Fatty Acid Desaturase 3 (FADS3) Is a Specific Δ13-Desaturase of Ruminant trans-Vaccenic Acid

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Abstract
In mammalian species, the Fatty Acid Desaturase (FADS) gene cluster includes FADS1 (Δ5-desaturase), FADS2 (Δ6-desaturase), and a third gene member, named FADS3. According to its high degree of nucleotide sequence homology with both FADS1 and FADS2, FADS3 was promptly suspected by researchers in the field to code for a new mammalian membrane-bound fatty acid desaturase. However, no catalytic activity was attributed to the FADS3 protein for a decade, until the rat FADS3 protein was shown in vitro to be able to catalyze the unexpected Δ13-desaturation of trans-vaccenic acid, producing the trans11,cis13-conjugated linoleic acid isomer. This review summarizes the recent investigations establishing the FADS3 enzyme as a reliable mammalian trans-vaccenate Δ13-desaturase in vivo and tries to identify further unresolved issues that need to be addressed.

Introduction
In 2000, the genomic structure and location of the Fatty Acid Desaturase (FADS) gene cluster was reported on chromosome 11 in humans [1]. This cluster was described to include both the FADS1 and FADS2 genes, arranged in a head-to-head orientation, and coding respectively for the Δ5- and Δ6-desaturases which are key enzymes involved in the biosynthesis of ω6 and ω3 polyunsaturated fatty acids (PUFA) [2–4]. A third gene, subsequently named FADS3, was also identified on this cluster, with a tail-to-tail orientation with FADS2, and with a high degree of nucleotide sequence homology with both FADS1 (62%) and FADS2 (70%) [1]. Since then, FADS3 gene orthologs, similarly clustered with FADS1 and FADS2 genes have been reported in many other mammalian species [5–8].

According to its high homology and structural similarity to both FADS1 and FADS2 genes (12 exons and 11 introns) suggesting a gene duplication event during evolution, FADS3 was promptly suspected by researchers in the field to code for an additional mammalian membrane-bound fatty acid desaturase with unknown activity. Indeed, the predicted structure of the FADS3 protein is composed of an N-terminal cytochrome b5-like do-
main characterized by an HPGG motif shown to be essential for the Δ6-desaturase activity of FADS2 [9] and a C-terminal fatty acid desaturase domain containing three histidine boxes (\(^{186}\)HDLGH, \(^{223}\)HFQHH, and \(^{387}\)QIEHH in the rat) characteristic of front-end desaturases [10]. Additionally, many researchers were at this time looking for a hypothetical second isoform of Δ6- or Δ5-desaturase in mammals. This hypothesis was supported by an association study carried out in humans suggesting that at least one single nucleotide polymorphism (SNP) located within the FADS3 gene (RS1000778) was associated with variations in the ratio of red blood cell arachidonic acid to linoleic acid in a context of coronary artery disease [11, 12].

Other Genome Wide Association Studies focusing on the human FADS gene cluster have shown potential associations of FADS3 SNP with variations in triglycerides, HDL-cholesterol, LDL-cholesterol plasma levels and with sphingolipid metabolism [13, 14], suggesting the possible implication of FADS3 protein in lipid homeostasis. However, other recent studies have additionally shown that human FADS3 variants were associated with pregnancy outcomes and more specifically with gestation duration [15] or with cold resistance [16]. Therefore, hypotheses concerning the clinical significance of FADS3 are difficult to establish from such a wide variety of results. In addition, knowledge of FADS3 gene regulation is very scarce, especially in humans, although FADS3 expression may be regulated by nutritional factors, like dietary PUFAs. This has been shown in baboon liver and human HepG2 cells, where the expression of the FADS3 transcripts was upregulated by a mix of docosahexaenoic acid (DHA) (C22:6 n-3) and arachidonic acid (C20:4 n-6), whereas other desaturase (FADS1 and FADS2) mRNA levels were downregulated [17].

A Decade of Negative Results on FADS3 Function

Looking for a possible desaturase activity, our team speculated first that FADS3 might be able to specifically catalyze the Δ6-desaturation of C24:5 n-3 to C24:6 n-3 in the Sprecher pathway, leading to the biosynthesis of DHA [18]. However, we found no catalytic activity of the recombinant rat FADS3 on C24:5 n-3 nor on C24:4 n-6 [unpubl. data]. Instead, we showed that the rat Δ6-desaturase encoded by the Fads2 gene was not only able to act on the 18-carbon PUFAs (C18:3 n-3 and C18:2 n-6) but also on the 24-carbon very-long-chain PUFAs (C24:5 n-3 and C24:4 n-6) in this biosynthesis [19]. This result was also obtained by de Antueno et al. [20] with the human Δ6-desaturase.

Additional negative results showed that the recombinant rat FADS3 displayed no Δ6-desaturase activity on C18:3 n-3, C18:2 n-6, C18:1 n-9, and C16:1 n-7. Moreover, no Δ5-desaturase activity was shown on C20:3 n-6 and C20:4 n-3, no Δ8-desaturase activity was measured on C20:2 n-6 and C20:3 n-3, and no Δ9-desaturase activity was detected on C18:0, C16:0, C14:0, and C12:0 [21]. A similarly disappointing lack of desaturase activity of the recombinant FADS3 protein was reported on a variety of PUFA substrates by the team of Tom Brenna [22], although alternative mRNA transcripts of the Fads3 gene were described in baboons and later on in mice [6, 23], leading to the hypothesis of tissue-specific role of FADS3. These negative results were, however, consistent with those obtained in Fads1- and Fads2-null mice [24–26], showing that FADS1 or FADS2 deficiency was not rescued by the presence of FADS3 in terms of PUFA composition of tissues.

Although it was clear that FADS3 was not functionally redundant with FADS1, FADS2, or with Stearoyl-CoA Desaturase (SCD) [21], no catalytic activity and no metabolic function was consequently attributed to the FADS3 protein for a long time.

In vitro Identification of FADS3 as a trans-Vaccenate Δ13-Desaturase

In 2012–2013, after an additional screening of many potential fatty acid substrates including unusual fatty acids, we demonstrated in vitro (Fig. 1a, b) the specific activity of the recombinant wild-type rat FADS3 to catalyze the Δ13-desaturation of trans-vaccenic acid (trans\(11\)-C18:1) [21]. This catalytic activity produced a little known octadecadienoic acid with Δ11,13 double bonds (Δ11,13-C18:2), belonging to the family of conjugated linoleic acid (CLA) isomers (Fig. 1c). In rat hepatocytes incubated with trans-vaccenic acid, we confirmed that native FADS3 mRNA silencing specifically reduced the synthesis of the Δ11,13-C18:2 [21].

Other results obtained at this period showed that recombinant chimeric mutants of the rat FADS3 protein with one amino acid (Leu\(^{188}\)) substituted with the corresponding residue of FADS1 and FADS2, inside the first \(^{186}\)HDLGH histidine box (FADS3-L\(^{188}\)F like FADS1 and FADS3-L\(^{188}\)Y like FADS2), kept the same substrate specificity toward trans-vaccenic acid and a similar rate of
substrate to product conversion (Fig. 1a, b). No switch of activity of these two mutants was shown toward the known substrates of ∆5- and ∆6-desaturase (data not shown). Later on, eight amino-acid residues that determine the substrate specificity of rat ∆5- and ∆6-desaturase were identified by Watanabe et al. [27]. It would now be interesting to revisit the FADS3 sequence to measure the effect of such a substitution of these corresponding eight amino acids (Ser 215, His 217, Arg 222, Val 241, Ala 242, Ser 250, Ser 251, and Pro 349) on its trans-vaccenate ∆13-desaturase activity.

Fig. 1. Trans-vaccenate is ∆13-desaturated by rat FADS3 and produces the trans 11,cis13-18:2. a COS-7 cells were transfected with the plasmids pCMV empty (control cells), pCMV/FADS3 (wild-type), pCMV/FADS3-L 188F (mutant like FADS1), and pCMV/FADS3-L 188Y (mutant like FADS2), and incubated with trans-vaccenic acid (200 µM) for 24 h. The gas chromatography-mass spectrometry profiles of FAMEs extracted from COS-7 cells show the synthesis of the trans 11,cis13-18:2 in cells expressing wild-type and mutant FADS3 compared to control cells. The identity of each important fatty acid is indicated above its respective peak. b Immunoblot was assessed on COS-7 cell extracts transiently expressing the recombinant wild-type and mutant FADS3 proteins detected with a polyclonal antibody against FADS2/3. c Metabolic pathway of trans-vaccenate conversion into trans 11,cis13-18:2 catalyzed by the Δ13-desaturase activity of FADS3.
Reasonable Questions and Controversies about the Reliability of this trans-Vaccenate Δ13-Desaturase Activity

This in vitro unexpected trans-vaccenate Δ13-desaturase activity raised many questions that needed to be further addressed.

First, the identity of trans-vaccenic acid as the single substrate of this FADS3-catalyzed Δ13-desaturation was both surprising and intriguing. Other trans fatty acids like trans-palmitoleic acid (trans-9-C16:1, two carbons shorter than trans-vaccenic acid with a similar n-7 double bond), trans11-C20:1 (two carbons longer than trans-vaccenic acid with a n-9 double bond) and rumenic acid cis9,trans11-C18:2 (with an additional n-9 double bond) were not desaturated by the recombinant rat FADS3 protein [21]. Trans-vaccenic acid is well described to be present in dairy products and in partially hydrogenated vegetable oils. In ruminant species, it is produced as an intermediate from linoleic (C18:2 n-6) and α-linolenic acid (C18:3 n-3) from oleic acid (C18:1 n-9) using Δ12- and Δ15-desaturases [36], mammals typically lack these “methyl-end” fatty acid desaturases, but are able to use “front-end” desaturases (Δ6- and Δ5-desaturases) to introduce new double bonds into these essential dietary fatty acids that serve as precursors for the synthesis of longer PUFAs of the n-6 and n-3 series. FADS3 appeared therefore as an exception to the consensus pattern that higher animals, including humans, are devoid of desaturases inserting double bonds in the methyl-end of fatty acids. Overall, these in vitro data had at the time not been replicated in vivo, and the ability of mammals to produce the trans11,cis13-CLA by FADS3-catalyzed Δ13-desaturation of trans-vaccenic acid remained therefore elusive since its physiological reliability was reasonably questioned.

In addition, Fads3-knockout mice were successfully generated by the team of Tom Brenna [37, 38]. As opposed to Fads1- and Fads2-knockout mice which had been previously characterized [24–26], no overt phenotype was associated with the Fads3 gene deletion, and no difference with wild-type animals was shown on viability, growth rate, fertility, food intake, and physical appearance. Differences in brain and liver PUFA composition were detected immediately after birth, including less DHA in the brain of newborn knockout mice at specific postnatal days, but these small differences did not remain constant over time. Some of the results obtained in this study therefore suggested that FADS3 may have a role during early development, probably in maintaining C22:6 n-3 synthesis in the liver during the brain growth spurt, which corresponds to an intense period of PUFA demand for accretion, especially when dietary n-3 PUFAs are limited. However, the absence of a robust metabolic phenotype associated with the Fads3 gene deletion made it difficult to conclude and predict a potential function of FADS3. Moreover, when the mice were fed with trans-vaccenic acid, no in vivo Δ13-desaturation of the dietary trans-vaccenic acid was observed in control mice compared with Fads3-knockout mice [38].

In vivo Confirmation of the Ability of Ruminant and Nonruminant Mammals to Produce the trans11,cis13-CLA from Dietary trans-Vaccenic Acid

In order to address the questions raised by this unexpected in vitro trans-vaccenate Δ13-desaturase activity for FADS3, investigations were carried out in ruminant and nonruminant species.
In ruminant species, the \textit{trans}11,\textit{cis}13-CLA detected in milk was, until now, suspected to be exclusively of rumen origin [29, 39]. Ruminal biohydrogenation can indeed convert dietary \alpha-linolenic acid (\textit{cis}9,\textit{cis}12,\textit{cis}15–18:3 or \textit{cis}15:3) to \textit{cis}9,\textit{trans}11,\textit{cis}15-conjugated triene [28], which is subsequently metabolized to \textit{trans}11,\textit{cis}15-18:2 by incomplete bacterial hydrogenation. The last step corresponding to the isomerization of \textit{trans}11,\textit{cis}15-18:2 to \textit{trans}11,\textit{cis}13-CLA has been shown to be catalyzed in vitro by specific \textit{Butyrivibrio fibrisolvens} strains [40].

In a first study [41], we confirmed that the FADS3 gene is present in ruminant mammal genomic sequence databases. We also demonstrated that the \textit{Δ11,13}-CLA found in ruminant milk fat and the highly probable \textit{trans}11,\textit{cis}13-CLA isomer produced by rodent FADS3 possess exactly the same structural characteristics, based on gas chromatography-mass spectrometry analysis of their 4,4-dimethyloxazoline (DMOX) derivatives, and correspond therefore to the same fatty acid. In particular, the DMOX derivative of the \textit{trans}11,\textit{cis}13-CLA synthesized by FADS3-catalyzed \textit{Δ13}-desaturation of \textit{trans}-vaccenic acid and that of the \textit{Δ11,13}-CLA present in cow milk fat exhibited both a very specific and similar mass spectrum pattern, with their ion at m/z 250 amu (atomic mass unit) being less abundant than their ion at m/z 262 amu. Then, we showed that bovine mammary epithelial cells expressed both FADS3 and SCD1 (stearoyl-CoA desaturase 1) mRNA and were able to synthesize both the suspected \textit{trans}11,\textit{cis}13-CLA and \textit{cis}9,\textit{trans}11-CLA (rumenic acid) isomers when incubated with \textit{trans}-vaccenic acid. Finally, the concomitant presence of the suspected \textit{trans}11,\textit{cis}13-CLA isomer with Fads3 mRNA was shown in goat mammary tissue, whereas both were conversely very low or even absent in goat liver. Therefore, this first study provided several lines of evidence that, by analogy with the dual origin of rumenic acid, which derives partly from ruminal biohydrogenation of \alpha-linoleic acid and mainly from endogenous host \textit{Δ9}-desaturation of \textit{trans}-vaccenic acid, the \textit{trans}11,\textit{cis}13-CLA may also originate both from ruminal biohydrogenation and from direct FADS3-catalyzed \textit{Δ13}-desaturation of \textit{trans}-vaccenic acid in ruminant mammary tissue.

A second study aimed at investigating further the putative in vivo synthesis of \textit{trans}11,\textit{cis}13-CLA from dietary \textit{trans}-vaccenic acid in rodents [42]. Considering the previous results showing the presence of this specific fatty acid in milk fat and suggesting its synthesis in the ruminant mammary gland [41], investigations were focused mainly on the lactating mammary tissue of female rats. During 1 week of pregnancy and 2 weeks postpartum, Sprague-Dawley female rats were fed two diets either high (10.0% of fatty acids and 3.8% of energy intake) or low (0.4% of fatty acids and 0.2% of energy intake) in \textit{trans}-vaccenic acid. The \textit{trans}11,\textit{cis}13-CLA was specifically detected, formally identified, and reproducibly quantified (0.06% of total fatty acids) in mammary gland phospholipids of lactating female rats fed the high \textit{trans}-vaccenic acid-enriched diet. This result was consistent with Fads3 mRNA expression being significantly higher in the lactating mammary gland than in the liver, showing a correlation between Fads3 mRNA and \textit{trans}11,\textit{cis}13-CLA.

These two studies therefore provided several lines of evidence of the endogenous ability for lactating mammary tissue to generate in vivo the \textit{trans}11,\textit{cis}13-CLA from dietary \textit{trans}-vaccenic acid. This physiological conversion demonstrated the proof of concept for this new pathway and established the FADS3 enzyme as a reliable mammalian \textit{trans}-vaccenate \textit{Δ13}-desaturase in vivo. To explain the discrepancy between these results and the absence of in vivo \textit{Δ13}-desaturation of the dietary \textit{trans}-vaccenic acid in control mice compared with Fads3 knockout mice obtained by Zhang et al. [38], one should consider that the design of the study was markedly different. Indeed, mice were provided a chow diet mixed with 5 mg \textit{trans}-vaccenic acid/day, which is very low compared to the \textit{trans}-vaccenic acid supplementation realized in our study (about 500 mg TVA/day in the high-TVA diet for 3 weeks) [42]. Furthermore, only male mice were considered and not lactating females [38]. Consequently, only the liver and heart, and not the mammary gland, were collected for fatty acid composition analysis.

\textbf{Conclusion and Further Unresolved Issues}

In this review, we report findings obtained during the last 20 years of investigation into FADS3 protein function, since the description of the FADS3 gene in 2000. To pool all of the results together, FADS3 protein function does not appear to be as crucial as FADS1 (\textit{Δ5}-desaturase) and FADS2 (\textit{Δ6}-desaturase) protein products are for PUFA biosynthesis and metabolism. This conclusion is supported by the demonstration that global Fads3-knockout mice are viable [38]. It is also consistent with the only described catalytic \textit{Δ13}-desaturase activity of the FADS3 protein on \textit{trans}-vaccenic acid [21], which does not alter the current paradigm of mammalian PUFA dependence on essential dietary fatty acids (\alpha-linoleic and \alpha-linolenic acids). Available evidence indicates that the FADS3-catalyzed \textit{Δ13}-desaturation of \textit{trans}-vaccenic acid is proba-
bly not a major pathway, which is in agreement with its late discovery. The physiological importance of this catalytic activity is still questionable and its link with the indirect impact of FADS3 on brain DHA levels in knockout/wild-type mice [38] is not understood. One can speculate that competition of FADS3 and SCD1 for their common trans-vaccenic acid substrate may modulate the synthesis of rumenic acid (cis9,trans11-CLA). Since rumenic acid is itself known to be a substrate of Δ6-desaturase [43, 44] and potential competitor of the global conversion of α-linolenic acid to DHA, availability of endogenously synthesized rumenic acid may in turn modulate DHA synthesis.

FADS3 may also be physiologically more important in females than in males, as shown by the specific synthesis of trans11,cis13-CLA in the mammary gland of lactating female rats fed with a diet enriched in trans-vaccenic acid [42]. In addition, Fads3 mRNA is highly expressed at the embryo implantation site in the mouse uterus [45] and FADS3 may therefore play an important role during mouse early pregnancy [46], although the link with its Δ13-desaturase activity is not established.

To further increase the knowledge on FADS3 protein, it would be first interesting to confirm in vivo that the synthesis of trans11,cis13-C18:2 is fully inhibited in Fads3 knockout mice and/or activated in mice overexpressing Fads3, when fed with different levels of dietary trans-vaccenic acid. Studying the recombinant human FADS3 protein activity on trans-vaccenic acid may also be interesting to compare the affinity and specificity with the rodent FADS3. Indirect results in humans may be obtained by analyzing the presence of the trans11,cis13-C18:2 in maternal milk from lactating women with high dairy intake, since trans-vaccenic acid accounts for 1.5–4% of total fatty acids in ruminant milk. Additionally, it remains a key question as to whether the trans11,cis13-CLA has a functional role. Further nutritional studies focusing on this very particular fatty acid may show that it becomes important under specific physiological conditions.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

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Author Contributions

P.L. and V.R. wrote the manuscript.

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