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A novel FADS2 isoform identified in human milk fat globule suppresses FADS2 mediated Δ6-desaturation of omega-3 fatty acids

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Abstract

Introduction.—The only known non-pharmacological means to alter long chain polyunsaturated fatty acid (LCPUFA) abundance in mammalian tissue is by altering substrate fatty acid ratios. Alternative mRNA splicing is increasingly recognized as a modulator of protein structure and function. Here we report identification of a novel alternative transcript (AT) of fatty acid desaturase 2 (FADS2) that inhibits production of omega-3 but not omega-6 LCPUFA, discovered during study of ATs in human milk fat globules (MFG).

Methods.—Human breastmilk collected from a single donor was used to isolate MFG. An mRNA-sequencing library was constructed from the total RNA isolated from the MFG. The constructed library was sequenced using an Illumina HiSeq instrument operating in high output mode. Expression levels of evolutionary conserved FADSAT were measured using cDNA from MFG by semi-quantitative RT-PCR assay.

Results.—RNA sequencing revealed >15,000 transcripts, including moderate expression of the FADS2 classical transcript (CS). A novel *FADS2* alternative transcript (*FADS2AT2*) with 386

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Conflict of interest

All authors declare no conflict of interest.

amino acids was discovered. When *FADS2AT2* was transiently transfected into MCF7 cells stably expressing FADS2, delta-6 desaturation (D6D) of alpha-linolenic acid 18:3n-3 → 18:4n-3 was suppressed as were downstream products 20:4n-3 and 20:5n-3. In contrast, no significant effect on D6D of linoleic acid 18:2n-6 → 18:3n-6 or downstream products was observed. *FADS2*, *FADS2AT1* and 5 out of 8 known *FADS3AT* were expressed in MFG. *FADS1*, *FADS3AT3*, and *FADS3AT5* are undetectable.

Conclusion.—The novel, noncatalytic FADS2AT2 regulates FADS2CS-mediated Δ6-desaturation of omega-3 but not omega-6 PUFA biosynthesis. This spliced isoform mediated interaction is the first molecular mechanism by which desaturation of one PUFA family but not the other is modulated.

Keywords

milk fat globule; polyunsaturated fatty acid; desaturase; elongase; alternative transcript

Introduction

Both long chain polyunsaturated fatty acids (LCPUFA) structural families with 18 or more carbon atoms, n-6 (omega-6) and n-3 (omega-3) are required for a wide range of physiological functions in humans. Preformed LCPUFA can be acquired in the diet but all human populations retain the capacity to synthesize them from precursors linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) [1], to yield LCPUFA at varying levels under basal conditions [2]. Since about 2001, the biochemical pathways for LCPUFA synthesis in a wide range of organisms have been revised as a result of the wide availability of molecular tools that produce clearer results than traditional methods restricted to radiotracers and semi-purified fractionated organelles. Among these are the pathways to omega-3 docosahexaenoic acid (22:6n-3) which is now known to proceed via Δ4-desaturation in a wide variety of organisms throughout the phylogenetic tree, including humans, established by gain-of-function experiments upon transformation or transfection of genes into cells [3, 4]. Such methods are particularly crucial for establishing biochemical pathways for fatty acids because of the multiple pathways such as β-oxidation and carbon recycling that can render interpretation of tracer studies ambiguous.

The fatty acid desaturase (*FADS*) gene cluster consists of a family of three genes located on human chromosome 11q12–13.1 that yield enzymes catalyzing the insertion of double bonds in PUFA, monounsaturated fatty acids (MUFA) and at least one saturate, palmitic acid (16:0) [5–7]. *FADS2* codes for a classical transcript that results in a promiscuous multi-functional protein most active as a Δ6-desaturase but also mediating Δ8 [8] and Δ4 desaturation [3], and with at least 10 substrates all competing for the active site [6]. *FADS1* codes for a Δ5-desaturase leading directly to the key signaling precursor omega-6 arachidonic acid (20:4n-6) and to omega-3 eicosapentaenoic acid (20:5n-3) from precursors 20:3n-6 and 20:4n-3, respectively. The *FADS1* protein is also a Δ7-desaturase [9]. Based on the metabolic conversion of precursor PUFA, certain mammalian cell lines have long been known to Δ6-desaturate one family of PUFA but not the other [10]. *In vivo* and *in vitro* evidence suggest that some metabolic states cause concentrations of LCPUFA that share a common step and, by the accepted pathways, a common enzyme to change reciprocally [11,

12]. Despite these well-established classical results, substrate competition is the only widely recognized mechanism for modulating the relative activity of desaturases toward n-6 vs n-3 fatty acids that defines total PUFA composition of tissues, and is normally interpreted as influencing human health via the balance, or ratio, of PUFA diet intake [13].

We first reported the existence of alternative transcripts (AT) of the fatty acid desaturase genes [14], and others have reported protein products [15]. Alternative splicing is increasingly recognized as a mechanism by which molecular function is modulated [16, 17]. While all three *FADS* genes produce AT [18], the only AT to show biological activity is FADS1AT1 which enhances Δ6-desaturase of 18:2n-6(→18:3n-6) [19].

Lipids constitute half the energy of human breast milk and carry key LCPUFA 22:6n-3 and 20:4n-6 [20] required for neural development among many other functions. Less appreciated is that milk contains a wide range of bioactives that may well retain optimal activity when directly transferred to the infant gut at body temperature, the normal means of transfer of milk in mammals. Human milk lipids are configured as colloidal assemblies within milk fat globules (MFG) [21, 22]. Earlier studies have shown MFG to be a source of mammary RNA, representative of the mammary epithelial cell (MEC) transcriptome [23–25]. mRNA in bovine milk is highly stable and specifically resistant to acidic conditions and RNase treatment, and can withstand industrial processing, though less is known of the properties of human milks [26].

Here we report functional characterization of the second AT of FADS2, FADS2AT2, with respect to desaturation activity toward the precursor PUFA 18:2n-6 and 18:3n-3, discovered in the course of an investigation of the human milk transcriptome.

Materials and Methods

Sampling

Human breastmilk sample collected from a single donor was used for RNA extraction and mRNA sequencing library construction. For amplifying ORF samples were pooled from four donors, RNA extracted and cDNA prepared. The study was approved by the Cornell University Institutional Review Board on Human Subjects research and afforded exemption 4 because the sample(s) were collected and anonymized at human milk banks.

RNA extraction and Sequencing

Human breastmilk sample was centrifuged at 835 g for 10 min at 4°C, and the low density top cream fraction containing MFG was collected. Total RNA from the cream fraction was isolated by using QIAshredder and RNeasy kit (QIAGEN, MD). The concentration of RNA was measured by a Qubit® 2.0 Fluorometer (Invitrogen, CA) and 63 μg of RNA was used for sequencing library preparation after testing for RNA integrity. In brief, mRNA was purified by Dynabeads® mRNA Purification Kit (Invitrogen, CA) according to the manufacturer's instructions. The purified mRNA was fragmented at 94°C for 8 min. First strand cDNA synthesis reaction was catalyzed by SuperScript® III Reverse Transcriptase (Invitrogen, CA) with random primers, and the second strand cDNA was synthesized with dUTP (Thermo Scientific, PA). The cDNA fragments were then blunt-ended by end-repair

mix (Enzymatics, MA). An 'A' base was added at the 3' end of the blunt phosphorylated DNA fragments using Klenow (3' to 5' exo-) (Enzymatics, MA). TruSeq adapters were ligated to the fragments and sample was size selected for 200 bp by Agencourt AMPure XP beads (Beckman Coulter, CA). Followed by uracil DNA glycosylase (Enzymatics, MA) digestion, the adapter-ligated DNA fragments were amplified for 14 PCR cycles. The library was purified by Agencourt AMPure XP beads (Beckman Coulter, CA) and quality was checked with an Agilent Bioanalyzer. The library was sequenced at Cornell University Biotechnology Resource Center using an Illumina HiSeq instrument operating in high output mode.

Bioinformatic Analysis

The raw data generated by Illumina sequencing were initially checked for low quality reads and "N" bases. The low quality reads were removed and reads with no more than three mismatches were retained and were mapped to the human genome (UCSC version: hg19) using TopHat2 software [27]. Raw RNA-Seq data have been deposited at NCBI SRA database under the accession number SRR1039806.

We used Integrative Genomics Viewer (IGV) software for visual exploration of our data set [28]. IGV became available recently for high-performance genomics data visualization and exploration. It has assisted in identifying novel splicing signatures, transcription initiation site, INDEL and SNP and rare genetic variants in tumor tissues [29–32]. Reads per kilo base per million (RPKM) value was calculated for each transcript and it was used to represent transcript abundance [33]. We developed an in-house PERL script to detect alternative splicing events within the genes expressed in MFG.

Cell Culture

MCF7 cells were grown in MEM-α with 10% FBS, 10 mM HEPES buffer and 0.5 mg/ml geneticin in a humidified environment at 37 °C with 5% CO_2. HepG2 (hepatocellular carcinoma) cells were grown in MEM/EBSS media with 10% FBS in a humidified environment at 37 °C with 5% CO_2. The cell pellets of HepG2 cells were used to isolate RNA and cDNA synthesis.

FADS2AT2 Open Reading Frame (ORF) Amplification

To amplify the full length ORF of *FADS2AT2* we designed a forward primer within the 5'UTR and reverse primer within the intron 10–11.

Forward: AGCCGTCTGTGCAGCGAGCA

Reverse: CTATGACCAAGTGGCACAGCAT

PCR reactions were carried out using EmeraldAmp GT PCR Master Mix (Clontech, CA) in a final volume of 25 μl using cDNA from pooled MFG and HepG2 cells. cDNA was synthesized by using iScript cDNA Synthesis Kit (Bio-Rad, CA). Thermal cycling conditions were as follows: initial denaturation at 95°C for 5 min followed by 42 cycles of denaturation at 95°C for 30 s, annealing at 66°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, visualized, gel extracted and sequenced.

Transfection Assay

The ORF synthesis of FADS2AT2 and its cloning into a pcDNA3.1 expression vector was carried-out at GenScript (GenScript USA Inc., NJ). For transfection studies, MCF7 cells stably transformed with *FADS2* classical transcript/FADS2CS (MCF7-F2) or empty vector (MCF7-C) were transfected with *FADS2AT2* DNA or empty vector (control) DNA using jetPRIME transfection reagent (Polyplus-transfection Inc., NY). The jetPRIME transfection reagent worked best in our hands and showed no off-target effects. Twenty-four hours after transfection, the cells were supplemented with 50 μM of albumin-bound 18:3n-3 or 18:2n-6 fatty acid and incubated for additional 24 hours (h). After incubation, the cells were washed twice with 1×PBS and removed by trypsinization. Fatty acid extraction was performed on the collected cell pellets.

Fatty Acid Analysis

Fatty acid methyl esters (FAME) preparation, structural identification and quantification of FAME were carried out as described earlier [3]. Briefly, FAME preparation was carried out using a modified one-step hydrolysis, extraction and methylation procedure of Garces and Mancha [34]. Structures of the FAME were identified by covalent-adduct chemical ionization tandem mass spectrometry on a Saturn 2000 ion trap mass spectrometer (Varian, Inc., Walnut Creek, CA). Quantitative analysis was performed with GC coupled to a flame ionization detector using an equal weight mixture to derive response factors for each FA [3]. The peak areas were normalized to 18:1n-7 because it did not change between treatments.

Semiquantitative RT-PCR

Expression levels of evolutionary conserved *FADSAT* were measured using cDNA from MFG and HepG2 cells. HepG2 cells cDNA was used as control. Semiquantitative RT-PCR analysis was performed using bridged primers designed from unique regions specific for each AT [14, 35, 36]. PCR primer sequences are presented in supplemental table 1. PCR reactions were carried out using EmeraldAmp GT PCR Master Mix (Clontech, CA) in a final volume of 25 μl. Thermal cycling conditions were as follows: initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 65°C to 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 visualized under UV light.

Results and Discussion

A total of 52 million reads were sequenced, showing expression of 15605 transcripts in MFG (Supplemental table 2). Overall, 67% of the sequenced reads were mapped to the human genome. Transcript levels of each gene were quantified using the RPKM method [33]. Based on the RPKM value the genes were grouped as low abundance (<10 RPKM; number of genes 11987), moderate abundance (10–100 RPKM; number of genes 3203) and high abundance (>100 RPKM; number of genes 415) [37]. More than >3500 genes having alternative splice sites were detected (Supplemental Table 3).

Using IGV [28], we explored the *FADS* genes splicing signatures to check for the evolutionarily conserved alternative transcripts in MFG. *FADS2* reads expressed in MFG included five reads within intron 10–11 (Figure 1). Knowledge of an earlier FADS3AT with intron retention [14] led us to hypothesize the presence of a novel isoform, and we designed primers flanking the 5' UTR and the sequence within intron 10–11. Using these primers and cDNA from HepG2 cells revealed a PCR product. The absence of a band appearing in our analysis of the single donor breastmilk sample may be due to low abundance of this transcript, as only five reads were found in intron 10–11; however, a clear PCR product was found in a separate pooled breast milk sample. The PCR products generated from HepG2 and pooled MFG yielded the same sequence, identified as the novel *FADS2* isoform (*FADS2AT2*), the second AT identified for FADS2 [36]. FADS2AT2 has a termination codon within intron 10–11 due to intron retention. ORF finder predicts a 386-amino acid protein retaining the conserved cytochrome b5 domain and all three histidine motifs characteristic of PUFA desaturases except for a change of histidine to glutamine "QIEHH" to "QIEHQ" within the last histidine motif. In the classical transcript (FADS2CS), the last histidine repeat consists of QIEHH amino acid sequence, and the nucleotides (CAC) coding for the last histidine amino acid "H" are split between exons 10 (CA) and 11 (C). In FADS2AT2 the termination codon is within the intron 10–11 due to the loss of the last two exons 11 and 12. This results in the replacement of histidine "H" with glutamine "Q" (QIEHQ). The nucleotides (CAG) code for the glutamine amino acid "Q", which is split between exon 10 (CA) and intron 10–11 (G) followed immediately by a termination codon (Figure 2).

FADS2AT2 matches GenBank Accession# BC009011.1. A single isolated report shows in the sea bass (*Dicentrarchus labrax* L.) genome a PUFA desaturase splice variant with the QIEHQ sequence [38], named *D6D-V*, with no function identified to date. D6D-V is highly expressed in the anterior intestine, followed by posterior intestine, brain and pyloric caeca [38]. This transcript is highly expressed in fish intestines. Fish may ingest high amounts of omega-3 long chain PUFA depending on food availability, and we speculate that this transcript may play a role in modulating the amount of endogenously desaturated omega-3 that eventually is transferred to the bloodstream. Because of the very high correlation between breastmilk omega-3 and dietary intake, the breastmilk may have a mechanism to reduce endogenous desaturation of omega-3 which then emerges in a milk-based transcript.

Functional Studies.

We used MCF7 human breast cancer cells with no native Δ6-desaturase activity as a model for testing activity. As in our previous studies, stable MCF7-F2 and MCF7-C are treated with albumin bound fatty acids and/or transiently transfected with exogenous FADS2AT2 DNA are used for functional studies [3, 19].

The gas chromatograms of MCF7-C only or MCF7-C transiently transfected with FADS2AT2 (MCF7-C + FADS2AT2) show no activity toward 18:2n-6 or 18:3n-3 (Figures 3 and 4). MCF7-F2 transiently transfected with control DNA (MCF7-F2 + control) or FADS2AT2 (MCF7-F2 + FADS2AT2) and incubated with 18:2n-6 shows biosynthesis of 18:3n-6 (Figure 3). The biosynthesis of 18:3n-6 is similar in both MCF7-F2 + control and

MCF7-F2 + FADS2AT2 transfections (Figure 3) indicating no modification of activity toward 18:2n-6. In contrast, MCF7-F2 + FADS2AT2 incubated with 18:3n-3 shows reduction in the synthesis of downstream products (18:4n-3, 20:4n-3 and 20:5n-3), when compared to MCF7-F2 + control (Figure 4).

MCF7-F2 + control incubated with 18:2n-6 Δ6-desaturate via 18:2n-6→18:3n-6. MCF7-F2 + FADS2AT2 yields similar results both qualitatively and quantitatively (Figure 5A). In contrast, MCF7-F2 + FADS2AT2 incubated with 18:3n-3 show a 30% reduction in the Δ6-desaturation product (18:3n-3→18:4n-3) compared to MCF7-F2 + control (Figure 5B). Moreover, the downstream LCPUFA 20:4n-3 and 20:5n-3, products of sequential elongation and desaturation of 18:4n-3, are reduced by about 50%, confirming the expectation from the known pathway 18:3n-3→18:4n-3→20:4n-3→20:5n-3. These data demonstrate inhibition of n-3 but not n-6 LCPUFA synthesis in cells expressing FADS2AT2. They are consistent with a mechanism in which FADS2AT2 non-catalytically binds 18:3n-3 but not 18:2n-6, effectively reducing the amount of 18:3n-3 available for desaturation, thus limiting downstream n-3 product formation a mechanism of function for FADSAT that we previously suggested [39]. Sequestration of 18:3n-3 would affect both desaturation and elongation reactions, while also disrupting processes such as fatty acid incorporation into membrane phospholipids.

Co-expression studies show that splice variants can exert a dominant negative effect on the wild type protein [40, 41]. We showed that an alternatively spliced isoform of FADS1 enhances the activity of FADS2 [19]. Thus, the splice variants can either reduce or enhance the activity of the classical forms possibly by interacting with the fatty acid substrates.

RNASeq failed to detect 5’ ends of FADS1 and FADS3 transcripts, possibly because they are CG-rich (Supplemental Figure S1 and Supplemental Figure S2). We performed semiquantitative RT-PCR to examine for the presence of previously characterized evolutionarily conserved FADSATs in MFG. The human HepG2 cells served as control. FADS2, FADS2AT1 and 5, out of 8 known FADS3AT, were expressed in MFG (Figure 6). These data show for the first time evolutionarily conserved AT in MFG; FADS1, FADS3AT3, FADS3AT5 were undetectable in MFG. Maningat et al, [42] reported interindividual variability in FADS1 expression in MFG; in their samples FADS1 expression was detectable in 3 out of 5 subjects.

To our knowledge, FADS2AT2 is the first human gene product to differently alter the endogenous biosynthesis of LCPUFA based on double bond structure. The desaturation of 18:3n-3 but not 18:2n-6 is reduced, leading to a reduced production of downstream LCPUFA such as 18:4n-3, 20:4n-3 and 20:5n-3. Since the two PUFA families compete for inclusion in membranes and elsewhere, presumably FADS2AT2 would be expressed when n-3 LCPUFA are in metabolic excess and n-6 LCPUFA are in demand. However, functions for the other *FADS* AT are not yet assigned, and the net effects of the AT milieu may be complex compared to the effects of one gene product.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

FADS	fatty acid desaturase
MFG	milk fat globule
PUFA	polyunsaturated fatty acid
RPKM	reads per kilobase of exon per million mapped sequence reads
IPA	ingenuity pathway analysis
IGV	integrative genomics viewer

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Highlights

- We show expression of 15,605 transcripts in human milk fat globule (MFG) from a single donor.
- A novel FADS2 alternative transcript (FADS2AT2) was discovered in MFG.
- FADS2AT2 is non-catalytic but inhibited FADS2 Δ6-desaturation of omega-3 but not omega-6 PUFA.
- FADS2AT2 is the first human molecular species that inhibits biosynthesis of one PUFA family but not the other.

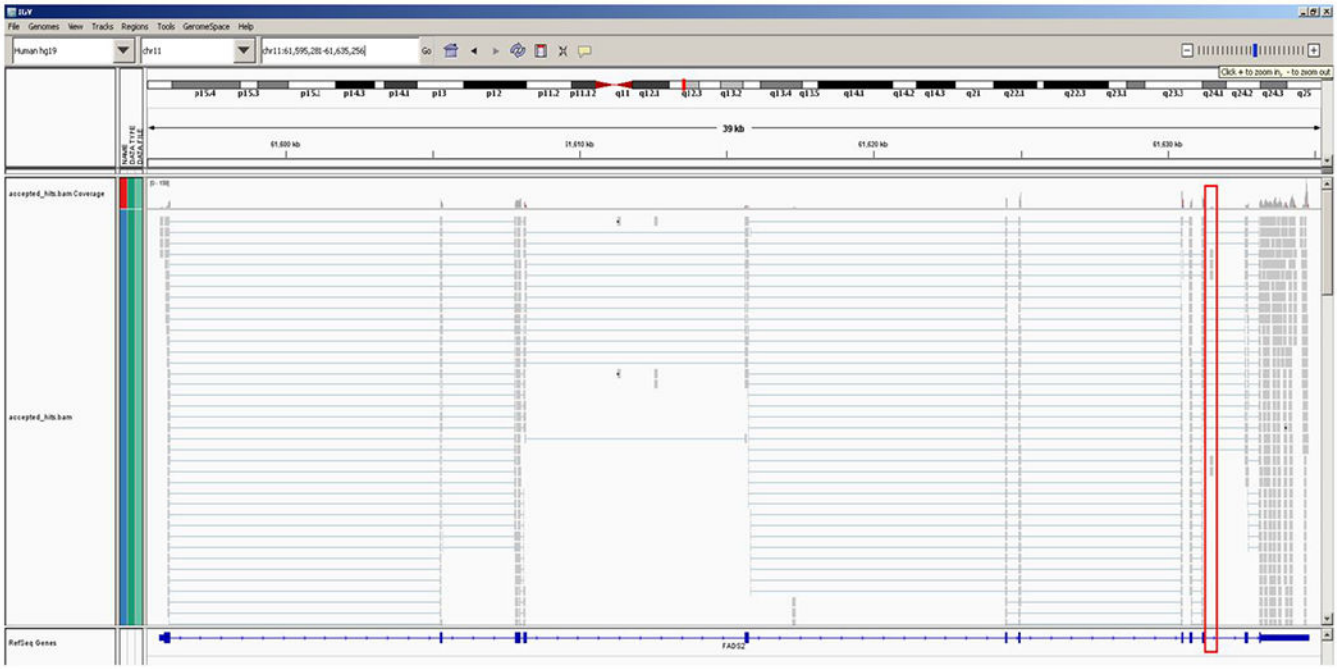

Figure 1:

FADS2 reads expressed in human milk fat globule. Screen shot image from the Integrative Genomics Viewer (IGV). In red box are the five reads found in intron 10–11. Primer pair designed flanking the 5' UTR and the sequence within intron 10–11 resulted in the identification of novel *FADS2AT2*.

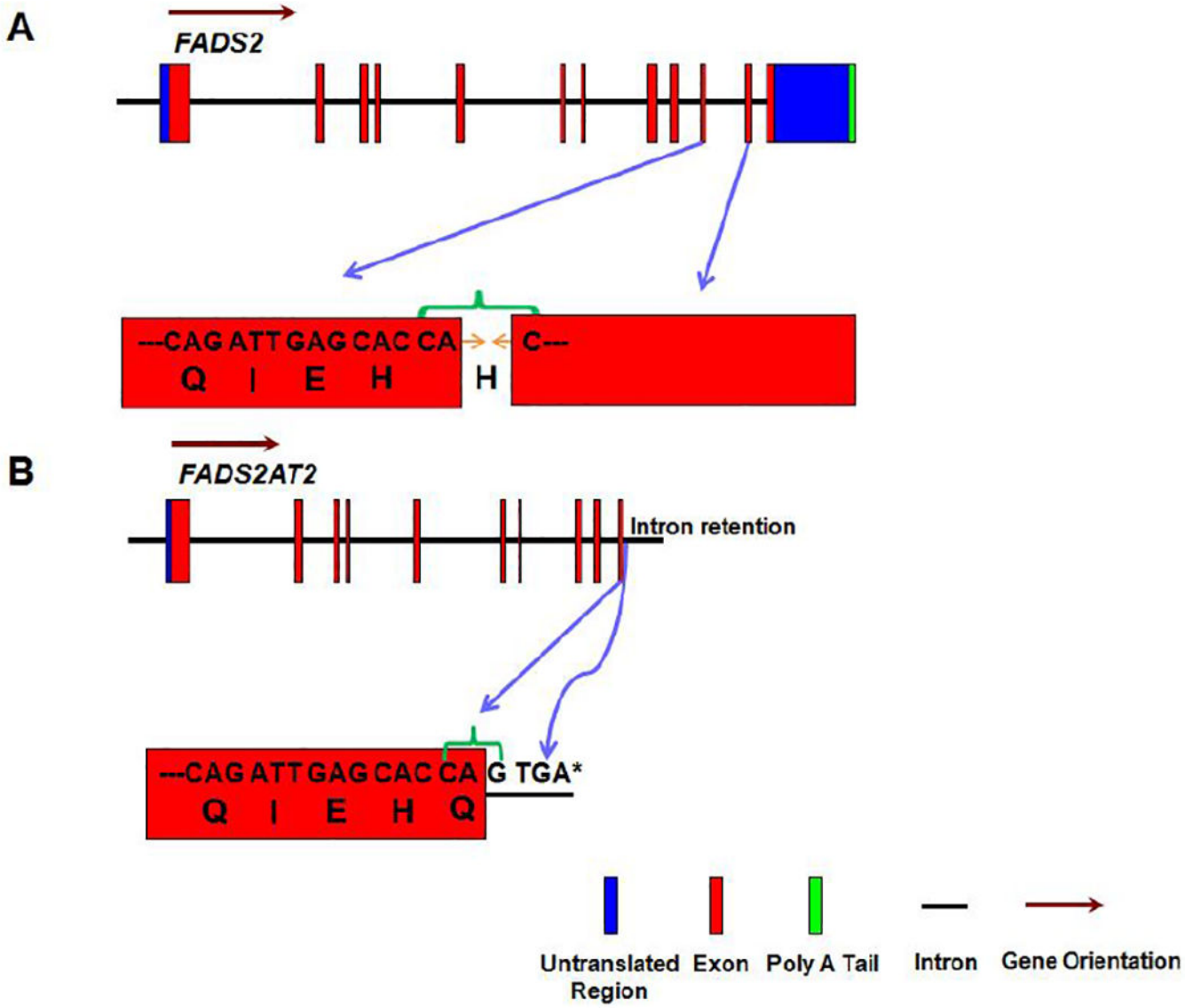

Figure 2:

Graphical representation of FADS2 and FADS2AT2. A) FADS2 shows 12 exons, zoomed in are exons 10 and 11. Front end desaturases are characteristic of having three well conserved histidine rich motifs, QIEHH represents the third motif. The nucleotides (CAC) coding for the last histidine amino acid “H” gets split between exons 10 (CA) and 11 (C). FADS2 codes for a 444 amino acid protein. B) FADS2AT2 is generated by the loss of exons 11 and 12 with intron retention after exon 10, zoomed in are exon 10 and intron 10–11. The nucleotides (CAG) code for the glutamine amino acid “Q” which gets split between exon 10 (CA) and intron 10–11 (G) followed immediately by termination codon. FADS2AT2 codes for a 386 amino acid protein.

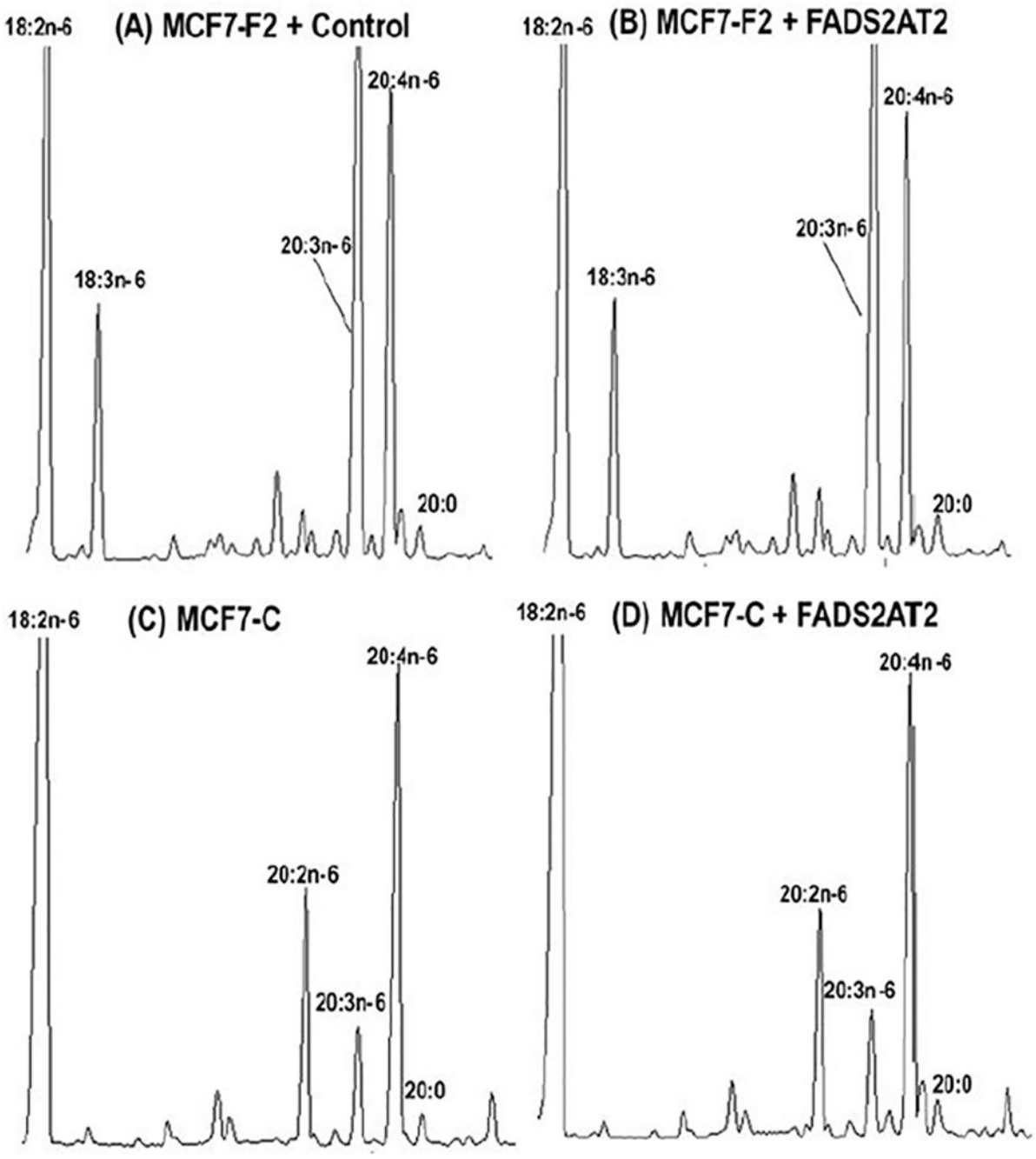

Figure 3:

GC results of transfection assay of stable MCF7-F2 cells using 50 μM of albumin-bound 18:2n-6. (A) MCF7-F2 + control shows 18:3n-6 and 20:3n-6 products synthesis (B) MCF7–2 + FADS2AT2 compared to (A) shows no change in the synthesis of 18:3n-6 and 20:3n-6 products (C) MCF7-C only cells or (D) MCF7-C + FADS2AT2 shows no desaturation activity.

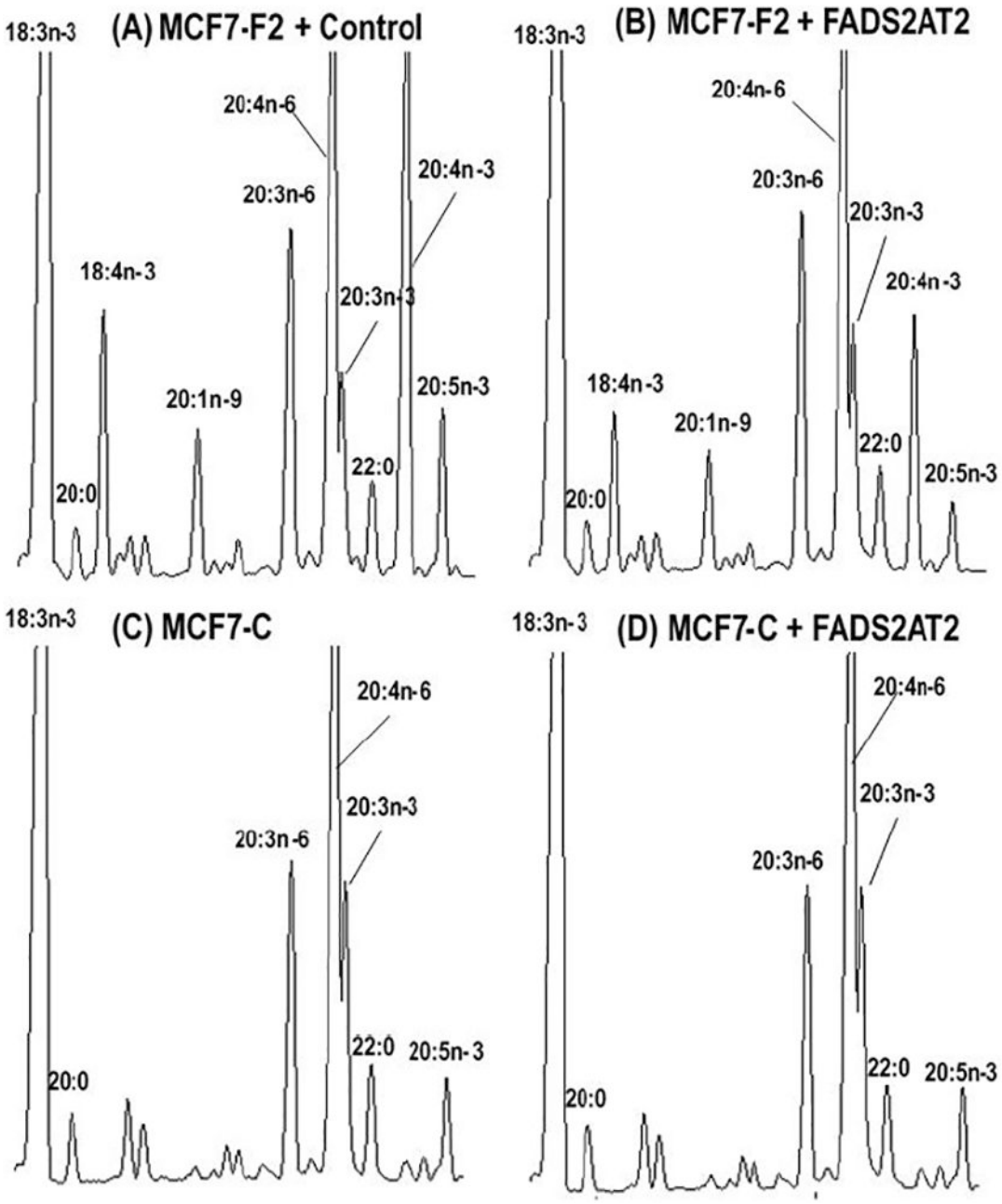

Figure 4:

GC results of transfection assay of stable MCF7-F2 cells using 50 μM of albumin-bound 18:3n-3. (A) MCF7-F2 + control shows 18:4n-3, 20:4n-3 and 20:5n-3 products synthesis (B) MCF7-F2 + FADS2AT2 shows reduction in the synthesis of 18:4n-3, 20:4n-3 and 20:5n-3 products (C) MCF-C only cells or (D) MCF-C + FADS2AT2 shows no desaturation activity.

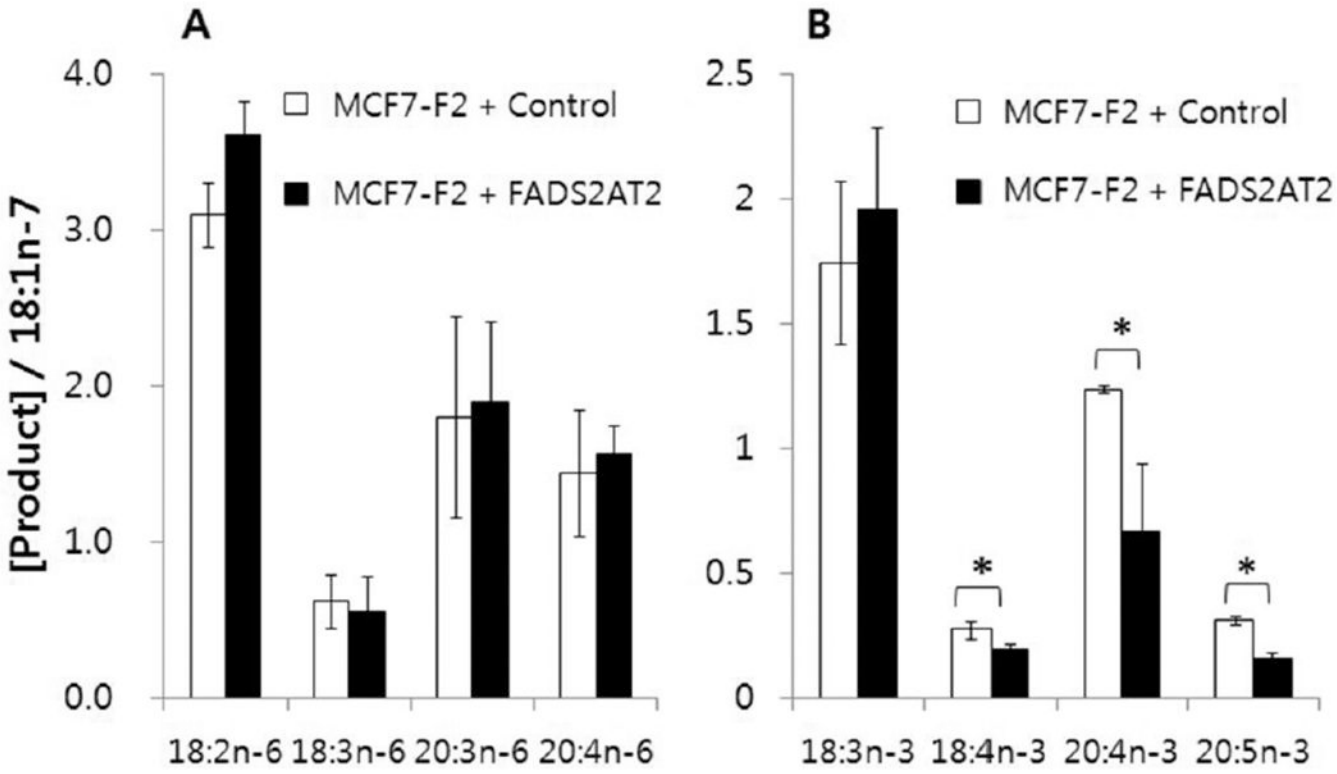

Figure 5:

Cells incubated with 18:2n-6 or 18:3n-3. **5A**: MCF7-F2 + FADS2AT2 or MCF7-F2 + control incubated with 18:2n-6. No changes in products were found between *FADS2AT2* and control. Data is representative of two independent experiments and are shown as mean ± SD ($P < 0.05$, Student's t test). **5B**: MCF7-F2 + FADS2AT2 or MCF7-F2 + control incubated with 18:3n-3. *FADS2AT2* suppress synthesis of 18:4n-3 and its downstream 20:4n-3 and 20:5n-3 fatty acids when compared to control. Data is representative of two independent experiments and are shown as mean ± SD ($P < 0.05$, Student's t test).

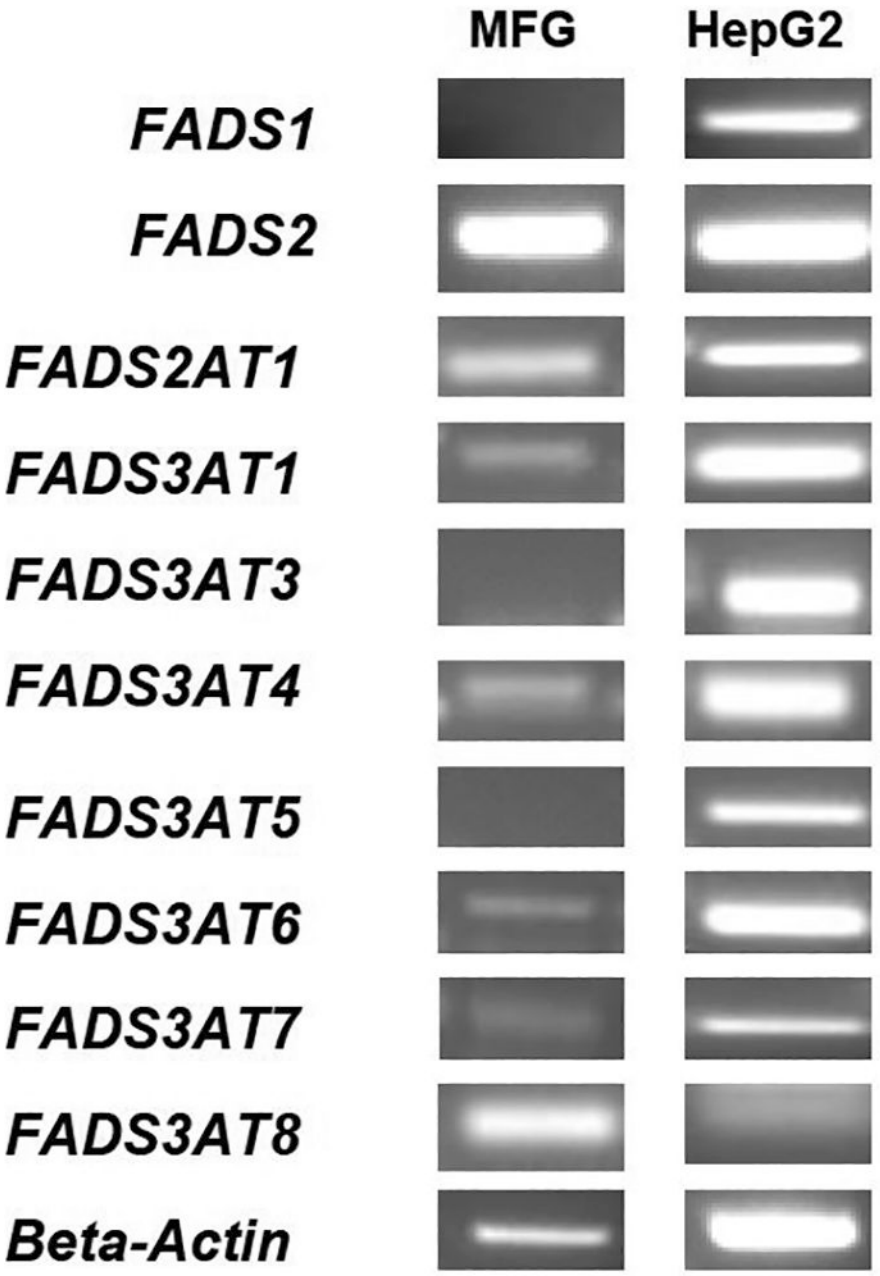

Figure 6:

Agarose gel image of FADS transcripts expressed in MFG. cDNA from hepatocellular carcinoma cell line (HepG2) is used as control. FADS and their ATs are all expressed in HepG2 cells. *FADS1*, *FADS3AT3* and *FADS3AT5* are undetectable in MFG.