynthesis (D'Andrea et al., 2002). The *FADS2* gene product catalyzes the ∆6-desaturation of 16:0 (palmitic acid) to 16:1n-10 (*cis*-6-16:1, sapienic acid) when expressed natively in human skin sebocytes (Ge et al., 2003), and when COS-7 cells were transfected with rat *FADS2* they acquired the ability to ∆6-desaturate 16:0 (Guillou et al., 2003). There are, however, no previous reports of *FADS2* gene product activity toward 20 carbon PUFA.

The alternative synthetic pathways to arachidonic acid from 20:2n-6 are either by sequential action of a ∆8-desaturase and a ∆5-desaturase, or vice versa. Initial ∆8-desaturation yields the eicosanoid precursor 20:3n-6, whereas initial ∆5-desaturase activity yields 5,11,14-20:3. There are numerous reports showing that 11,14-20:2 is ∆5-desaturated to 5,11,14-20:3 (Takagi, 1965; Ullman and Sprecher, 1971; Sprecher and Lee, 1975; Naval et al., 1993; Chen et al., 2000). However, no clear evidence for the conversion of 5,11,14-20:3 to 20:4n-6 has been found (Ullman and Sprecher, 1971; Sprecher and Lee, 1975; Chen et al., 2000). Sprecher and coworkers have studied the desaturation of 11,14-20:2 with isotope labeling *in vitro* and *in vivo*, and consistently find that rat liver does ∆5-desaturate it to 5,11,14-20:3, but they find no evidence of Δ8-desaturation activity on this product (Ullman and Sprecher, 1971; Sprecher and Lee, 1975). Fourteen day feeding of 5,11,14-20:3 led to the accumulation of this PUFA in liver phosphoglycerides where it

decreased 20:4n-6, while feeding of 11,14-20:2 did not alter 20:4n-6 levels (Chen et al., 2000). The production of 5,11,14-20:3 and 5,11,14,17-20:4 in human leukemia K562 cells has been reported in which the Δ5-desaturase is the only active desaturase operating because of the lack of Δ6-desaturase activity in these cells (Naval et al., 1993). Consistent with this report, we recently found significant amounts of 7,11,14-20:3, 7,11,14,17-20:4, and 9,13,16,19-22:4 in the liver lipids of chow-fed *FADS2* null mice, all of which may be synthesized by action of ∆5-desaturase, coded by *FADS1*, on 18:2n-6 or 18:3n-3, followed by prompt elongation (Stroud, Lawrence, Brenna, Nakamura, 2008, unpublished observations). These data support the hypothesis that the ∆5-desaturase acts on PUFA only when its preferred substrate is not available, which may well imply that its products found in experimental studies are not relevant *in vivo* under normal conditions.

Reports of ∆8-desaturase activity in rodent and human testes have appeared (Albert and Coniglio, 1977; Albert et al., 1979), and the most recent study shows stable isotope labeling best explained by direct conversion of 11,14-20:2 to 20:4n-6 via ∆8-desaturation, albeit as a minor pathway (Chen et al., 2000), but there are no existing molecular data to implicate a specific gene responsible for coding for vertebrate ∆8-desaturase activity. Δ8-Desaturation has been shown unequivocally in unicellular organisms where the gene has been cloned and is active when expressed in *Arabidopsis thaliana* (Qi et al., 2004). A Δ8-desaturase was first reported in the single cell protist *E. gracilis* (Wallis and Browse, 1999) and later, along with a Δ9PUFA-elongase, in *Isochrysis galbana* (Qi et al., 2002) as well as the free living amoeba *A. castellanii* (Sayanova et al., 2006). The present report is the first to show that

a vertebrate gene product introduces a double bond at the Δ8 position, demonstrating an alternative pathway to LCPUFA biosynthesis.

The competition experiments provide insight as to whether ∆8-desaturase activity of the *FADS2* protein product can be important *in vivo*. The synthesis of 20:4n-3 dominates by 3.1-fold over 20:3n-6, consistent with long-established observations for ∆6-desaturase preference for 18:3n-3 over 18:2n-6 (Brenner and Peluffo, 1966; Christiansen et al., 1968; Cook et al., 1991). These observations are also are consistent with *in vitro* work showing that the biosynthesis of n-6 PUFA is strongly suppressed by <2% of calories of 18:3n-3, whereas nearly 10 times as much 18:2n-6 is required to equally suppress n-3 PUFA biosynthesis (Holman, 1998) and indicating that the affinity of the biosynthetic apparatus favors n-3 PUFA. Our competition experiments (Table 1.2) also establish that the *FADS2* gene product exhibits both ∆6-desaturase and ∆8-desaturase activities when both substrates are available. As expected, the *FADS2* gene product showed higher ∆6-desaturase activity by acting on the 18:2n-6 substrate to generate 7-fold more product than for the 20:2n-6. The relative action ∆6/∆8 activity toward the n-3 was much greater, at 23-fold, indicating that the conventional pathway would be strongly favored when both substrates are available.

In conclusion, baboon *FADS2* gene cloned into *S. cerevisiae* causes gain of Δ8-desaturase activity, in addition to coding for Δ6-desaturase activity. ∆8-desaturase activity on 20:2n-6 leads directly to 20:3n-6, the immediate precursor of PGE1 and of 20:4n-6. All available evidence indicates that Δ8-desaturation is a minor pathway, but further study may show that it becomes

important when there is high demand for eicosanoid synthesis such as in inflammation or vasodilation, particularly in situations in which specialized tissues require 20:3n-6 as a precursor to prostaglandins E1 and F1α, hydroxyeicosatrienoic acids, or thromboxane B1. This alternative pathway to the eicosanoid precursors may explain data suggesting that 20:2n-6 levels are related to human health.

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CHAPTER 3

NOVEL FATTY ACID DESATURASE 3 (*FADS3*) TRANSCRIPTS GENERATED BY ALTERNATIVE SPLICING[2]

3.1. Introduction

Long chain polyunsaturated fatty acids (LCPUFA), especially docosahexaenoic acid (DHA; 22:6n-3), eicosapentaenoic acid (EPA; 20:5n-3) and arachidonic acid (ARA; 20:4n-6) are ubiquitous in mammalian tissue and are indispensable for health, development and cognition (Carlson, 2001). They are essential constituents of phospholipid components of the biological membranes, are precursors for signaling eicosanoids and docosanoids that are major drug targets (e.g., COX-1, COX-2 inhibitors, leukotriene receptor antagonists), and are also known to regulate transcription of genes involved in lipid metabolism (Jump, 2002; Schmitz and Ecker, 2008). They can be obtained from the diet or are endogenously synthesized in the liver among many tissues, from dietary essential fatty acids (EFA) precursors, alpha-linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6) by an alternating series of position-specific desaturation and carbon chain-elongation reactions.

Fatty acid desaturases are enzymes that catalyze the introduction of *cis* double bonds at specific positions in a fatty acid chain. *FADS1, FADS2, and FADS3* are members of fatty acid desaturase gene family that arose evolutionarily from gene duplication. They share high degree of sequence

[2] Based on the reference of Gene, 2009 Oct 1;446(1):28-34.

homology, are clustered within the 100 kb region on the long arm of human chromosome 11q12-13.1, have the same exon/intron organization with 12 exons and 11 introns, and are similarly organized on mouse chromosome 19 (Nakamura and Nara, 2004). The gene products of *FADS1* and *FADS2* have well defined functions as front end PUFA desaturases required for LCPUFA biosynthesis in mammals however no function has emerged for *FADS3.*

Most known desaturase genes involved in PUFA biosynthesis contain the N-terminal cytochrome b5 domain (HPGG) as electron donor and three histidine motifs “HXXXH, HXXHH and QXXHH”, conserved from human to microalgae (Sperling et al., 2003), however, there are exceptions (Sayanova et al., 2006). *FADS1* codes for a 5-desaturase, and *FADS2* codes for a 6-desaturase with many substrates, and has recently been shown to have 8-desaturase activity (Park et al., 2009). Both genes are considered to yield a protein product(s) thought to operate on both n-3 and n-6 PUFA. The ∆6-desaturase (*FADS2*) operates on 18:3n-3 and 18:2n-6, resulting in the synthesis of 6,9,12,15-18:4 and 6,9,12-18:3 (gamma-linolenic acid), respectively. This step is rate-limiting, and is followed by elongation to 8,11,14,17-20:4 and 8,11,14-20:3 (dihomo-gamma-linolenic acid). A rapid ∆5-desaturation (*FADS1*) on these PUFA produces EPA and ARA. EPA can be further elongated and desaturated to yield DHA, which is accepted as the mammalian pathway (Voss et al., 1991). These steps are certainly localized to the endoplasmic reticulum (ER), and a beta-oxidation step is localized to the peroxisomes, but have been suggested over the years to occur in mitochondria (Infante and Huszagh, 1998), or via a ∆4-desaturase as demonstrated in *Thraustochytrium* (Qiu et al., 2001). Expression of *FADS1* and *FADS2* were detected in several human tissues

with greatest expression in liver for *FADS1*; *FADS2* showed highest expression in the brain followed by liver, lung and heart. Only a single transcript has been identified for both genes (Cho et al., 1999a; Cho et al., 1999b).

FADS3 was cloned by Marquardt et al (Marquardt et al., 2000). It spans 17.9 kb of genomic DNA, shares a high degree of homology with *FADS1* (52%) and with *FADS2* (62%), and encodes a putative protein of 445 amino acids (aa) with a molecular mass of 51.1 kDa. *FADS3* is among the six most highly expressed genes at the implantation site of fertilized mouse embryos in the interimplantation region (prior to implantation), suggesting a crucial role in the initiation of pregnancy (Ma et al., 2006). However, the function of the *FADS3* gene product remains unknown.

To date, there are no unambiguous reports of AT for any of the FADS genes. Two isoforms for *FADS3* were detected with Northern blotting but an attempt to confirm them using RT-PCR was not successful (Marquardt et al., 2000). In the course of investigation of the role of *FADS3* in LCPUFA biosynthesis, we performed RT-PCR analysis using baboon neonate cDNA and detected multiple AT generated by alternative splicing events. We report here characterization of *FADS3* AT, their expression in twelve baboon neonate tissues and in human undifferentiated and differentiated neuroblastoma cells.

3.2. Materials and Methods

Total RNA extraction and preparation of cDNA

High quality neonate baboon tissues obtained at necropsy, treated with RNAlater, and maintained at -80°C, were used to isolate RNA. Total RNA from 30 mg tissue homogenates was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA). Total RNA yield was assessed by 260 nm UV absorption. RNA quality was analyzed by 260/280 nm ratios of the samples and by agarose gel electrophoresis to verify RNA integrity. One microgram of DNase-treated total RNA was reverse-transcribed into first strand cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The resulting cDNA was used as template for PCR-reactions.

RT(Reverse Transcription)-PCR and sequence analysis of *FADS3* genes

The protein coding region of baboon *FADS3* and *FADS3* AT were generated from primers synthesized using human *FADS3* (NM_021727) and monkey *FADS3* (XR_014740) mRNA sequences. Primer pairs are presented in Supplementary Table 2.1. PCR primers were ordered from Integrated DNA Technologies (IDT, Coralville, IA) and were amplified by PCR with baboon liver, retina and spleen cDNA as templates and Ampli Taq Gold (Applied Biosystems, USA) in a 30 μl reaction volume containing 1µM of each primer, 0.25 mM dNTPs, 10x PCR buffer, and 1.5 mM $MgCl_2$ using Eppendorf gradient thermal cycler. Touch down PCR was performed for *FADS3* amplification reactions, wherein annealing temperature was set between 72°C and 69°C for 1 min for 40 cycles. In addition to the band of expected size ~1.4 kb, some discrete bands were obtained in agarose gels. The products were gel

purified, cloned in pGEM T-Easy vector (Promega, USA) and sequenced using T7 forward and SP6 reverse universal primers. DNA sequencing was performed at Cornell University Life Sciences Core Laboratories Center using the Applied Biosystems automated 3730 DNA analyzer.

Mammalian cell culture and sample preparation

SK-N-SH neuroblastoma cells (ATCC, USA) were grown at 37°C in a humidified environment with 5% CO2 using DMEM/F-12 with 10% FBS, 2mM L-alanyl-glutamine, and 15 mM HEPES for undifferentiated cells and routine passaging. Cell differentiation was carried out in serum free DMEM/F-12 with 1X N-2 supplement (Invitrogen, USA) containing 100 mg/L human transferrin, 5 mg/L recombinant full chain insulin, .0063 mg/L progesterone, 16.1 mg/L putrescine, and .0052 mg/L sodium selenite (Bottenstein, 1992; Dong and Lim, 1996). Cells were seeded in parallel and grown to 70% confluence in regular growth media with 10% FBS. One flask of undifferentiated cells was then harvested for RNA extraction, while the remaining cells were switched to chemically defined N-2 supplemented media for an additional six days in order to halt growth and induce differentiation before harvesting. RNA was isolated using the QIAshredder and RNeasy Mini kit (Qiagen, Valencia, CA).

Expression of *FADS3AT in* baboon tissues and human cells

To analyze the expression levels of each AT, primer pairs specific for each transcript bridging the deleted parts of the exons were designed (Supplementary Table 2.2). Tissue distribution of each transcript was measured using 12 normal tissues from a baboon neonate (12 weeks old) and

undifferentiated (embryonic stage) and differentiated SK-N-SH neuroblastoma cells by RT-PCR. For *FADS3* AT PCR amplification reactions, annealing temperature was performed between 60-65°C for 30-40 cycles. The PCR fragments generated from AT were cloned and sequenced. Amplicon size of each AT is presented in Supplementary Table 2.2.

3.3. Results and Discussion

Sequence analysis of baboon *FADS3*

The sequencing of the expected ~1.4 kb PCR product (Baboon *FADS3*) revealed the presence of an open reading frame of 1,338 base pairs, encoding a putative protein of 445 aa and a stop codon. It shares a high degree of homology with other FADS genes (51% identity with baboon *FADS1* (EF531577) and 62% identity with baboon *FADS2* (EU780003)). Like other front-end desaturases, baboon FADS3 protein also has "HPGG" (at aa positions 55-58) characteristic of Cytochrome b5 and three conserved histidine motifs "HXXXH, HXXHH and QXXHH". The human and baboon *FADS3* mRNA sequences align at a level of 97% identity along the entire coding region. Analysis and comparison of aa sequence of baboon FADS3 showed 99% identity and 99% similarity with human FADS3 (NP_068373), and 54% identity and 70% similarity with bifunctional zebrafish desaturase (AAG25710).

Sequence analysis of baboon *FADS3AT*

When PCR products were separated on a 2% agarose gel, discrete bands of various sizes were detected in addition to the cDNA of the expected ~1.4 kb

size. All of the PCR products were gel purified, cloned and sequenced. Seven AT for baboon *FADS3* were identified in addition to the classical transcript. The generated splice variants correspond to the absence of a portion of exon, complete absence of one or several exons, and retention of an intron. The *FADS3* AT sequences are deposited at GenBank and were assigned the following GenBank Accession numbers EU780002, EU780004, FJ641198, FJ641199, FJ641200, FJ641201, FJ641202, and FJ641203.

Using sequence analysis data with aid from ORF Finder, we identified the putative coding regions for eight AT for *FADS3* with 1.34 kb (classical splicing), 1.14 (AT1), 0.77 (AT2), 1.25 (AT3), 0.51 (AT4), 0.74 (AT6), and 1.11 (AT7). Figure 3.1 presents the structure of these AT. In addition we identified an AT of 0.51 kb (AT5) length that has a termination codon within intron 8-9. Predicted protein sequences of the *FADS3* AT indicate that three variants, AT1, AT3 and AT7, would produce shorter versions of the protein resulting from missing exon(s), whereas AT2, AT4, AT5 and AT6 would produce truncated proteins. AT1 lacks exon 3 due to an in-frame loss of 66 aa and AT3 lacks a portion of exon 3 resulting from an in-frame loss of 28 aa. Both AT1 and AT3 retain all the conserved motifs characteristic of front end desaturases ("HPGG" Cytochrome b5 motif and three histidine repeats "HDLGH, HFQHH, QIEHH). The absence of a portion of exon 8 together with exons 9-10 (AT7) results in an in-frame loss of 76 a.a and also results in the loss of the last histidine repeat "QIEHH".

In AT2, the skip of exon 6 causes a reading frameshift that results in the loss of the last histidine repeat "QIEHH", and generates a truncated protein of 257

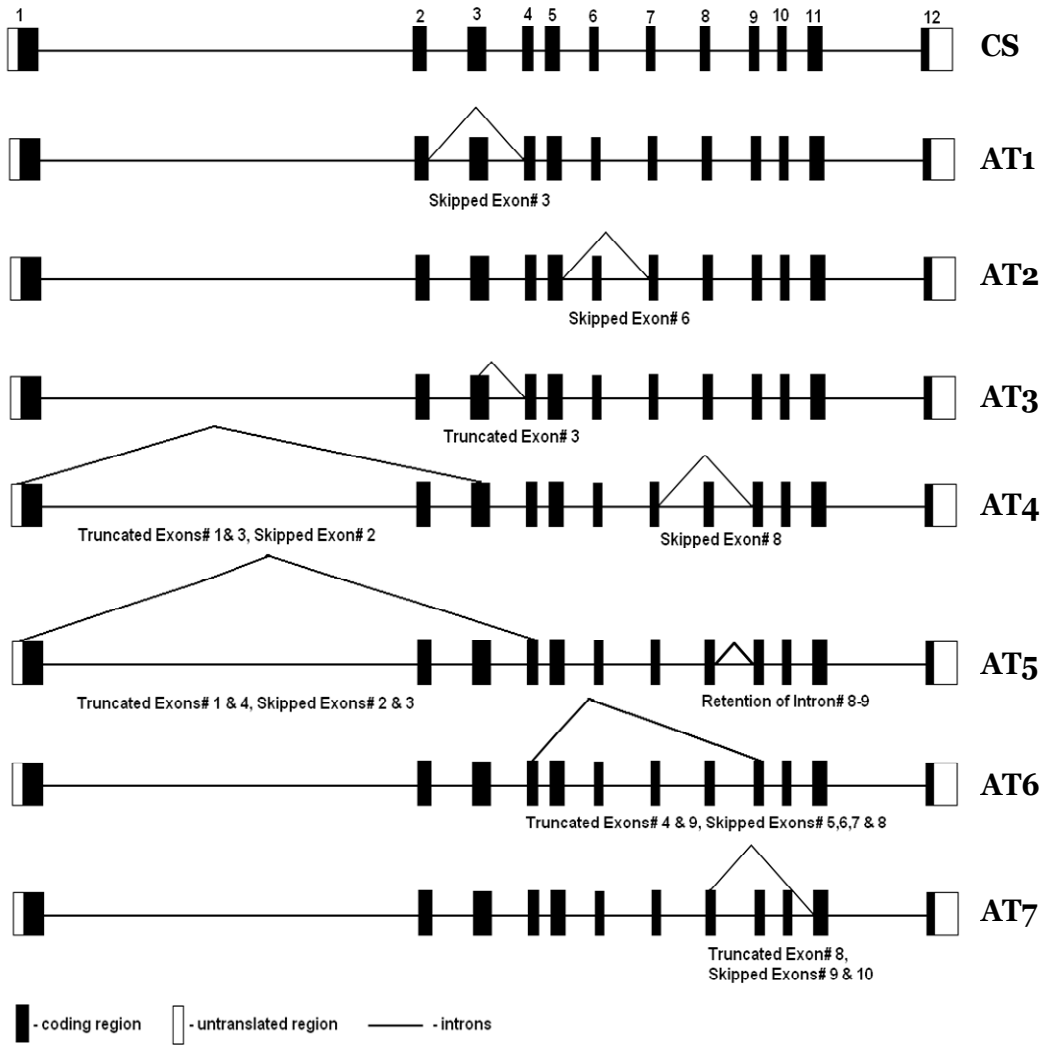


Figure 3.1. Baboon *FADS3* and *FADS3* AT. Missing spans within AT are shown. Numbers 1 to 12 are exons; CS, classical splicing; AT1-AT7 denote alternative transcripts.

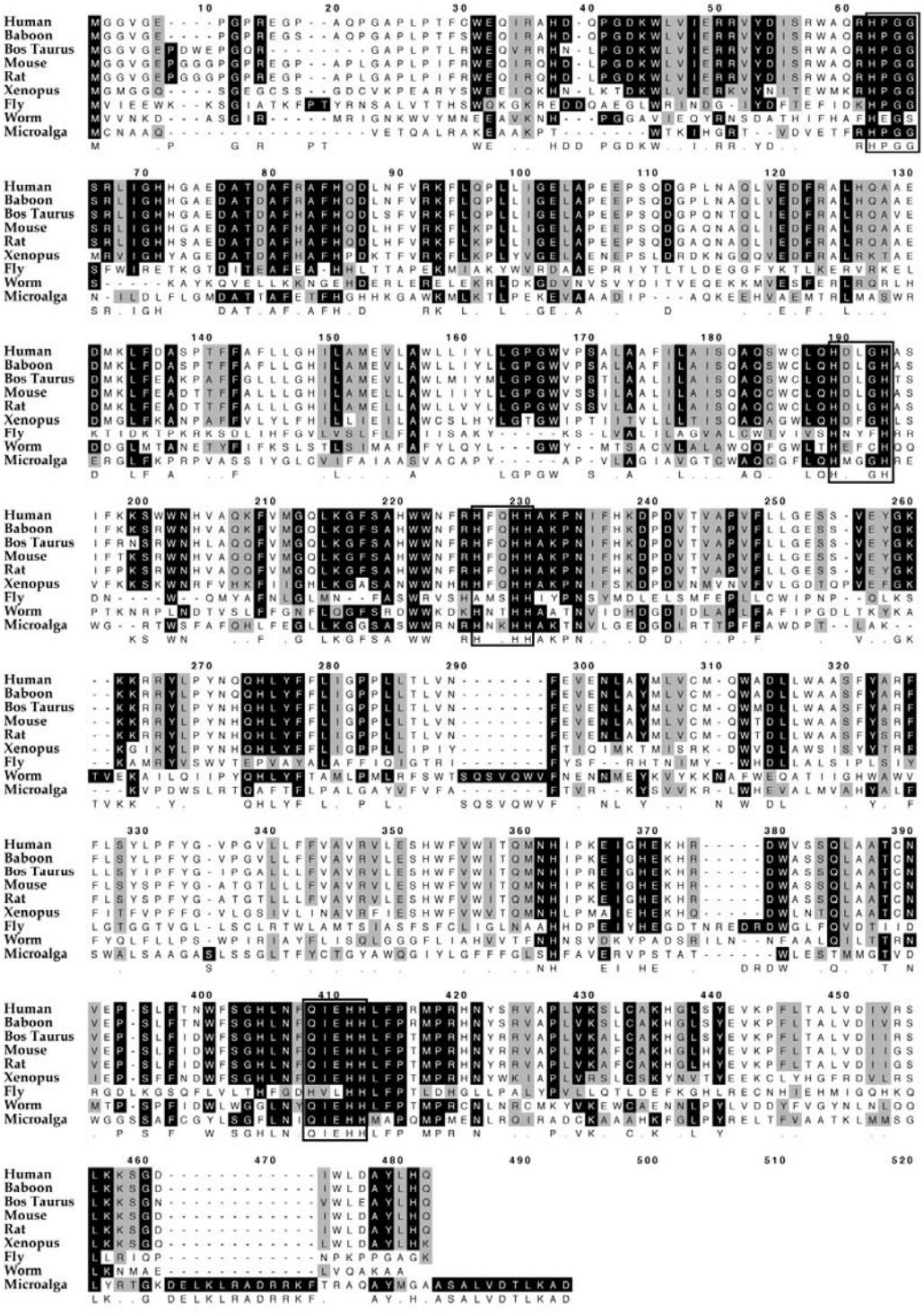
aa. AT4, AT5, and AT6 result from multiple exon deletions. AT4 lost HPGG and QIEHH, AT5 lost HPGG, HDLGH, and QIEHH, whereas, AT6 retains only HPGG while all the histidine repeats are lost from splicing events. Hastings et al. reported that substrate specificity can vary depending upon the differences in the sequence of a desaturase even though the catalytic activities are identical (Hastings et al., 2004). This raises the possibility that *FADS3* AT might have different substrate specificities even though some of them share all the conserved catalytic domains.

FADS3 conservation and putative subcellular localization of baboon *FADS* genes

Figure 3.2 shows the alignment of *FADS3* and its putative homologs from various species. It can be seen that the "HPGG" characteristic of Cytochrome *b*5 and three conserved histidine motifs "HXXXH, HXXHH and QXXHH" are well conserved from human to microalgae.

We used the Proteome analyst computational tool to query the subcellular localization of the FADS genes (Lu et al., 2004). Amino acid sequence information is entered and the software provides an estimate of the probability that the protein is localized to a specific organelle. FADS1, FADS2, and FADS3 were all found to be localized to ER with a probability score of 1.000; FADS1 and FADS2 are known to code for ER proteins. Similarly an equivalent test for the mitochondria shows that FADS1 and FADS2 had probability scores of 0.529 and 0.562, respectively, whereas FADS3 was found to have a probability score of 0.999 for mitochondrial localization. This result suggests that FADS3 contains domains that are more commonly found in the

Figure 3.2. Alignment of amino acid sequences of FADS3. Using MacVector software and ClustalW alignment, FADS3 aa sequences were aligned from various species. Well conserved motifs common for desaturases are depicted in boxes. The “HPGG” characteristic of a cytochrome b5 domain and three conserved histidine motifs “HXXXH, HXXHH and QXXHH” are shown in boxes.


Human
Baboon
Bos Taurus
Mouse
Rat
Xenopus
Fly
Worm
Microalga

mitochondria compared to FADS1 and FADS2, and may point to a mitochondrial role for one or more proteins for which the FADS3 codes.

Expression of *FADS3AT* in baboon tissues and human cells

Although *FADS3* was cloned in 2000 (Marquardt et al., 2000), and was shown to be expressed in human heart, liver, lung, uterus and brain tissues, nothing is known about its function in LCPUFA biosynthesis. We determined the tissue distribution and expression patterns of *FADS3* AT in a baboon neonate and also determined the regulation patterns in human NB cells. To study the tissue distribution of *FADS3* AT we performed semiquantitative RT-PCR using primer pairs bridging the deleted parts of the exons specific for each transcript, as shown in Supplementary Table 3.2. Figure 3.3 shows that all the variants are expressed in the 12 baboon neonate tissues studied. The AT2 variant was present in reproducibly smaller amounts in all 12 tissues. The primer pair designed to amplify AT3 also revealed the presence of a shorter amplicon (AT8?). AT8 did not initially appear in our cloning screens, but is also expressed in all tissues. The sequencing of the shorter amplicon (~50 base pairs) showed deletion within exon 4. Notably, the AT3 primer pair amplifies only AT8 in the occipital lobe region of the brain, whereas the remaining 11 tissues express both AT3 and AT8 (Figure 3.3). However, AT3 is highly expressed in the thymus, compared to other tissues. The highest levels of AT8 were observed in occipital cortex lobe, pancreas, skeletal muscle and heart,

with much lower levels in thymus. Our RT-PCR analysis using primers specifically designed to amplify AT5 also confirms that intron 8-9 remains in the transcript, and it is strongly expressed in immune tissues (thymus and

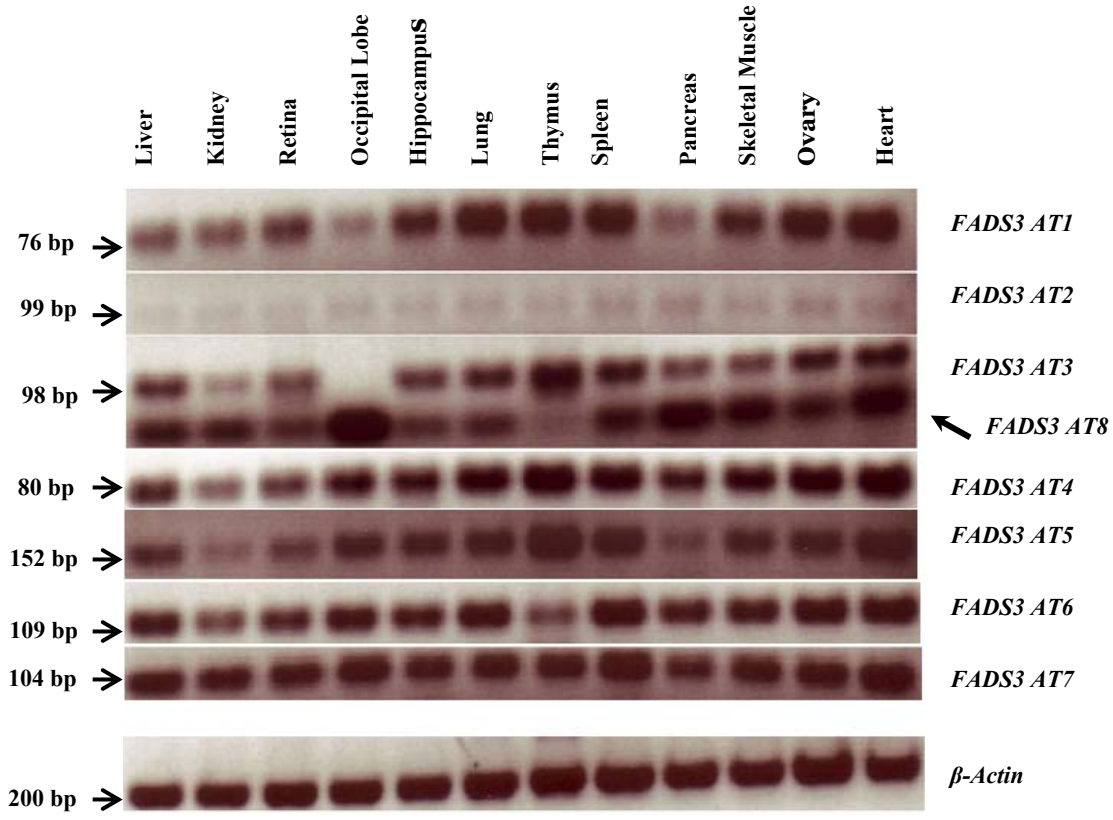


Figure 3.3. *FADS3* AT expression in 12 Baboon Neonate Tissues. RT-PCR analysis of *FADS3* AT shows expression in 12 baboon neonate tissues. The amplified products were resolved on a 2% agarose gel, visualized with ethidium bromide and a negative image is shown. Legend: bp, base pair.

spleen) and the heart. These data support the hypothesis that FADS3 AT encode functionally important proteins.

Expression of *FADS3* AT in response to cell differentiation was also investigated using human SK-N-SH neuroblastoma (NB) cells. Figure 4 shows that all the variants are found to be expressed in these cells. However, they showed reciprocal increases and decreases in expression pattern in response to cell differentiation (Figure 3.4). One of the most striking observations is the significant down-regulation of AT5 expression in differentiated and significant up-regulation in undifferentiated NB cells. Different patterns of expression of AT show that these transcripts are under specific control during the NB cell differentiation. Our data demonstrate that the *FADS3* AS events widely detected in baboon tissues are also conserved in human cells. A recent estimate shows splicing level differences of 6-8% between orthologous human and primate (chimpanzee) transcripts and 4% in genes having one or more cassette alternative exons, compared to 80% differences between human and mouse genes (Calarco et al., 2007).

FADS-coded PUFA desaturases and putative isoforms

AS, first proposed by Gilbert (Gilbert, 1978), is rapidly gaining importance as an essential molecular mechanism whereby a single gene transcript can give rise to a number of spliced mRNAs to generate remarkable protein diversity.

Blencowe suggested that AS may be the most extensively used posttranscriptional modification of genes (Blencowe, 2006). Modrek et al.

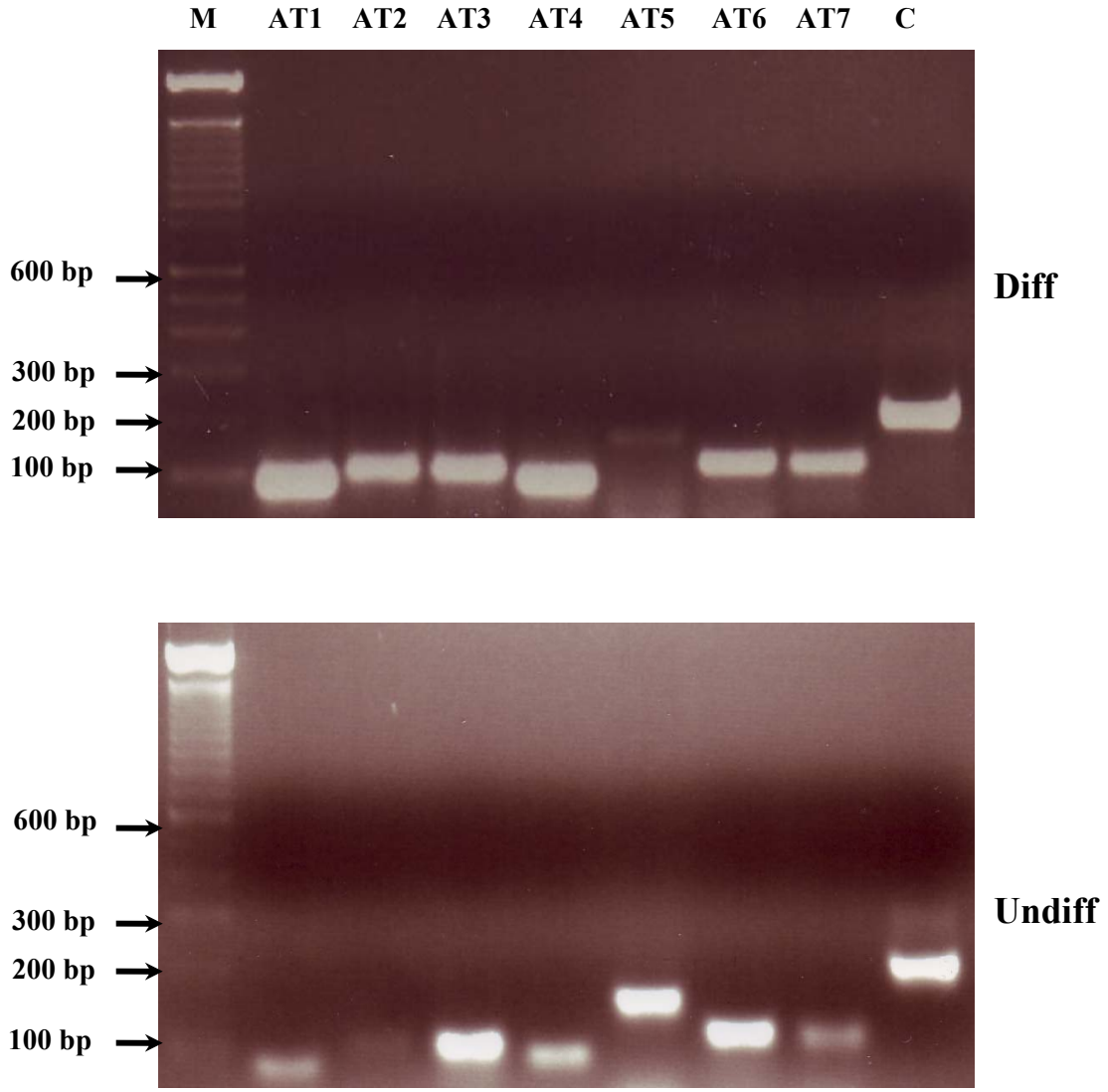


Figure 3.4. *FADS3* AT expression in human SK-N-SH Neuroblastoma (NB) Cells. RT-PCR analysis of *FADS3* AT shows expression in human SK-N-SH neuroblastoma (NB) cells. M represents a 100 base pair molecular size marker; AT1-AT7 represent alternatively spliced variants; C-Beta-actin Control; Diff, differentiated cells, Undiff, undifferentiated cells.

performed genome-wide analysis to test AS events using a human EST database analysis and found that the majority of splicing events (74%) occur in coding regions, followed by 5'-UTR (22%) and 3'-UTR (4%) (Modrek et al., 2001).

Modrek et al. (Modrek et al., 2001) assessed the systemic functions affected by AS events in human genes and found that many were specific to immune and nervous systems. LCPUFA are highly concentrated in membranes of the nervous system and serve as substrates for signaling molecules. Diets rich in n-6 PUFA induce various physiological and metabolic changes leading towards proinflammatory status with production of series 2 prostaglandins and series 4 leukotrienes, whereas n-3 PUFA have opposite effects (Benatti et al., 2004). The well known, pioneering 1963 work of Mohrhauser and Holman showed that rat liver PUFA composition is related to the ratio of 18:3n-3 and 18:2n-6 in the diet (Mohrhauer and Holman, 1963a; Mohrhauer and Holman, 1963b). Vertebrate desaturase and elongation enzymes operate on both the n-3 and n-6 PUFA families. However, alternative hypotheses involving n-3 or n-6 specific desaturases have been proposed from time to time which suggest that the competition may, at least in part, be "apparent". A compelling example is the reciprocal changes in concentration of 22:6n-3 and 22:5n-6 that are found associated with specific metabolic conditions, for instance in tissues of Zellweger's syndrome (Martinez, 1992) or adrenoleukodystrophy (ALD) patients(Martinez, 1990), acyl CoA knockout (Acox ko) mice (Infante et al., 2002), and in vitamin A deficient mice (Zhou et al., 2004). In Zellweger's, ALD, and Acox ko, 22:6n-3 drops while 22:5n-6 rises; however, in vitamin A deficient mice, the opposite is found: 22:6n-3 rises and 22:5n-6 drops. The

latter paper (Zhou et al., 2004) specifically suggests, as we have (Infante et al., 2002), that common enzyme pathways for 22:6n-3 and 22:5n-6 cannot explain these results. Plausible hypotheses are that isoforms based on FADS AT exist that operate with high selectivity on specific PUFA and are differently regulated, and/or that the unknown function(s) of *FADS3* are PUFA-specific. A vertebrate 5-desaturase with all the characteristics of microsomal desaturases, including 63% identity with the human 5-desaturase (*FADS1*), and with significant activity only towards n-3 PUFA (20:4n-3) has been reported in Atlantic salmon (Hastings et al., 2004). An early study of PUFA synthesis in mammalian cells concluded that separate enzymes may exist for the n-3 and n-6 PUFA (Maeda et al., 1978). A mitochondrial pathway involving separate PUFA desaturases for n-6 and n-3 fatty acids was postulated to explain observed features of LCPUFA biosynthesis (Infante and Huszagh, 1998).

Many isoforms of stearoyl CoA desaturase (SCD) are known in many species, expressed in a tissue specific manner (Dridi et al., 2007), but thusfar no AT of any of the PUFA-specific desaturases are known, other than the ones that we present in this study. The data of figures 3 and 4 show that AT of PUFA-specific desaturases exist in primates, and support the hypothesis that AT have different roles because of their differential expression. Molecular regulation of PUFA desaturases produced by classical splicing of *FADS1* and *FADS2*, respectively, are well studied, but nothing is known about *FADS3* regulation. Both *FADS1* and *FADS2* are regulated by macro and micronutrients, hormones and multiple transcription factors (Brenner, 1981; Zolfaghari and Ross, 2003; Nakamura and Nara, 2004). Identifying the substrate specificities of these novel *FADS3* transcripts and functions

associated with them will help in better understanding the cellular processes mediated by PUFA and their metabolites.

3.4. Conclusion

This is the first finding of AT of *FADS* genes. We present *in vivo* and *in vitro* evidence that at least 9 novel *FADS3* AT, including AT8, are generated by splicing events. The *FADS3* AT are expressed in many tissues and showed changes in abundance in response to human neuronal cell differentiation. *FADS3* AT function is as yet unknown but their structure, and high sequence homology to functional FADS1 and FADS2, strongly implies that they are involved in LCPUFA biosynthesis. LCPUFA are ubiquitous components of cells and tissues that can be, but are not always, obtained in the diet. Intense interest in LCPUFA metabolism is related to the wide range of human intakes compared to requirements for optimal health. The most recent frontiers include diseases of aging (e.g., Alzheimer's disease) and psychiatric/affective disorders. Determining the function of *FADS3* AT, conserved among species, provides a putative mechanism for understanding of LCPUFA biosynthetic regulation, and how it differs among individuals.

APPENDIX

Supplementary Table 3.1.
Primer pairs used to generate protein coding regions of Baboon FADS3 and *FADS3* alternative transcripts

FADS3 Primers	Forward Primer	Reverse Primer	Amplify Transcripts
Primer Pair1	ATGGGCGGCGTCGGGGAGCCCGGT	TGGTTGCTGGTGCCCTGAG	CS, AT1, AT2, AT3, AT6 and AT7
Primer Pair2	TGCTCGGACCTCGGCCACCGCCT	TGGTTGCTGGTGCCCTGAG	AT4
Primer Pair3	TCTTGCTCGGACCTCGGCCACCGCCT	TGGTTGCTGGTGCCCTGAG	AT5

Primer pair 1: Forward primer was designed using monkey GenBank Accession (XR_014740) and reverse primer was derived from Human *FADS3* published by Marquardt et al. 2000 (Genomics 66, 175-83).

Primer pair 2: Forward primer was designed using human *FADS3* GenBank Accession (NM_021727) targetting 5' UTR region and reverse primer was derived from Human *FADS3* published by Marquardt et al. 2000 (Genomics 66, 175-83).

Primer pair 3: Forward primer was designed using human *FADS3* GenBank Accession (NM_021727) targetting 5' UTR region and reverse primer was derived from Human *FADS3* published by Marquardt et al. 2000 (Genomics 66, 175-83).

CS-Classical transcript

AT1-AT7-Alternative transcripts

Supplementary Table 3.2.
Primer pair's specific for each alternative transcript (bridging the deleted parts of the exons) of *FADS3*

FADS3 AT Primers	Forward Primer	Reverse Primer	Amplicon Size (Base Pairs)
AT1	TGAATGCTCAGTCCTGGTGTCTGCA	ACATGGTTCCACCGGGACTTCTTGA	76
AT2	ACCACGCCAAGCCCAACATCTT	AGCAGCGGCGGGCCGACTCGA	99
AT3	ATCCTGGCCATGGAGGCTCAGTCCTG	AACTTCTGGGCCACATGGTTCCACC	98
AT4	TGCAGTGGGCGGGTCCTGGAGAGCCA	TCATGGCCGATCTCCTTGGGGATGT	80
AT5	TCTTTGTTGCTGTCAGGTATGGCAGG	TCACATGATTCCGCCAGCAAGCCA	152
AT6	CTTCATCCTGGGCCAGCTCTCAGCT	AAGAGGTGGTGCTCGATCTGGAAGT	109
AT7	TTCTTATCCTACCTCTTCCCCAGGAT	TTCACTTCGTAGCTGAGGCCGTGCTT	104
Beta-Actin	ATTGCCGACAGGATGCAGAA	AAGCATTTGCGGTGGACGAT	200

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CHAPTER 4

ALTERNATIVE SPLICING GENERATES A NOVEL *FADS2* ALTERNATIVE TRANSCRIPT IN BABOONS[3]

4.1. Introduction

Fatty acid desaturases are enzymes that catalyze the introduction of *cis* double bonds at specific positions in a fatty acid chain. *FADS1* and *FADS2* are genes coding key desaturase enzymes for the biosynthesis of long chain polyunsaturated fatty acids (LCPUFA), and are referred as 'front end' desaturases (Marquardt et al., 2000; Nakamura and Nara, 2004). Fatty acid desaturase 2 (*FADS2*) codes for a protein that catalyzes the first and rate limiting step in the biosynthesis of LCPUFA from 18 carbon PUFA. In mammals, the *FADS2* gene product catalyzes 6-desaturation in at least five substrates, 18:2n-6, 18:3n-3, 24:5n-3, 24:4n-6, and 16:0 (D'Andrea et al., 2002; Guillou et al., 2004). Recently, we demonstrated that the primate *FADS2* gene product also catalyzes 8-desaturation, using substrates 20:2n-6 and 20:3n-3, which were previously thought to be dead end products (Park et al., 2009a).

We recently reported the first finding of alternate transcripts (AT) of the *FADS2* and *FADS1* paralog *FADS3* elsewhere (Park et al., 2009b). In this study, we

[3] Based on the reference of Mol Biol Rep. DOI: 10.1007/s11033-009-9750-9

report a transcript variant of *FADS2* in fetal baboon brain generated by alternative splicing, as well as evidence that it is widely expressed in baboon neonate tissues and in human SK-N-SH neuroblastoma (NB) cells.

4.2. Materials and methods

Fetal baboon brain tissue was obtained from banked tissue at the Southwest Foundation for Biomedical Research (San Antonio, Tx). Neonate baboon tissues originated from a 12 week old baboon fed an infant formula with no LCPUFA from a previous study (Hsieh et al., 2007). All tissues were maintained at -80°C since necropsy until RNA isolation.

RNA isolation and cDNA synthesis:

Total RNA was extracted from baboon tissue homogenates and SK-N-SH neuroblastoma (NB) cells using the RNeasy Mini kit (Qiagen, Valencia, CA). The yield of total RNA was assessed by 260 nm UV absorption. The quality of RNA was analyzed by 260/280 nm ratios of the samples and by agarose gel electrophoresis to verify RNA integrity. One microgram total RNA was reverse transcribed into first-strand cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The resulting cDNA was stored at -20°C until use.

PCR conditions and identification of alternative transcript:

We have earlier (Park et al., 2009a) reported the protein coding region of baboon *FADS2* (GenBank accession number EU780003) using primers *FADS2* forward: ATGGGGAAGGGAGGGAACCAGGGCGA and *FADS2*

reverse: TCATTTGTGAAGGTAGGCGTCCAGCCA. The above primers were used to perform polymerase chain reaction (30 µl) with baboon fetal brain tissue cDNA as template, 1 µM of each primer, 0.25 mM each of dNTPs, 1.5 mM $MgCl_2$, 5X PCR buffer, and high-fidelity Taq polymerase (Roche Diagnostics Ltd) in an Eppendorf gradient thermal cycler. Cycling conditions were: initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 72°C for 45 s and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. Two prominent bands were obtained when separated by electrophoresis on a 2% agarose gel. The two bands were gel purified, cloned into pGEM T-Easy vector (Promega, USA) and sequenced using T7 forward and SP6 reverse universal primers at the Cornell University Life Sciences Core Laboratories.

Mammalian cell culture and sample preparation:

SK-N-SH neuroblastoma (NB) cells (ATCC, USA) were grown in a humidified environment at 37°C with 5% CO_2 using DMEM/F-12 with 10% FBS, 2mM L-alanyl-glutamine, and 15 mM HEPES for undifferentiated cells and routine passaging. NB cell differentiation was carried out in serum free DMEM/F-12 with 1X N-2 supplement (Invitrogen, USA) containing 100 mg/L human transferrin, 5 mg/L recombinant full chain insulin, 0.0063 mg/L progesterone, 16.1 mg/L putrescine, and .0052 mg/L sodium selenite (Bottenstein, 1992; Dong and Lim, 1996). Cells were seeded in parallel and grown to 70% confluence in regular growth media with 10% FBS. One flask of undifferentiated cells was then harvested for RNA extraction, while the remaining cells were switched to the chemically defined N-2 supplemented media for an additional six days in order to halt growth and induce

differentiation before harvesting. RNA was extracted using the QIAshredder and RNeasy Mini kit (Qiagen, Valencia, CA).

Expression of *FADS2 and AT1 in* baboon tissues and human cells:

To analyze the expression of *FADS2 CS*, a forward primer was designed within the exonic region that is deleted in the alternative transcript. To amplify *FADS2 AT1*, the forward primer bridged the deleted parts of exons.

FADS2 CS Primer sequences:
Forward: AGGCCCAAGCTGGATGGCTGCAA
Reverse: AGTTGGCAGAGGCACCCTTTAAG

FADS2 AT1 Primer sequences:
Forward: AGAAGCATAACCTGTCTGTCTACA
Reverse: ATGATTCCACCAGTTGGCAGAG

The tissue distribution of each transcript was measured by RT-PCR using cDNA from 12 normal tissues from a baboon neonate (12 weeks old), and in undifferentiated (embryonic stage) and differentiated SK-N-SH neuroblastoma cells. PCR amplification reactions were performed using 1 μM of each primer, 0.25 mM each of dNTPs, 1.5 mM $MgCl_2$ and AmpliTaq Gold (ABI, Foster City, CA) in a final volume of 30 μl. Cycling conditions were: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 67°C for 45 s and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products were separated on 2% agarose

gels, and the PureLink™ Gel Extraction Kit (Invitrogen) was used to isolate the PCR bands. The amplicons were sequenced to confirm identity.

4.3. Results and Discussion

Previously, we cloned and sequenced the entire protein coding region of baboon *FADS2*, GenBank accession number EU780003, using baboon neonate liver cDNA. However, in addition to the expected size PCR band we noticed another prominent amplified product using baboon fetal brain cDNA (Figure 4.1). Product sequencing resulted in the identification of an 873 bp *FADS2* alternative transcript (*AT1*) with a C/T substitution at nucleotide position 327 (GenBank Accession# FJ901343). The splice variant has truncated exons 1 and 4 and skipped exons 2 and 3 (Figure 4.2). The putative coding region 0.9 kb for *AT1* resulting from an in-frame loss of 154 aa was identified using ORF finder <http://www.ncbi.nlm.nih.gov/projects/gorf/>. Two of the conserved motifs characteristic of front end desaturases ("HPGG" Cytochrome *b*5 motif and the first histidine repeat "HDYGH") are lost during the splicing event.

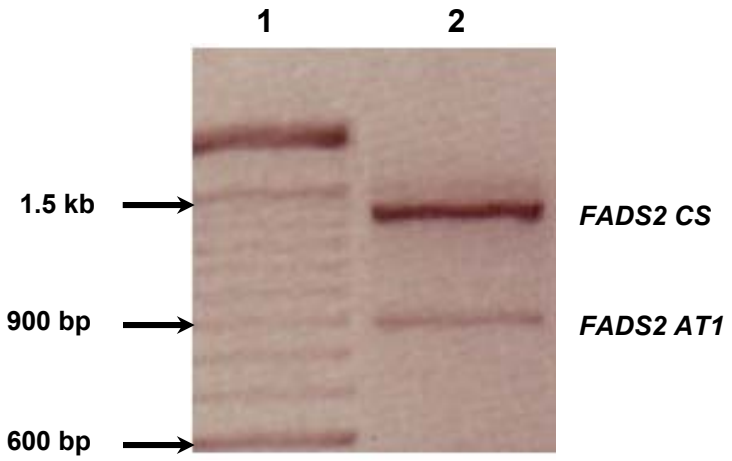

Figure 4.1. *FADS2 CS*- Classical splicing, *FADS2 AT1* is alternative splicing. The products were separated on 2% agarose gel, visualized with ethidium bromide and a negative image of photograph is shown. Lane 1: 100 base pair molecular weight marker and Lane 2: PCR products amplified by RT-PCR

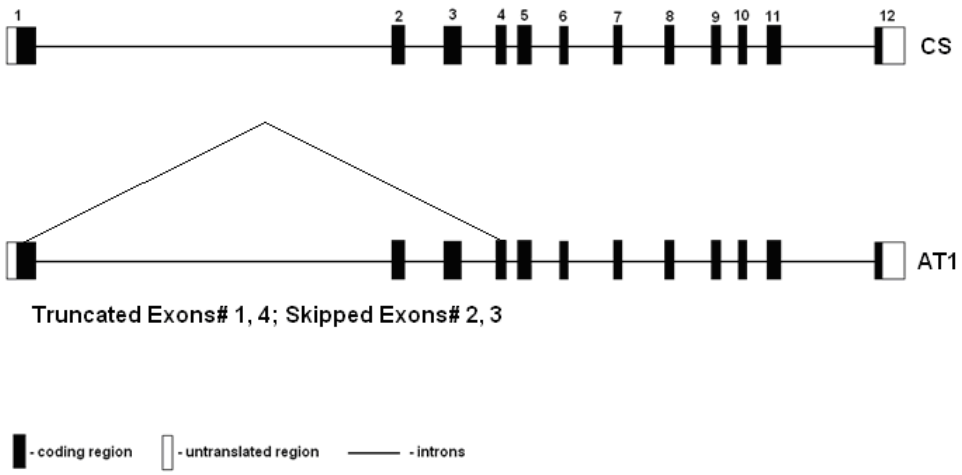

Figure 4.2. *FADS2 CS*- Classical splicing, *FADS2 AT1* is the novel alternative transcript. Missing spans within AT1 are shown. Numbers 1 to 12 are exons, CS- Classical Splicing, AT1- alternative transcript

Expression profiling:

To investigate if *AT1* expression is limited to the fetus, we studied the expression of *FADS2* classically spliced (*FADS2 CS*) and *AT1* in 12 neonate baboon tissues and in human SK-N-SH neuroblastoma (NB) cells (figure 4.3 and figure 4.4). *FADS2 CS* expression was greater than *AT1* in all tissues examined, and displayed relatively little variability, with noticeably lower expression only in kidney. *AT1* expression was highly variable, showing greater expression in liver, retina, occipital lobe, hippocampus, spleen, and ovary, than in other tissues. *FADS2 CS* and *AT1* were also expressed in undifferentiated and differentiated NB cells with no obvious difference in expression levels.

Putative subcellular localization:

FADS2 CS and FADS2 AT1 putative subcellular localization prediction was achieved using the web-based tool, Proteome Analyst Specialized Subcellular Localization Server *(PA-SUB)* (Lu et al., 2004). The software takes amino acid sequence information in FASTA format and provides an estimate of the probability that the protein location within a cell. Both FADS2 CS and FADS2 AT1 were found to be localized to endoplasmic reticulum (ER) with a probability score of 1.000 and to be localized to mitochondria with probability scores of 0.562 and 0.999, respectively. FADS2 CS is known to code for ER proteins. However, the above results suggest a mitochondrial role, as well as an ER role, for FADS2 AT1.

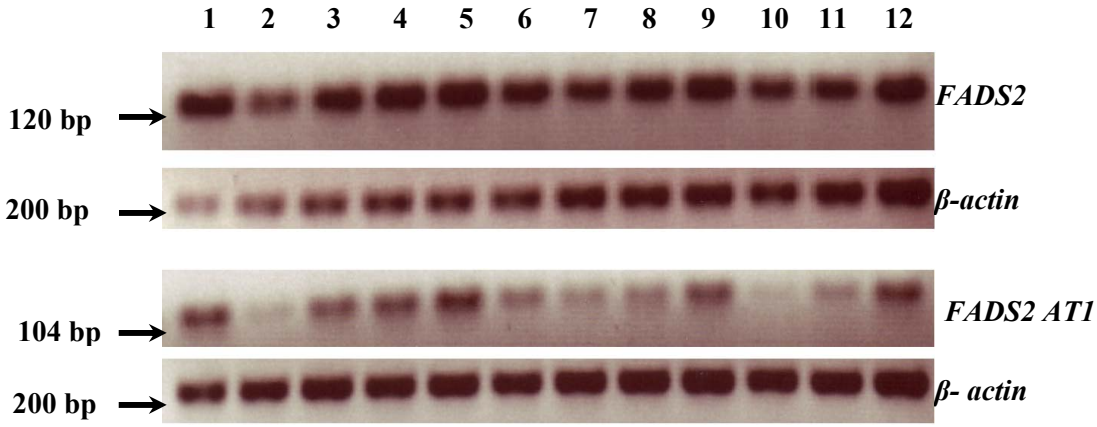

Figure 4.3. *FADS2* and *AT1* expression in 12 neonate baboon tissues (lane 1: liver, lane 2: kidney, lane 3: retina, lane 4: occipital lobe, lane 5: hippocampus, lane 6: heart, lane 7: skeletal muscle, lane 8: lung, lane 9: spleen, lane 10: thymus, lane 11: pancreas, lane 12: ovary)

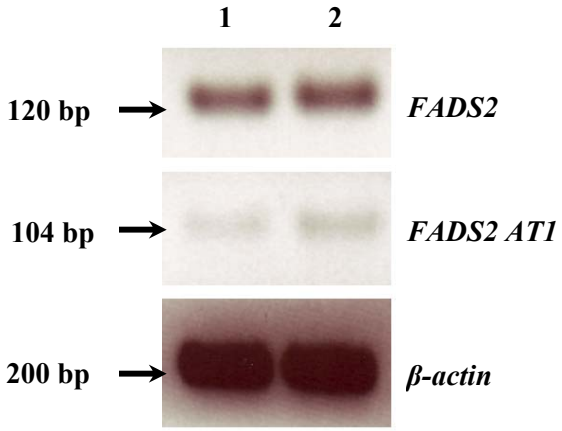

Figure 4.4. *FADS2* and *AT1* expression in human SK-N-SH neuroblastoma (NB) cells (lane 1: undifferentiated NB cells, lane 2: differentiated NB cells)

Functional characterization studies remain to be implemented to understand the physiological significance of the co-expression of *AT1* along with *FADS2*. Although *FADS2 AT1* is missing apparently conserved domains, in the flowering plant *Anemone leveillei*, a desaturase has been described without the cytochrome *b*5 domain catalyzed synthesis of non-methylene-interrupted (non-conjugated) PUFA. This observation shows that the four common domains are not required for production of functional protein (Sayanova et al., 2007). Qualitatively, expression levels appear to follow the tissue concentrations of 22 carbon LCPUFA. Confirmation of this observation would suggest a role in the regulation or biosynthesis of LCPUFA from precursors.

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CHAPTER 5

ALTERNATIVE TRANSCRIPTS OF FATTY ACID DESATURASE (FADS) GENES

5.1. Introduction

The interconversion of polyunsaturated fatty acids (PUFA) in mammals has long been known to be mediated by desaturation, elongation, and β-oxidation activities. Biosynthesis of long chain PUFA (LCPUFA) from the eighteen carbon precursors linoleic acid (LA, 18:2n-6) and linolenic acid (ALA, 18:3n-3) is especially important because of the prevalence of 18:2n-6 and 18:3n-3 in the human food supply.

Figure 5.1 is an outline of the currently accepted pathways for LCPUFA biosynthesis starting from 18:2n-6 and 18:3n-3, with genes and associated activities shown. In vertebrates, desaturation and elongation takes place in the endoplasmic reticulum (ER), apart from a report of 5-desaturase activity in the nuclei (Ves-Losada and Brenner, 1995). β-oxidation activity is found in the peroxisomes and mitochondria, including a critical step leading directly to 22:6n-3 biosynthesis from 24:6n-3 (Sprecher et al., 1995). β-oxidation catalyzes chain shortening interconversion, while the fatty acid chain elongation system operating on PUFA via three genes (ELOVL2, 4, and 5) (Jakobsson et al., 2006) chain lengthens.

PUFA desaturation activity is mediated by the Fatty Acid Desaturases (*FADS*) gene cluster located within a 100 kb region of the long arm of human chromosome 11q12-13.1, with the homologous genes located on mouse chromosome 19 (Nakamura and Nara, 2004). *FADS*1, 2, and 3 are consist of 12 exons and 11 introns, and include 3 conserved his box motifs and a cytochrome *b*5 motif. *FADS1* (Cho et al., 1999a) gene products were first established as having ∆5-desaturase activity catalyzing 20:3n-6 → 20:4n-6 (arachidonic acid, ARA) and 20:4n-3 → 20:5n-3 (eicosapentaenoic acid, EPA). *FADS2* (Cho et al., 1999b) gene products have ∆6-desaturase activity catalyzing 18:2n-6 → 18:3n-6 and 18:3n-3 → 18:4n-3; based on *in vitro* assays (Su and Brenna, 1998), ∆6-desaturation is generally considered the rate limiting step for LCPUFA biosynthesis. *FADS3* (Marquardt et al., 2000) shares similar sequence homology to *FADS1* and *FADS2* as the latter two genes are to one another. Despite the congruence of sequence similarity, no function has emerged for *FADS3*.

Recent studies have detected associations between specific single nucleotide polymorphisms (SNP) in *FADS* genes and blood fatty acid levels in normal adults (Schaeffer et al., 2006; Gieger et al., 2008; Rzehak et al., 2008), adults with cardiovascular disease (Malerba et al., 2008), pregnant and lactating women (Xie and Innis, 2008), and in attention-deficit/hyperactivity disorder (ADHD) (Brookes et al., 2006). More than 90% of SNPs that are associated with fatty acid concentrations are located in the untranslated regions, in introns, and in the intergene regions in CpG islands. Mechanisms that may be influenced by these non-coding regions are differential promoter binding and changes in methylation patterns that alter transcription.

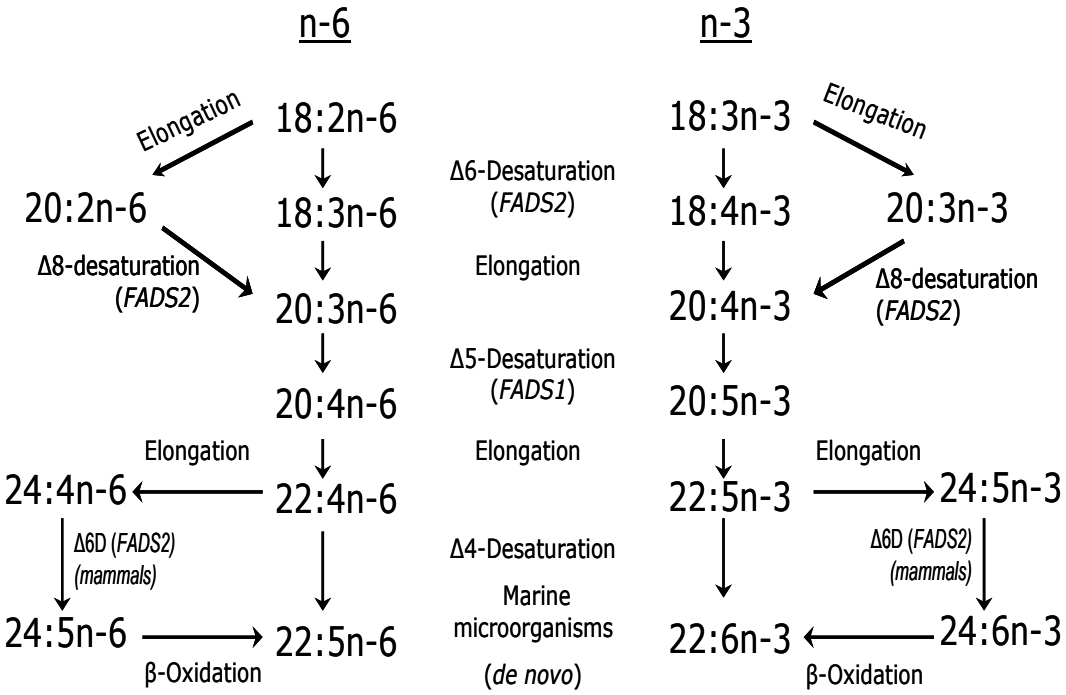


Figure 5.1. Biosynthesis of LCPUFA with emphasis on the role of *FADS* genes, as generally accepted. β-oxidation may chain shorten any LCPUFA. Not shown are 16:2n-6 and 16:3n-3 which can be elongated to 18:2n-6, and 18:3n-3, respectively.

Another possibility is genome-wide alternative splicing (AS) events that yield alternative transcripts (AT) (Gilbert, 1978). The majority of AT result from full or partial exon deletion or intron retention. AT may code for modified proteins with different specificities than the protein coded by the transcript resulting from classical splicing (CS), or serve some regulatory role. AT serve to expand the range of products for which a genome can code. It has been estimated that 90% of multi-exon genes have alternative transcripts. In a remarkable example of AS, the Drosophila gene *Down syndrome cell adhesion molecule* (Dscam) potentially generates over 38,000 transmembrane proteins of the immunoglobulin superfamily (Hattori et al., 2007) that function in the development of neural circuitry, and specifically in dendritic self-avoidance (Matthews et al., 2007). Metabolic regulation of AT expression has also been reported. For instance, ABCA1, a gene required for lipid transport across the plasma membrane has at least three AT that are regulated in a tissue and diet specific manner (Singaraja et al., 2005).

Recently, we have re-examined various aspects of the pathways of LCPUFA biosynthesis, showing, for instance, that the *FADS2* gene product catalyzes the ∆8-desaturation of 20:2n-6 → 20:3n-6 and 20:3n-3 → 20:4n-3 (Park et al., 2009a). In the course of these molecular studies we discovered AT associated with *FADS3* (Park et al., 2009b) and, most recently, *FADS2* (Park et al., 2009c). We discuss here salient aspects of these first reports of FADS AT and their possible implications for LCPUFA biosynthesis.

Table 5.1 outlines the number of transcripts, proteins, and activities reported for the mammalian *FADS* genes. *FADS1* function is limited to ∆5-desaturation

Table 5.1. Current knowledge of primate PUFA desaturases.

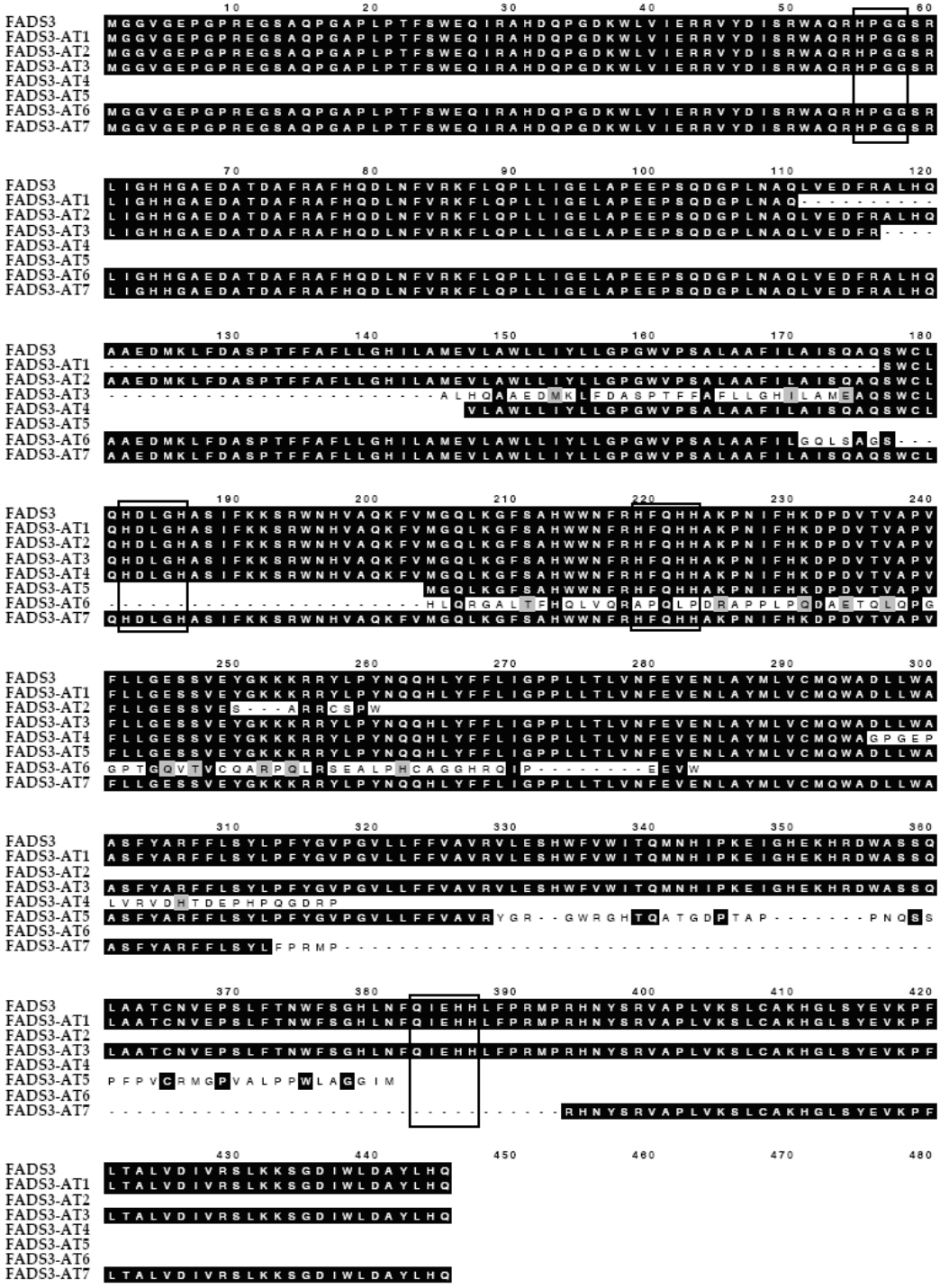
Gene	*FADS1*	*FADS2*	*FADS3*
Transcript(s)	1	2	8 (9?)
Protein	*Not characterized*		
Activities	Δ5-desaturation 20:3n-6→20:4n-6 20:4n-3→20:5n-3	Δ6-desaturation 18:2n-6→18:3n-6 18:3n-3→18:3n-3 24:5n-3→24:6n-3 24:4n-6→24:5n-6 16:0→16:1n-10 Δ8-desaturation 20:2n-6→20:3n-6 20:3n-3→20:4n-3	*Not identified*

of the 20 carbon precursors of ARA and EPA. *FADS2* has a much wider substrate spectrum. Its microsomal activity is about one quarter that of ∆5-desaturation (Su and Brenna, 1998), and it prefers n-3 to n-6 PUFA. It also shows small ∆8-desaturation activity toward 20 carbon PUFA in the presence of 18 carbon substrates (Park et al., 2009a). Finally, *FADS2* apparently mediates the biosynthesis of sapienic acid (16:1n-10), the most abundant unsaturated fatty acid on human skin, though the activity on 16:0 as a substrate is low (Ge et al., 2003). *FADS3* has no known substrates. Analogous PUFA desaturase genes reported in other species, including fish (Hastings et al., 2004), *C. elegans* (Spychalla et al., 1997), and many plant species (e.g. (Sayanova et al., 2007)) have a much wider range of substrates and specificities than those of mammals.

4.2. FADS3 AT

We recently described the detection of 7 AT of *FADS3* in 12 tissues of three month old baboons (Park et al., 2009b). AT are numbered sequentially according to the order in which they were discovered. Figure 5.2 is an alignment of predicted amino acid sequences for the *FADS3* CS and AT. AT1 and AT2 are missing exons 3 or 6, respectively. FADS3 AT3 has a truncated version of exon three; AT4 has a truncated exon 1, truncated exon 3, and is missing exons 2 and 8; AT5 has a truncated exons 1 and 4 and is missing exons 2 and 3, as well as retaining intron 8-9; AT6 skips the span from part of exon 4 through part of exon 9; AT7 skips part of exon 8 and all of exons 9 and 10. Predicted protein sequences indicate that AT1, AT3, and AT7 would code

Figure 5.2. Alignment of Baboon *FADS3* alternative transcripts using MacVector software and ClustalW alignment. Well conserved motifs common for desaturases are depicted in boxes. The “HPGG” characteristic of Cytochrome b5 is boxed in red, whereas three conserved histidine motifs “HDLGH, HFQHH and QIEHH” are shown in blue boxes.


FADS3
FADS3-AT1
FADS3-AT2
FADS3-AT3
FADS3-AT4
FADS3-AT5
FADS3-AT6
FADS3-AT7

for shorter proteins; AT1 and AT3 retain all conserved motifs characteristic of PUFA desaturases (HPGG cytochrome *b*5 and three histidine repeats "HDLGH, HFQHH, QIEHH"). The deletion of part of exon 8 and all of exons 9-10 result in the loss of the last histidine repeat QIEHH in AT7. AT2, AT4, AT5, and AT6 would produce truncated proteins missing one or more motifs.

The FADS3 AT were cloned into yeast (*Saccharomyces cerevisiae*) and mammalian cells, but showed no activity toward a variety of PUFA substrates (our unpublished data, 2009). Further studies were carried out to detect FADS3 AT expression in baboon tissue and cultured human neuroblastoma cells using AT-specific PCR primers bridging deleted parts of exons. Remarkably, all seven AT, were detected in all baboon tissues and in human cells (Park et al., 2009b). Figure 5.3 shows expression relative to β-actin for 12 tissues. Summed expression of all AT was highest in hippocampus and more than double that in pancreas; summed expression in all tissues was highest for AT6, and more than triple that of AT2. Correlation analysis of these data were performed using JMP 7.0 (SAS Institute, Cary, NC, USA) for the 21 possible pairs of 7 different AT. Results are shown in Table 5.2 for the significant correlations ($p<0.05$). Six of the seven correlations are among *FADS3* AT4, 5, 6, and 7. These results imply coordinated expression of these

AT in tissues, and also imply that expression of the other AT are independent. *FADS3* AT4,5,6,and 7 putatively code for truncated proteins that, individually, are all missing one or two of the four conserved motifs, and all are missing the QIEHH at the 3' end. It is tempting to speculate that simultaneous translation of all these ATs would code for proteins that could bind to form one or more

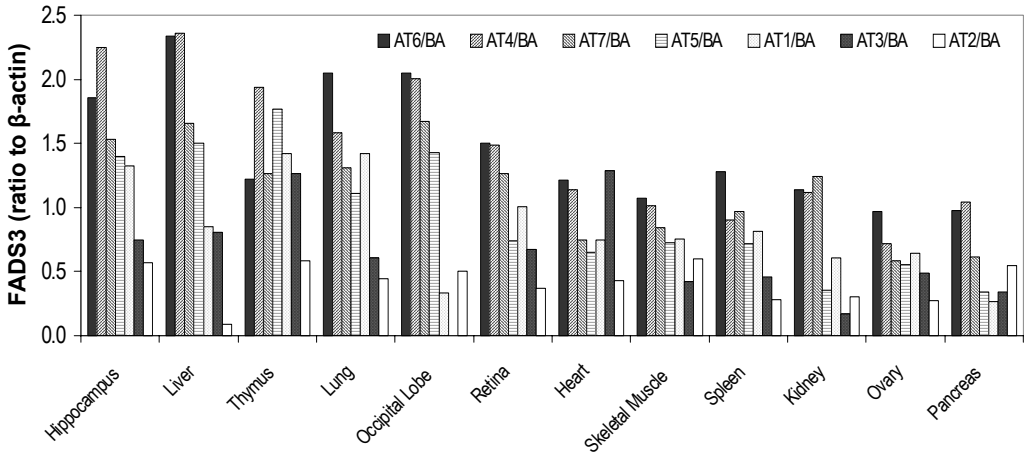


Figure 5.3. **Expression of the seven** *FADS3* **AT in 12 tissues of a 12 week old baboon.** Data are arranged left to right in order of total expression relative to β-actin. Gels were scanned and quantified manually using ImageJ <http://rsbweb.nih.gov/ij/>.

Table 5.2. Significant correlations between AT in various tissues[4].

	r^2	P
AT7 – AT4	0.79	0.0001
AT5 – AT4	0.77	0.0002
AT7 – AT6	0.74	0.0003
AT4 – AT6	0.68	0.0010
AT5 – AT7	0.55	0.0055
AT5 – AT6	0.46	0.0150
AT3 – AT1	0.37	0.0352

[4] Calculated with JMP 7.0 <http://www.jmp.com/>, "Multivariate Methods >Multivariate" menu and activation of "pairwise correlations" to obtain p values

functional desaturases, possibly to enable activity toward multiple substrates or for purposes of regulation.

5.3. FADS2 AT

Figure 5.4 presents quantitative data on the expression of *FADS2* CS and AT1 in the same twelve organs of baboon neonate as in Figure 5.3. Liver expression of *FADS2* CS is 4-fold that of the average expression of the other organs. The range of expression in the other organs is 2-fold, from the highest in retina to the lowest in skeletal muscle. In contrast, the range for *FADS2* AT1 in these same tissues varies over a factor of 20, from highest again in occipital lobe to the lowest in thymus. The dramatic difference in expression suggests a difference in function and independent regulation.

Preliminary observations on species conservation of FADS AT. The conservation of alternatively spliced transcript variants of *FADS* AT was examined in several animal species, as shown in Figure 5.5. Using the *baboon-specific primers* (Park et al., 2009c) for each of the *FADS AT*, we performed RT-PCR analysis using liver cDNA from mice, pig (porcine), dog, fox, chicken and horse. At least five of the seven *FADS3* AT and *FADS2* AT1 are reproducible in many species. These PCR products have not yet been confirmed by sequencing, but are consistent with the hypothesis that *FADS* AT perform some functionally important role, encoding proteins or performing a regulatory role.

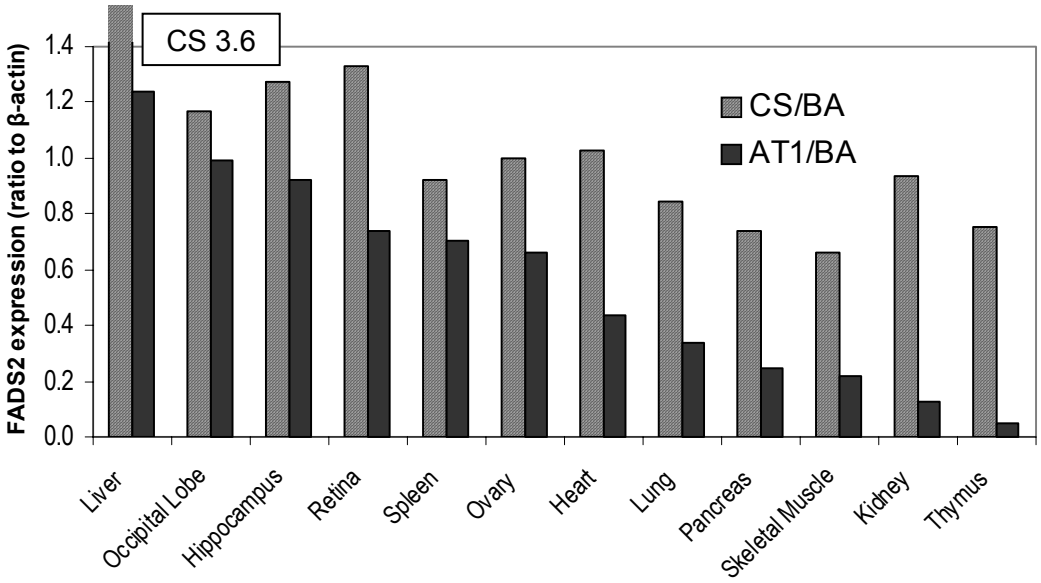


Figure 5.4. Expression of the *FADS2* classically spliced (CS) transcript and AT1 in various tissues of a 12 week old baboon. The y-axis is plotted with liver FADS2 CS off-scale because it is four-fold the average of the others. The range of expression for FADS2 AT1 is much greater among the 11 tissues (other than liver) than for the CS.

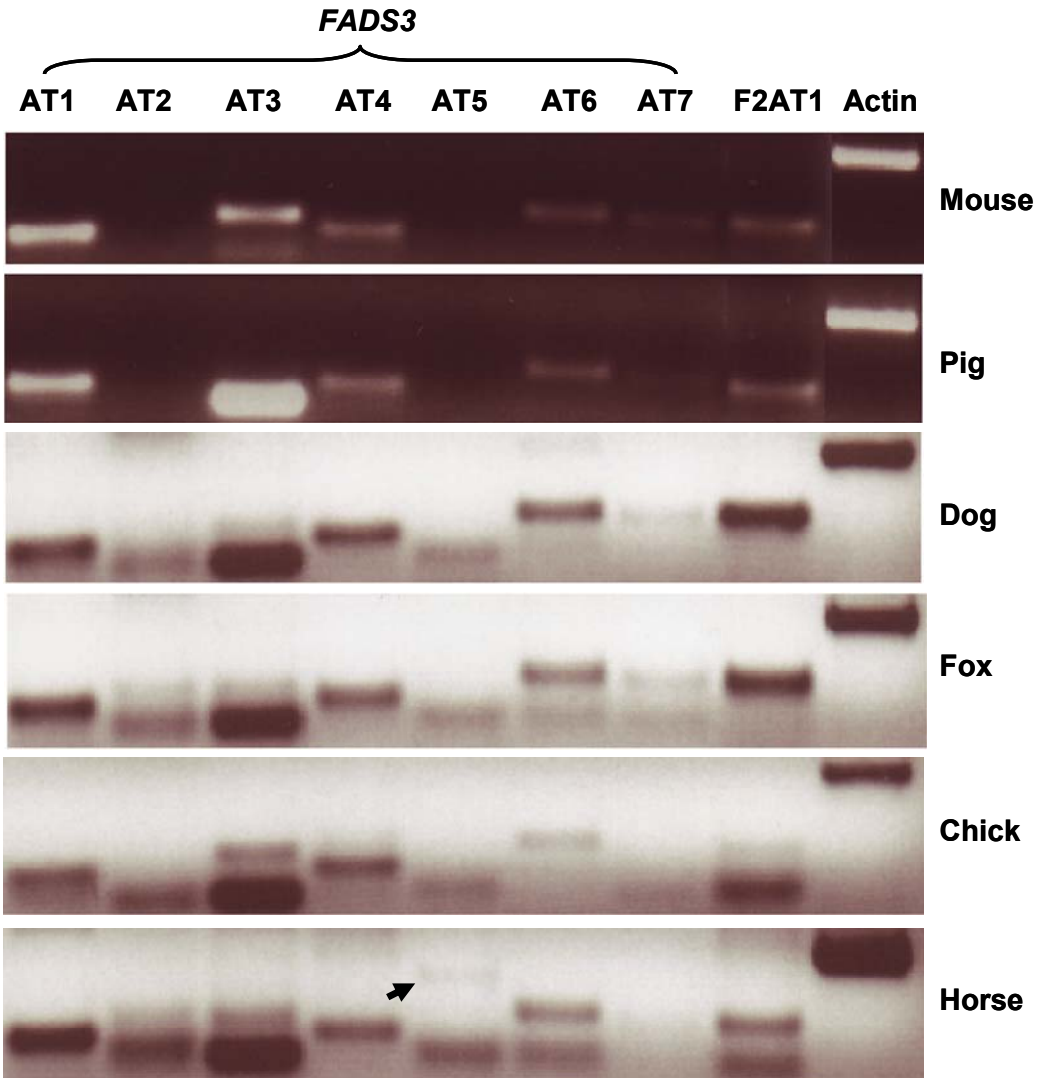


Figure 5.5. Expression of *FADS3* AT and *FADS2* AT1 in five mammalian species and in the chick, analyzed using baboon-specific primers. All but FADS3 AT2 and FADS3 AT5 are expressed in most of the species. FADS3 AT5 is detected in horse.

More than one investigator of desaturase function has suggested that the existence of at least two different 6-desaturases as a possible explanation of functional studies (Bourre and Piciotti, 1992; Marzo et al., 1996). To our knowledge, isozymes of a ∆6-desaturase have been described only in the arachidonic acid producing fungus *Mortierella* sp. and not for other organisms (Sakuradani and Shimizu, 2003; Nakamura and Nara, 2004). In previous work, we and others have also suggested that specific LCPUFA synthases would explain results of ours and others that are not easily accounted for by the accepted LCPUFA synthesis pathways (Infante and Huszagh, 1998; Infante et al., 2002). Although a function has yet to emerge for FADS3 and for the various AT that we have described, they represent a putative molecular basis on which alternative regulation and LCPUFA biosynthetic pathways could be based.

Hypothetically, FADS AT as mediators of LCPUFA biosynthesis or regulation could have implications for establishing variability in individual and population LCPUFA dietary requirements. Among the few studies that have reported on stable isotope tracers in humans, at least one in preterm infants showed wide variability to form 22:6n-3 and 20:4n-6 (Uauy et al., 2000). Differential expression of AT among individuals in response to diets could imply differential LCPUFA requirements. For instance, some populations subsisting on diets that provide only 18:2n-6 and 18:3n-3 as PUFA may require supplementary LCPUFA because of low expression of putative AT-related LCPUFA synthetic proteins, while others might synthesize sufficient LCPUFA from precursors. Group differences of this type have been suggested (Gibson and Sinclair, 1981) but no compelling data has been presented. The novel

findings of FADS AT suggest that investigations to elucidate molecular mechanisms and nutritional factors regulating their expression are required to elucidate factors mediating human PUFA desaturation.

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CHAPTER 6

CONCLUSION AND FURTHER STUDIES

6.1. Conclusion

Scientific and public interest in the relationship between dietary fatty acids, especially long chain polyunsaturated fatty acids (LCPUFA), and human health has been high for decades because of their nutritional importance throughout the development and aging. LCPUFA, namely DHA, EPA, and ARA are ubiquitous in the mammalian tissue, achieving highest concentrations in the membranes of neural and other excitable tissue, and contribute significantly to human and animal health, development, and cognition (Carlson, 2001; Brenna and Diau, 2007). The composition of these fatty acids affects the biological membrane integrity and fluidity, eicosanoid synthesis, critical molecules in eicosanoid signaling for physiological homeostasis, protein-lipid interactions, membrane bound receptor function and regulation of gene expression (Nakamura and Nara, 2004; Kothapalli et al., 2007). Especially, DHA and ARA are major LCPUFA of vertebrate central nervous system (CNS) and are known to be important in infant brain development and in maintenance of adult brains. Deficiency of omega-3 fatty acids results in decrease of DHA in the brain and retina, which in turn may lead to memory loss, learning disabilities, and impaired visual activity (Benatti et al., 2004; Hsieh and Brenna, 2009).

The parent PUFA 18:2n-6 and 18:3n-3 are components of all human diets,

and the LCPUFA can be biosynthesized from them, though the adequacy of biosynthesis is in question. Vertebrates are unable to biosynthesize LCPUFA *de novo* as they lack Δ12 and Δ15 desaturase enzymes responsible for converting 18:1n-9 (oleic acid) into 18:2n-6 and then into 18:3n-3 (Pereira et al., 2003). The essentiality of 18:2n-6 and 18:3n-3 in vertebrates has been known for decades. The main sources of 18:2 are sunflower oil, safflower oil, soybean, corn, and cottonseed, and 18:3 is derived from green leafy vegetables, canola, flaxseed, soybean, and walnuts (Simopoulos, 1999). LCPUFA are not readily available in many western diets and their endogenous synthesis from dietary precursors, 18:2n-6 and 18:3n-3 requires an alternating series of position-specific desaturation and carbon chain-elongation reactions. The focus of my research dissertation is desaturation steps.

FADS1 and *FADS2* genes which encode for Δ5 and Δ6 desaturation, respectively, are considered to yield protein product(s) thought to operate on both n-3 and n-6 PUFA. FADS2 catalyzes the rate limiting step acting on parent PUFA 18:2n-6 and 18:3n-3 in the biosynthetic pathway for LCPUFA biosynthesis. In addition to 18:2 and 18:3, FADS2 uses at least three more substrates, 24:6n-3, 24:5n-3 and 16:0. However, there are no previous reports of *FADS2* gene product activity toward 20 carbon PUFA. The existence of an alternative pathway for the biosynthesis of 20:4n-6 and 20:5n-3 was first observed in a protozoan (*Euglena gracilis*). In this microorganism 18:2n-6 and 18:3n-3 are first elongated to 20:2n-6 and 20:3n-3, followed by delta 8 desaturation and delta 5 desaturation, respectively to yield 20:4n-6 and 20:5n-3. The existence of delta 8 desaturation activity has been reported in rodent and human testes and the most recent study shows stable isotope labeling

best explained by direct conversion of 11,14-20:2 to 20:4n-6 via Δ8-desaturation, albeit as a minor pathway, but there are no existing molecular data to implicate a specific gene responsible for coding for vertebrate Δ8-desaturase activity. In mammals, 20:2n-6 and 20:3n-3 are widely considered dead-end products, in part because their conversion to LCPUFA has not been unequivocally established. We hypothesized that the primate *FADS2* gene product would have Δ8-desaturase activity and cloned baboon *FADS2* into *Saccharomyces cerevisiae*, an organism with no native PUFA biosynthetic capability, to test for gain of Δ8-desaturation activity. By using gas chromatography-covalent adduct chemical ionization tandem mass spectrometry (GC-CACI-MS/MS) and quantitatively analyzed by GC-flame ionization detection (GC-FID) we identified new product peaks in FADS2 transformed yeast cells incubated with 20:2n-6 and 20:3n-3 substrates. After establishing the Δ8-desaturase activity towards 20 carbon PUFA, we studied the relative activity in competition experiments. The Δ8-desaturase activity toward n-3 and n-6 fatty acids was investigated by supplementing media with a 1:1 mixture of 20:2n-6 and 20:3n-3 as substrates and we found a relative conversion efficiency of ~3.1-fold, favoring the n-3 PUFA. Similarly, 1:1 mixtures of 18:2n-6+20:2n-6 or 18:3n-3+20:3n-3 were added to media to test the relative Δ6-desaturase and Δ8-desaturase activities. We found that, Δ6-desaturation activity was preferred to Δ8-desaturation activity by 7-fold for n-6 (18:2n-6 over 20:2n-6) and by 23-fold for n-3 (18:3n-3 over 20:3n-3), respectively. Our results suggest that the conventional pathway would be strongly favored when both substrates are available and also establishes the affinity of the biosynthetic apparatus favoring n-3 PUFA. Our data established the unambiguous molecular evidence of the existence of Δ8-desaturation in

primates, suggesting alternative pathway for LCPUFA biosynthesis (Park et al., 2009a). From our results, we consider Δ8-desaturation to be a minor pathway; however, in individuals homozygous for the minor alleles for several SNPs in FADS gene cluster, especially FADS2 (Schaeffer et al., 2006) Δ8-desaturation may well be the only or major pathway. In these individuals 18:2n-6 desaturation product 18:3n-6 is decreased, whereas the elongation product 20:2n-6 is increased.

Alternative splicing (AS), first proposed by Gilbert (Gilbert, 1978), is now becoming well recognized as an important molecular mechanism for expanding protein diversity in higher eukaryotes. AS, initially thought to affect very small number of genes, but recent evidence shows at least 95% of multiexon genes have alternative transcripts (Calarco et al., 2009). *FADS* (*FADS1*, *FADS2* and *FADS3*) gene cluster is localized within a 100 kb region on the long arm of human chromosome 11q12-13.1, similar organization is reported on mice chromosome 19 (Nakamura and Nara, 2004). There are previous reports suggesting the existence of alternative transcripts (AT) for *FADS* genes, but none have been reported in the literature so far. In the course of investigation the role of *FADS* genes in LCPUFA biosynthesis, we performed RT-PCR analysis using baboon fetal and neonate cDNA and detected an AT for *FADS2* and multiple AT for *FADS3* generated by alternative splicing events (Park et al., 2009b; Park et al., 2009c). Using ORF finder, we also determined the putative coding regions of FADSAT.

At least 8 AT were identified for *FADS3* with 1.34 kb (classical splicing), 1.14 (AT1), 0.77 (AT2), 1.25 (AT3), 0.51 (AT4), 0.74 (AT6), and 1.11 (AT7). In

addition, we identified an AT of 0.51 kb (AT5) length that has a termination codon within intron 8–9. AT1 and AT2 lost exons 3 or 6, respectively. AT3 has a portion of exon three missing; AT4 has missing parts of exon 1 and exon 3 and complete loss of exons 2 and 8; AT5 has a truncated exons 1 and 4 and is missing exons 2 and 3, as well as retaining intron 8-9; AT6 skips the span from part of exon 4 through part of exon 9; AT7 skips part of exon 8 and all of exons 9 and 10. Predicted protein sequences indicate that AT1, AT3, and AT7 would code for shorter proteins; AT1 and AT3 retain all conserved motifs characteristic of PUFA desaturases (HPGG cytochrome *b*5 and three histidine repeats "HDLGH, HFQHH, QIEHH"). The deletion of part of exon 8 and all of exons 9-10 result in the loss of the last histidine repeat QIEHH in AT7. AT2, AT4, AT5, and AT6 would produce truncated proteins missing one or more motifs. *FADS2AT1* resulted from the partial or complete loss of first four exons causes in-frame loss of 154 aa and generates a coding region of 0.9 kb.

The *FADS2* AT and *FADS3* AT are expressed in twelve neonate baboon tissues and human neuronal cells. *FADS3* AT also showed changes in abundance in response to human neuronal cell differentiation. The correlation analysis of 7 *FADS3* AT showed that most significant correlations are among *FADS3* AT3 4, 5, 6, and 7, having implication that expression of those AT may be coordinated, however, other AT probably are independently expressed in tissues. Though the function of FASD3 is unknown, *in vivo* and *in vitro FADS3* AT gene expression, as well as structural and high sequence proximity to *FADS1* and *FADS2*, suggest that *FADS3* AT may relate to LCPUFA synthesis and/or regulation and have physiologically important roles.

The gene expression of *FADS2* classical transcript is higher than *FADS2*AT1 in all baboon tissues and showed very little variability. However, in the same tissues AT1 expression varies over a factor of 20, from highest in occipital lobe to the lowest in thymus. Qualitatively, expression levels appear to follow the tissue concentrations of 22 carbon LCPUFA. The dramatic difference in expression suggests a difference in function and independent regulation.

We also found that these AT are well conserved across several mammalian and avian species. Determining the function of these AT, conserved among species, provides a putative mechanism for understanding of LCPUFA biosynthetic regulation, and how it differs among individuals.

6.2. Further studies

The Δ8 desaturation alternative pathway to the eicosanoid precursors may explain data suggesting that 20:2n-6 levels are related to human health. All available evidence indicates that Δ8-desaturation is a minor pathway, but further study may show that it becomes important when there is high demand for eicosanoid synthesis, such as in inflammation or vasodilation, particularly in situations in which specialized tissues require 20:3n-6 as a precursor to prostaglandins E1 and F1α, hydroxyeicosatrienoic acids, or thromboxane B1.

The novel findings of FADS AT suggest that investigations to elucidate molecular mechanisms and nutritional factors regulating their expression are required to elucidate factors mediating human PUFA desaturation. Identifying

the functions for *FADSAT* is a uniquely important key to vertebrate physiology, and specifically the nervous system, which cannot function without polyunsaturated fatty acids.

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