The fatty acid desaturase 3 gene encodes for different FADS3 protein isoforms in mammalian tissues

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Abstract  In 2000, Marquardt et al. (A. Marquardt, H. Stöhr, K. White, and B. H. F. Weber. 2000. cDNA cloning, genomic structure, and chromosomal localization of three members of the human fatty acid desaturase family. Genomics. 66:176–183.) described the genomic structure of the fatty acid desaturase (FADS) cluster in humans. This cluster includes the FADS1 and FADS2 genes encoding, respectively, for the Δ5- and Δ6-desaturases involved in polyunsaturated fatty acid biosynthesis. A third gene, named FADS3, has recently been identified but no functional role has yet been attributed to the putative FADS3 protein. In this study, we investigated the FADS3 occurrence in rat tissues by using two specific polyclonal antibodies directed against the N-terminal and C-terminal ends of rat FADS3. Our results showed three potential protein isoforms of FADS3 (75 kDa, 51 kDa, and 37 kDa) present in a tissue-dependent manner. The occurrence of these FADS3 isoforms did not depend on the mRNA level determined by real-time PCR. In parallel, mouse tissues were also tested and showed the same three FADS3 isoforms but with a different tissue distribution. Finally, we reported the existence of FADS3 in human cells and tissues but different new isoforms were identified. To conclude, we showed in this study that FADS3 does exist under multiple protein isoforms depending on the mammalian tissues. These results will help further investigations to determine the physiological function of FADS3. —Pédrono, F., H. Blanchard, M. Kloareg, S. D’andréa, S. Daval, V. Rioux, and P. Legrand. The fatty acid desaturase 3 gene encodes for different FADS3 protein isoforms in mammalian tissues. J. Lipid Res. 2010. 51: 472–479.

Supplementary key words protein isoform • mRNA level • rat • human

PUFAs are key components involved in a variety of physiological functions (1). Some of them, belonging to the n-6 or n-3 families, have to be fulfilled from the diet or derived from the biosynthetic pathways resulting in the conversion of essential precursors to their respective elongated polyenoic products.

The availability of PUFA in mammalian cells greatly depends on the activity of enzymes involved in FA metabolism. In animals and humans, the Δ5- and Δ6-desaturases are the pivotal enzymes introducing de novo unsaturations in the carbon chain of precursors leading to the synthesis of long-chain PUFA (LC-PUFA). These enzymes were cloned 10 years ago from mammals (2–5). In parallel, Marquardt et al. (6) described the human genomic structure of the fatty acid desaturase (FADS) cluster including the FADS1 and FADS2 genes coding, respectively, for the Δ5- and Δ6-desaturases. A third gene, named FADS3, was identified, revealing 62% and 70% nucleotide sequence identity with FADS1 and FADS2, respectively. Further studies showed a significant correlation between FADS3 polymorphism and lipid metabolism markers such as PUFA, high-density or low-density-lipoprotein cholesterol, and triglyceride levels (7–10). The newly discovered gene was thereafter integrated into a serial analysis of gene expression and a DNA microarray succeeding in more physiological data. FADS3 was therefore found to be highly expressed at the implantation site of the embryo in mouse uterus (11) and downregulated during human neurogenic differentiation (12). More recently, Park et al. described, in baboon, different alternative transcripts of FADS3 generated by alternative splicing, which suggests the occurrence of multiple FADS3 gene products (13). This study also showed a different pattern of expression in response to human neuroblastoma SK-N-SH cell differentiation. All data together only concern the FADS3 gene with no description of the functional role of the putative FADS3 protein.

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Abbreviations: AT, adipose tissue; FADS, fatty acid desaturase; FCS, fetal calf serum; LC-PUFA, long-chain PUFA; RIPA, radio immuno precipitation assay.

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A protein database search identified FADS3 as a front end desaturase as well as FADS1 and FADS2. The predicted structure of FADS3 describes a membrane-bound desaturase composed of an N-terminal cytochrome b5-like domain and a C-terminal FA desaturase domain (Fig. 1A). These two domains are thought to be potentially involved in the regulation or the catalytic assignment of the desaturase as previously reported for FADS2 (14), suggesting an FA desaturase role of FADS3. It is therefore possible to assume that FADS3 exists in cells.

A recent study reported an enhanced mRNA expression of Fads3 in liver of Fads2−/− mice (15). Deletion of the Fads2 gene modifies the enzymatic pathways of LC-PUFA biosynthesis without impairing the normal lifespan of Fads2−/− mice but causing a variety of pathologies (16). FADS2 is known to catalyze the conversion of multiple FA substrates (17–19) and thus, to take part in many physiological functions. The close relationship between Fads2 and Fads3 in these knockout mice is thought to underline the putative involvement of Fads3 in the FA metabolism. No biological assignment has yet been attributed to that latest gene; no evidence has emerged concerning the translation from the transcript into the protein. Thus, the question is, does the FADS3 protein exist? To answer this query, we reported in this study the expression of Fads3 in mammalian tissues. We first analyzed the transcript level of Fads3 in rat tissues and then we investigated the occurrence of the FADS3 protein in mammalian cells or tissues.

MATERIALS AND METHODS

Materials

DMEM and antibiotics were purchased from Eurobio (Les Ulis, France). Fetal calf serum (FCS) was obtained from Lonza (Levallois-Perret, France). African green monkey kidney Cos-7 cell lines (from ECACC), HRP conjugated mouse anti-IgG, mouse...
monoclonal anti-GAPDH antibody, and chemicals were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France). Chemicals for PCR were from Eurogentec (Angers, France). pCMV/myc/cyt0 and pCMV•SPORT-βgal plasmids were from Invitrogen (Cergy Pontoise, France). Gene Pulser Xcel™ electrotransfection system and Kaleidoscope prestained standard were purchased from Bio-Rad (Marnes-la-Coquette, France). Customized human normal tissue blot was from Intercibble (Montluçon, France). HRP conjugated rabbit anti-IgG antibody and Immobilon detection kit were from Millipore (Guyancourt, France). HUH7 cells were a generous gift from the Laboratory of Genomics (Agrocampus Ouest, Rennes, France). BALB/c female mouse tissues were kindly given by V. Le Moigne (Agrocampus Ouest, Rennes, France).

Animals
Male and female Sprague-Dawley rats (150–200g body weight) were obtained from the Elevage Janvier breeding center (Le Genest Saint Isle, France). Animals had access to standard rodent chow (Scientific Animal Food and Engineering, Angy, France) and water ad libitum. Rats were fasted 24 h before euthanasia and bled by decapitation after anesthesia and analgesia using intraperitoneal injection of pentothal (75 mg/kg body weight). Fresh tissues were used to extract RNA and proteins. White adipose tissue (AT) was perirenal, intestine was from jejunum, and skeletal tissues were used to extract RNA and proteins. Male and female Sprague-Dawley rats (150–200g body weight) were obtained from the Elevage Janvier breeding center (Le Genest Saint Isle, France). Animals had access to standard rodent chow (Scientific Animal Food and Engineering, Angy, France) and water ad libitum. Rats were fasted 24 h before euthanasia and bled by decapitation after anesthesia and analgesia using intraperitoneal injection of pentothal (75 mg/kg body weight). Fresh tissues were used to extract RNA and proteins. White adipose tissue (AT) was perirenal, intestine was from jejunum, and skeletal tissues were used to extract RNA and proteins.

Cell culture
Cos-7, SH-SY5Y, and HUH7 cell lines were cultured in DMEM supplemented with 10% FCS and complemented with antibiotics (50 UI/ml penicillin, 50 µg/ml streptomycin, 10µg/ml gentamycin). Cells were maintained at 37°C in a humidified atmosphere of air (95%) and CO₂ (5%) up to 70–80% confluence. Cell extracts were prepared in radio immuno precipitation assay (RIPA) buffer containing PBS (150 mM NaCl, 0.95 mM NaH₂PO₄, 4.05 mM Na₂HPO₄; pH 7.4), 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 10 mg/ml aprotinin. Samples were also diluted in native lysis buffer (50 mM Tris-HCl pH 8, 1% Nonidet P40, 150 mM NaCl) when loading in native PAGE.

Rat Fads3 cloning and plasmid construction
The human FADS3 nucleotide sequence (GenBank accession number AF084560) was used to blast-search the rat genomic database (6). Designed from the Rattus norvegicus chromosome 1 genomic contig NW_047563.2, an oligonucleotide primer (5′-CAG CAA AGA GGA GTA TCC CAG TGG CAA-3′) was produced to screen the rat liver Marathon-ready cDNA library by Rapid Amplification cDNA Ends (RACE) reactions using the advantage-GC2 PCR kit (Clontech, Saint-Germain-en-Laye, France). The 5′ end of the rat Fads3 cDNA containing a candidate ATG start codon was obtained, as verified by the sequencing of the resulting PCR product (SCRIBE, Rennes, France). Oligonucleotide primers were then designed to PCR amplify the full-length rat Fads3 coding sequence from the rat liver Marathon-ready cDNA library. The forward primer (5′-CAT TCT TCA GAC CTC TGC CAC GTA-3′) was deduced from the former 5′ end sequence. The reverse primer (5′-CTG GGA AGA CAT GCT ATG CTC ACC-3′) was designed from a rat EST sequence (GenBank accession number AI178762) presenting a high sequence homology with the human FADS3 3′ flanking region. After the first-round PCR, a nested PCR amplification was performed using the cloning forward primer (5′-GTG TGC ATA GCG GCC AGC ATG GCC GT-3′) including the candidate translation start codon (in bold) and a Neot restriction site (underlined) and the cloning reverse primer (5′-ACT GCC GCC GCC TTC ACT GAT GGA GGT ACG C-5′) with the putative stop codon (in bold) and a Neot restriction site (underlined). The resulting 1530-bp PCR product was cloned into a pCMV/myc/cyt0 expression vector and the in-frame orientation was confirmed by DNA sequencing. The recombinant plasmid is referred to as pCMV/Fads3. The sequence of the full-length rat Fads3 cDNA is available in the GenBank database (accession number AJ494720).

Plasmid transfection
Rat recombinant FADS1, FADS2, and FADS3 were expressed after transfection of pCMV/myc/cyt0 plasmids containing the rat Fads1 (pCMV/Fads1), Fads2 (pCMV/Fads2), or Fads3 (pCMV/Fads3) gene inserts (17). Recombinant plasmids were transiently transfected into Cos-7 cells using electroporation. Briefly, 70% confluent cells were electroporated (250V-1500µF) and then cultured in DMEM with 10% FCS. After 48 h, cells were scraped off and protein extracts were diluted in RIPA buffer. The transfection efficiency was assessed by β-galactosidase colorimetric assay after cotransfection with pCMV•SPORT-βgal.

Quantification of the Fads mRNA level by real-time PCR
Total RNA was extracted from the tissues of three rats with Extract-All® (Eurobio, Les Ulis, France) and retrotranscribed in duplicates using SuperScript™ II reverse transcriptase (Invitrogen, Cergy Pontoise, France) according to the manufacturer’s instructions. Real-time PCR was performed in duplicates with the TaqMan Universal PCR Master Mix (Applied Biosystems, Courtaboeuf, France) containing 40 ng of retrotranscribed RNA, 0.5 µM of each primer, and 0.25 µM of TaqMan probe (Table 1). PCR was run using ABI Prism 7000 sequence detection system (Applied Biosystems, Courtaboeuf, France) as follows: 2 min at 50°C, 5 min at 95°C, 40 cycles of 10 s at 95°C, and 1 min at 60°C (SENAY, INRA, Saint-Gilles, France). Normalization was assessed using the Yakima Yellow®-Eclipse® DarkQuencher 18S rRNA control (Eurogentec, Angers, France). The PCR efficiency was estimated by calibration curves and by calculation using LinReg (20). The relative expression was evaluated as delta Cycle threshold (ΔCt = Ct_rads – Ct_rads). Rat Fads3 antibody production
Two polyclonal antibodies against Rattus norvegicus FADS3 were produced using a double-X 28-day protocol (Eurogentec, Angers, France). Briefly, two specific peptides corresponding to the N-terminal sequence (5′-QIRQHDLPGDKWL) and the carboxyl-terminal sequence (5′-PSPEIGHKHDWS) of rat FADS3 (Fig. 1B) were synthesized and coupled to Keyhole Limpet Hepocyanin; these target peptides display 85% and 100% identity with the human protein sequence respectively. Rabbits were then immunized with both peptides for 4 weeks. The two antibodies were then purified from the serum by affinity chromatography and were respectively referred to as anti-NtermFADS3 and anti-CtermFADS3. The specific reactivity of each antibody was checked by blot against the immunizing peptides and by Western blot against recombinant FADS3. A third antibody, referred to as anti-FADS2/3, was also punctually used because of its immunospecificity with rat recombinant FADS2 and FADS3. This third nonpurified polyclonal serum was produced by immunization of rabbits with two peptides as described in Fig. 1B (17).

Protein expression
Protein expression was assessed by Western blot after SDS-PAGE or native PAGE as described elsewhere (21). Rat FADS3
transcripts, as well as particularly represented. These data demonstrated that abundant especially in testis whereas were distributed in a tissue-specific and sex-dependent manner.

First, this experiment underlined a high significant effect of sex and tissue as well as their interaction.

Statistics

Data were analyzed using the R software (22). For each Fads transcript level, a linear model was applied on data to estimate the effect of sex and tissue as well as their interaction.

RESULTS

Expression of the Fads genes in rat tissues

The expression of the Fads3 gene was estimated by determining the tissue distribution of Fads3 transcripts as compared with the other genes of the cluster (Fig. 2). First, this experiment underlined a high significant effect of sex, tissue, and the interaction of sex and tissue on the mRNA level of each Fads gene (P < 10^-5). Second, our results showed that in somatic tissues, Fads1 and Fads2 displayed a similar mRNA profile (Fig. 2A, B) with the highest transcript amounts in the liver, kidney, brain, lung, and aorta. Fads3 mRNAs were differently distributed and mainly present in the lung, white AT, aorta, spleen, heart, and kidney (Fig. 2C). The pancreas and skeletal and abdominal muscles shared the lowest transcript level of Fads whereas the aorta, lung, and kidney represented tissues with the highest transcript level commonly found for each Fads gene. Moreover, our data showed a differential Fads mRNA amount according to gender. For instance, Fads3 transcripts were significantly more abundant in females than in males, particularly in the aorta, brown AT, and skeletal and abdominal muscles. In sexual organs, Fads2 mRNA and, to a lesser extent, Fads1 mRNA were highly abundant especially in testis whereas Fads3 mRNA was not particularly represented. These data demonstrated that Fads3 transcripts, as well as Fads1 and Fads2 transcripts, were distributed in a tissue-specific and sex-dependent manner.

Immunospecificity of anti-NtermFADS3 and anti-CtermFADS3 antibodies

In order to assess the occurrence of FADS3 in rat tissues, we produced antibodies specifically targeted against rat FADS3. Different peptides were thus designed as described in Fig. 1B. The first successful antibody was produced after rabbit immunization with two long peptides. This polyclonal antibody was tested on rat recombinant FADS1, FADS2, and FADS3. Our results showed that this antibody recognized a protein at 48 kDa corresponding to recombinant FADS3, but also a protein corresponding to recombinant FADS2 at 45 kDa (Fig. 3). This antibody was named anti-FADS2/3. Therefore, the nonspecificity toward FADS3 resulted in the production of new antibodies targeted against smaller peptides (Fig. 1B). Of seven antibodies, only two were successfully produced. The specificity of antibodies was tested on recombinant FADS1, FADS2, and FADS3 (Fig. 3). Our results showed that both antibodies detected FADS3 but neither FADS1 nor FADS2. Thus, the antibody directed against the N-terminal end of FADS3 was named anti-NtermFADS3 and the other one targeted toward the carboxyl-terminal end of FADS3 was named anti-CtermFADS3.

Identification of the FADS3 protein in rat tissues

We then investigated the protein level of FADS3 in the same selected tissues as previously described for the mRNA level in rat tissues using the two FADS3 antibodies, anti-NtermFADS3 and anti-CtermFADS3 (Fig. 4). The predicted molecular mass of rat FADS3 is 51.5 kDa according to the cDNA sequence. Our results showed a band around 51 kDa essentially found in the liver and kidney and to a lesser extent in the heart and brown and white AT (Fig. 4A). This 51 kDa band was also present in a few other tissues but more than 40 µg of protein extracts were needed to visualize it by Western blot (data not shown). A similar result was obtained with the anti-NtermFADS3 antibody but the 51 kDa protein was also identified by the anti-CtermFADS3 antibody. Different bands were also observed around 75 kDa and 37 kDa depending on the antibody. The anti-NtermFADS3 antibody recognized a 75 kDa protein in several organs, especially in the lung but also in the spleen, thymus, and aorta. The anti-CtermFADS3 antibody revealed a 37 kDa protein, highly abundant in skeletal and abdominal muscles, abundant in the brain and minor in the heart, eye, and thymus. Another band was also found below 37 kDa, for example, in the lung, stomach, and liver. This band may also be suspected in muscles under the extended signal as observed in diluted samples (data not shown). These data were representative for male rats but similar results were also obtained in females (data not shown).
shown). In sexual organs, the major protein was detected at 37 kDa in the ovary (Fig. 4B). The 51 kDa protein was equivalently present in all tissues except in the prostate, whereas no protein was observed at 75 kDa. This data showed three potential protein isoforms of FADS3, one of which was expected at 51 kDa. When tissues were presented in the decreasing order of the mRNA level determined in Fig. 2C, we showed that the occurrence of FADS3 was independent of the transcription level. Thus, our results have demonstrated that the Fads3 mRNA level is not correlated with the FADS3 protein level.

We further investigated these three isoforms of FADS3 in native PAGE by focusing on the lung, heart, kidney, and skeletal muscle (Fig. 4C). When the 51 kDa and 75 kDa isoforms from the lung, heart, and kidney were studied in native conditions, two bands were observed. On the contrary, the 37 kDa isoform from skeletal muscle migrated as a single band.

Occurrence of FADS3 in mouse tissues

The expression of FADS3 was also tested on several tissue extracts from mice using our two rat FADS3 antibodies (Fig. 5). Our results showed a similar profile with three protein isoforms. The 75 kDa protein was only observed in the lung. Liver and kidney presented the 51 kDa protein together with the 37 kDa protein, which is particularly abundant as compared with the rat tissues (Fig. 4A). Heart only contained the 51 kDa protein at a very low level and no 37 kDa protein as anticipated. The most unexpected result appeared in skeletal muscle, which displayed no protein, whereas it corresponded to the main tissue expressing the 37 kDa protein in rat. Thus, these results confirmed the presence of three isoforms of FADS3 distributed differently according to the tissues and depending on the animal type.

Occurrence of FADS3 in human cells and tissues

We then studied the natural FADS3 protein on human cells (SH-SY5Y and HUH7) and tissues using both anti-NtermFADS3 and anti-Cterm-FADS3 (Fig. 6). The target peptides designed to produce these antibodies share 85%
and 100% homology with the human protein sequence, respectively; therefore, these antibodies conceived to detect the rat FADS3 protein can also interact with the human one.

Our results on human cells showed different isoforms of FADS3 detected with the anti-CtermFADS3 antibody (Fig. 6A). In SH-SY5Y cells, we found three proteins with apparent molecular masses of 61 kDa, 56 kDa, and 37 kDa. The 37 kDa isoform was previously found in rat and mouse tissues in comparison with the two other proteins. The 61 kDa isoform was a protein commonly found in SH-SY5Y and HUH7 cells and commonly detected with both anti-NtermFADS3 and anti-CtermFADS3 (data not shown).

In human tissues, first, we found the same 56 kDa isoform located ubiquitously and also another slightly heavier one at 59 kDa, but only in the lung, kidney, brain, and skeletal muscles (Fig. 6B). Second, the 37 kDa isoform and a second one, just above at 39 kDa, were also detected in the same tissues as observed in rat (Fig. 4A). Pancreas did not display any of these isoforms as previously observed in rat, whereas kidney presented both isoforms undetected in rat. Third, the 75 kDa isoform especially present in the rat lung was not observed in the human lung with the anti-NtermFADS3 (data not shown) but this antibody revealed a new isoform of FADS3 at 28 kDa, mainly in the lung, kidney, pancreas, and ovary and to a lesser extent in the brain and liver.

These results together showed that FADS3 is expressed in human cultured cells and tissues under different isoforms commonly found in the murine tissues or specific to humans.

**DISCUSSION**

This study presented the cloning of Fads3 and, for the first time, the occurrence of the natural FADS3 protein in mammalian tissues or cells, which demonstrated that Fads3 is not a pseudogene.

The analysis of rat tissues confirmed that Fads3 is transcribed in an organ-specific manner. This data was previously exhibited in various human tissues. Marquardt et al. (6) showed, by Northern blot, a consistent level of FADS3 transcripts in the heart, brainstem, and liver as compared with the uterus and lung. We did not obtain the same result in rat using real-time PCR. First, we found the highest transcription level of Fads3 particularly in the lung but also in the heart. Our result was confirmed by Northern blot (data not shown). Second, the most unexpected result lies in the fact that the level of Fads3 mRNA is weak in the liver, which is considered the main organ of FA desaturases such as Fads1 and Fads2 (5, 23, 24). Moreover, the Fads3 mRNA profile in rat tissues was different from those determined for Fads1 and Fads2 suggesting a differential function of Fads3 in lipid metabolism.

Thereafter, taking the translation into consideration, we further investigated the expression of FADS3. We used different specific antibodies directed against the N-terminal and carboxyl-terminal sequences of rat FADS3, corresponding respectively to the cytochrome b5-like domain and the FA desaturase domain (Fig. 1). We found three potential protein isoforms (37 kDa, 51 kDa, and 75 kDa) differently distributed according to the species (rat and mouse). The 51 kDa molecular mass corresponds to the predicted size of murine FADS3 considering the cDNA sequence.
In native PAGE, the 51 kDa and 75 kDa proteins were detected as oligomers or protein complexes, contrary to the 37 kDa protein found as a single protein. That additional information on native proteins argues in favor of a different physiological involvement of FADS3 depending on the isoform. The 37 kDa protein was detected in mammalian tissues with only the anti-CtermFADS3, suggesting it would correspond to the putative FA desaturase domain. This short molecular mass could be explained by protein degradation or cleavage; one is known in the VLA\textsuperscript{\textbullet}AL motif, which separates the cytochrome b5-like domain from the FA desaturase domain, but that cleavage would generate a 33 kDa protein. We can also hypothesize the possibility of an alternative translation initiation process. In that case, a 37.3 kDa fragment would be produced if the translation is initiated by the second Met found in the primary sequence of FADS3, even though the sequence conditions of such a process do not appear optimal here (26). Such a 37 kDa size was also observed for the Δ9-desaturase from rat liver using antibodies against C terminus of rat SCD1 and SCD2 (27). Thus, the 37 kDa protein could present the same structure as the purified Δ9-desaturase naturally devoid of fused cytochrome b5-like domain.

The three potential isoforms of FADS3 identified in rat were also detected in mouse using the same rat FADS3 antibodies. The tissue distribution was, nevertheless, different notably in muscles where no 37 kDa protein was observed in mouse tissues as compared with rat tissues. This unexpected result could be explained by the fact that the experiment was conducted on female mice. Indeed, we previously showed a significant difference between male and female rats on the transcript level of Fads3 especially in skeletal muscles. To confirm this distinction between genders, we looked at the occurrence of the 37 kDa protein in rat tissues and specifically in skeletal muscles but no difference was observed (data not shown). Thus, FADS3 is thought to be located variably depending not only on the tissues but also on the murine species or strains. This may be extended to humans, as demonstrated in our results on human cultured cells where we also identified various isoforms of FADS3 differently distributed according to the cell types (neuroblastoma and hepatoma cells) collected from males (HUH7) or females (SH-SY5Y).

The tissue distribution of FADS3 was also tested on human samples and our results underlined the presence of multiple isoforms of the natural protein. We did not observe the predicted protein at 51 kDa but two heavier ones, which molecular mass probably results from a posttranslational modification. The most unexpected result came from the new isoform at 28 kDa only detected with the anti-NtermFADS3 antibody. This short isoform corresponds to the N-terminal end of FADS3 containing the cytochrome b5-like domain. No explanation can be postulated on the nature of such a size with regard to the known putative cleavage sites, but this may be the product of an alternative transcript with truncated and skipped exons as described by Park et al. (13). This isoform would be, thus, an enzymatic cofactor or a short protein involved in the regulation of the FA metabolism.

Alternatively, if different natural isoforms of FADS3 were present in humans, these results obtained on a commercial tissue blot have nevertheless to be considered as the result on one sample of one human being. Further experiments have to be investigated to confirm these results from
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