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Molecular Cloning of a Functional *Fads2* Promoter from Zebrafish

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ABSTRACT

The FADS2 catalyzes the first rate-limiting step in the long chain-polyunsaturated fatty acids (LC-PUFAs) biosynthesis pathway by converting α-linolenic acid and linoleic acid into stearidonic acid and γ-linolenic acid via the ω-3 and ω-6 pathways respectively. In mammals, PPARα and SREBP-1c have been implicated in the polyunsaturated fatty acids (PUFAs) mediated transcriptional activation of FADS2 promoter. However, in zebrafish, not much is known regarding the regulation of fads2 transcriptional regulation. Here, in this study, five vectors containing different promoter regions were constructed in order to analyse putative promoter activities. Through truncation analysis, it was found that the 1.2 kb promoter was able to drive luciferase activity to an approximate 40-fold in HepG2 cells. Upon mutagenesis analysis, three sites which are the putative NF-Y, SREBP and PPAR binding sites were found to be essential in driving the promoter activity. Lastly, the 1.2 kb *fads2* promoter was able to direct EGFP expression specifically to the yolk syncytial layer (YSL) when transiently expressed in microinjected zebrafish embryos.

INTRODUCTION

Mammalian desaturases can be regulated by various transcription factors; however, two most prominent *trans*-factors affecting the expression of fatty acid desaturase 2 (*FADS2*) would be peroxisome proliferator-activated receptor α (PPARα) and sterol regulatory element-binding proteins 1c (SREBP-1c) [1,2]. Although, polyunsaturated fatty acids (PUFAs) are catalytic products of desaturases, its involvement in a feedback regulatory function via the PPARα and SREBP-1c on desaturases expression has been noted previously. Other than PPARα and SREBP-1c, key *cis*-acting elements and their corresponding trans-acting factors like nuclear transcription factor Y (NF-Y), specificity protein 1 (Sp1), GATA, CCAAT-enhancer-binding proteins (C/EBPα), retinoid X receptor (RXR) and carbohydrate-responsive element-binding protein (ChREBP) have been implicated in desaturases transcriptional activation. In fact, NF-Y and SREBP sites were remarkably conserved as previously identified in Salmon and Cod *FADS2* promoter [3].

In teleost like zebrafish, localization of high level of transcript in the liver and intestine in adult suggested that these were the primary PUFAs biosynthesis sites [4]. In early embryonic stages, localization of *fads2* transcripts in the primitive brain further proves the significance of PUFAs during early vertebrate neural development. As embryos progressed into the 72 hpf stage, the primary biosynthesis role was taken over by the now extensively differentiating hepatocytes in the primitive endodermal layer as shown through *in situ* hybridization [5]. This demands an extensive coordination among the brain, primitive liver and intestinal tissues. In this aspect, it is possible that different subsets of transcription factors are required to fully integrate the responses needed for tissue specific desaturation of PUFAs to occur in the continuously growing vertebrate.

In view of the apparent conservation of the biosynthesis pathway for PUFAs between mammals and teleost, it is reasonable to postulate that similar transcriptional regulation mechanism governs the expression of *fads2* in zebrafish. Due to the many desirable features of using zebrafish as a model such as the optical transparency and non-invasive procedures in gene

introduction, it is believed that its usage may shed more insight to the current paradigm regarding *fads2* transcriptional regulation. Hence this study serves as an initial step towards dissecting the molecular mechanism which governs *fads2* transcriptional regulation in zebrafish. In this part of the study, a 5' promoter region of zebrafish *fads2* was cloned and characterized. The relative importance of *cis*-elements present in this fragment was addressed using deletion and site-directed mutagenesis analyses. Lastly, the ability of the *fads2* promoter to drive tissue-specific expression was examined in zebrafish embryos by transient transgenesis.

MATERIALS AND METHODS

Cloning and sequence analysis

RNA ligase mediated-rapid amplification of cDNA ends (RLM-RACE) was performed using GeneRacer™ Kit (Invitrogen, USA) to amplify the full length of the 5' end of *fads2* cDNA and thus map the transcriptional start site of the gene. The primer sets used for nested PCR have been listed in **Table** 1. Fragments generated were then cloned into pGEM-T Easy vector (Promega, USA). A PCR based method was employed to amplify the promoter region of zebrafish *fads2* promoter region. The approximately 2.3 kb of promoter, upstream of the start codon was amplified using the iTaq™ DNA polymerase (iNtRON Biotechnlogy, Inc., Korea) according to the manufacturer's instructions.

Construction of promoter-reporter plasmids

In order to investigate the promoter activity of zebrafish *fads2* gene, eight different sequences of the *fads2* promoter region were amplified using primers specified in **Table** 1 and subcloned into *KpnI* and *XhoI* restriction sites of pGL3-basic luciferase reporter plasmid (Promega). Resulting plasmids were denoted as P2T (-1282 to +70), P23 (-1282 to +851), 485 (-485 to +1852), 167 (-167 to +1852), 95 (-95 to +1852), D47 (+47 to +1852), D570 (+570 to +1852) and D1003 (+1003 to +1852). Some constructs were designed to include partial sequence of the 1.7 kb intron I to assess the potential regulatory sequence present within this region.

Mutagenesis experiments were carried out using QuickChange™ II Site-Directed Mutagenesis kit (Stratagene, USA) according to the manufacturer's protocol with the following primers, 5'-ATGCTCGTTTGATGCAGGTA*CCT*GATTATTTCCCTTCACAAC-3' for NF-Y, 5'-CTAATTTTTACACATGAATT*CTT*TCACCGAGTGCACTTTTTAC-3' for PPAR and 5'-ATTGGCTGCTGGAGCTCGA*CCT*ATCTGTTCGGAATTCAGCGA-3' for SREBP. All promoter constructs were placed upstream of the luciferase reporter gene. For large scale preparation of plasmid DNA, the Qiagen® Plasmid Midi Kit was used to purify up to 100 µg of ultrapure plasmid which was used for transient transfection analysis.

Table 1. Sequence of oligonucleotides used in promoter-reporter constructs and the expected size of promoter fragments amplified.

Primer name	Forward primer (5'-3')	Reverse primer (5'-3')	Usage
GeneRacer™ 5'	GCACGAGGACACUGACAUGGACUGA	CCAGCATAGTGTCCGAGGAT	RLM-RACE
GeneRacer™ 5' Nested	GGACACTGACATGGACTGAAGGAGTA	TGCACCTCCTCCCAGGTGTAGC	RLM-RACE
P2T	GGTACCGTGCTTTTACAAACGATGTAT	CTCGAGTTGACTCACCGTCTCAGCAG	Promoter-reporter construct
P23	GGTACCGTGCTTTTACAAACGATGTAT	CTCGAGTAGGCATCATTTCCCCAAG	Promoter-reporter construct
485	GGTACCTGAACGTTTACAGTTAATTTTCGAG	CTCGAGTGCACCTCCTCCCAGGTGTAGC	Promoter-reporter construct
167	GGTACCCGCCCTTCCAAAGTTCTCTC	CTCGAGTGCACCTCCTCCCAGGTGTAGC	Promoter-reporter construct
95	GGTACCTTGGCTGCTGGAGCTCGAAT	CTCGAGTGCACCTCCTCCCAGGTGTAGC	Promoter-reporter construct
D47	GGTACCTGCCTGCTGAGACGGTGAGT	CTCGAGTGCACCTCCTCCCAGGTGTAGC	Promoter-reporter construct
D570	GGTACCTGTCAGGTGGCTTGCTTTGG	CTCGAGTGCACCTCCTCCCAGGTGTAGC	Promoter-reporter construct
D1003	GGTACCTGTGCCTCAAATTGATTTTATATGC	CTCGAGTGCACCTCCTCCCAGGTGTAGC	Promoter-reporter construct

Cell culture and transient transfection

The human HepG2 hepatoma cell lines was maintained in 1× MEM medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS), 1× non-essential amino acid and 1 mM sodium pyruvate. Cells were maintained at 37°C in 5% CO_2 with penicillin (100 U/ml) and streptomycin (100 mg/ml) added to the culture media. Cells were grown to 40-60% confluency prior to seeding for transfections or routine passages.

Transient transfection was carried out using Lipofectin® Reagent (Invitrogen, USA) as described by the manufacturer. Prior to the day of transfection, 500 µl of 2 X 10^4 cells/ml were seeded in each of the wells of 24-well plates. The cells were then incubated overnight at 37°C in a CO_2 incubator until it reached 40-60% confluency. For each transfection sample, 0.5 µl of Lipofectin® Reagent was first added to 24.5 µl of OptiMEM® I Serum Free Medium in a microcentrifuge tube and incubated for 45 mins at room temperature. Meanwhile, a second solution was prepared, which consisted of 1.0 µg of reporter-construct and 0.1 µg of pRL-TK plasmids diluted in 25 µl of OptiMEM® I Serum Free Medium. Transfections were performed in 250 µl OptiMEM® I Serum Free Medium for 24 h, and then cell medium was replaced with 500 µl fresh 10% FBS MEM medium. Cell lysates were collected 24 h post-transfection and used for the measurement of relative promoter activity of each construct with Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's protocol. The measurement was performed with a TD-20/20 Turner Designs luminometer (Turner Designs, USA) and luminescence reading taken. Data were presented as the mean ± S.E. of results from four independent transfections. The transfections were repeated at least once to ensure the reproducibility of the data.

Microinjection of EGFP plasmid into zebrafish embryos

In order to visualize the specificity of the *fads2* promoter in driving gene expression, promoter was further cloned into the pZsGreen1-1 plasmid (Clontech, USA). The *fads2* promoter-EGFP construct (25 ng/ml) was then diluted in 1× Danieau's buffer and 0.25% phenol red. Microinjection was carried out under a dissection microscope using a Nanoliter 2000 microinjector (World Precision Instrument, USA). Embryos were monitored for GFP expression under a fluorescent microscope (Leica, Germany) from 30 hpf onwards.

RESULTS

Conserved *cis*-acting elements are identified in zebrafish *fads2* promoter

When 2.0 kb of zebrafish *fads2* 5' flanking sequence derived from genbank (CU694371.13) was compared to salmon (AY736067.2), mouse (AC135670.3), and human *fads2* (AP002380.3), with regard to the *cis*-elements predicted by MatInspector (www.genomatix.de/matinspector.html), conserved modules of binding sites for transcription factors PPAR, SREBP, RXR and NF-Y factors were evident (**Fig.** 1). At least two NF-Y response elements which lie in close proximity to each other while lying proximal to TSS +1 were identified in the 5' promoter of all species (**Fig.** 1). In all species observed, at least two PPAR response element (PPRE) seems to be present within the 5' promoter region and the location of these binding sites seems highly conserved with at least one PPRE within 500 bp of upstream of TSS +1 (**Fig.** 1).

In this study, it was also found that SREBP binding site lies in close proximity to NF-Y which suggest co-binding may be involved in transcription regulation (**Fig.** 1). Two putative

TATA boxes as indicated by MatInspector software have been identified within the 2.0 kb promoter sequence whereby the distal is located at -293/-289 position while the proximal located at -31/-27 position relative to the TSS +1 site (**Fig.** 1). Collectively, the *in silico* comparison of the zebrafish *fads2* promoter to human, mouse, and salmon revealed highly conserved *cis*-elements that are likely to play a role in modulating their expression.

RACE-PCR of the 5'-end identified a product of the size 174 bp which was then cloned into pGEM-T Easy vector (Promega, USA) and sequenced. The result was then blasted for confirmation and aligned with 99% of identity to the initial sequence of *fads2* revealed by Hastings *et al*., 2001 (result not shown). This ruled out the possibilities of a second transcription start site (TSS +1) which frequently occurs in vertebrates.

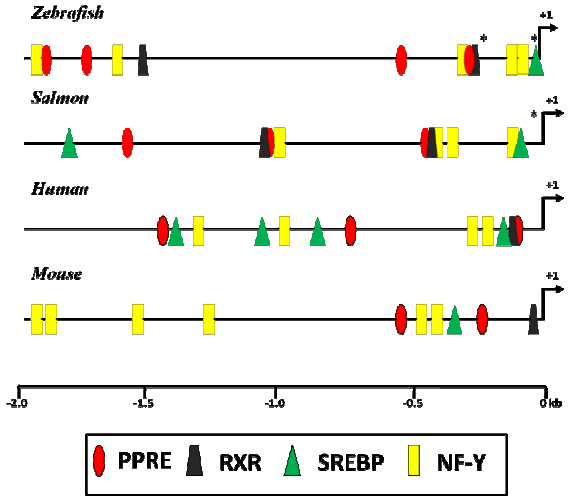

Fig. 1. Conservation of *cis*-elements predicted in the 5' flanking promoter region of zebrafish *fads2* (CU694371.13) in comparison to the equivalent 2.0 kb region in the gene promoter of salmon (AY736067.2), human (AP002380.3), and mouse (AC135670.3) derived from genbank. The location of putative TATA boxes identified in proximity to the transcription start site +1 are indicated by asterisk (*).

Deletion analysis of *fads2* promoter in HepG2 cell line

Eight promoter fragments of different sizes were PCR-generated in order to determine the functional region of the zebrafish *fads2* promoter. The purified fragments were subsequently cloned into pGL3 vector and tested for its transcriptional activity in HepG2 cell line, which endogenously expresses high level of PPARα transcript [6-8]. These plasmids containing the corresponding promoter fragments upstream to the luciferase reporter gene were transiently transfected into HepG2 cells after which cells were harvested for dual-luciferase assay.

As shown in **Fig.** 2, the 1282 bp of *fads2* promoter conferred the highest level of luciferase activity which was able to activate the luciferase reporter to a level approximately 40-fold of the promoterless pGL3 Basic vector. Since *fads2* gene possesses a 1.7 kb in length of Intron I, part of the region was also included in the deletion analysis. The inclusion of 851 bp downstream from TSS +1 was found to significantly reduce the promoter activity to approximately 5-fold possibly indicating a negative regulator binding site. Further truncation of the zebrafish *fads2* promoter to 485 fragment as compared to P23 fragment did not affect the reporter gene activity significantly (**Fig.** 2). Even when the distal PPAR binding sites (upstream of -485) were deleted, the luciferase activity derived from them was comparable to that of the P23 fragment. However, deletion analysis revealed that the loss of a fragment between position -485 and -167 render the loss of transcriptional activity near to its basal level. This can probably be attributed to the loss of the distal TATA binding site which serves as the assembly site for basal transcriptional machinery [9]. This may also suggest that critical transcriptional complexes binding sites may lie within this region of the 1.2 kb promoter. Taken together, these results indicate that the 485 bp fragment of the *fads2* promoter is sufficient to drive the basal promoter activity and that the distal PPRE is dispensable for basal promoter activity. Collectively, transient transfection studies in HepG2 demonstrated the functionality of the 1.2 kb *fads2* promoter in a mammalian cell line.

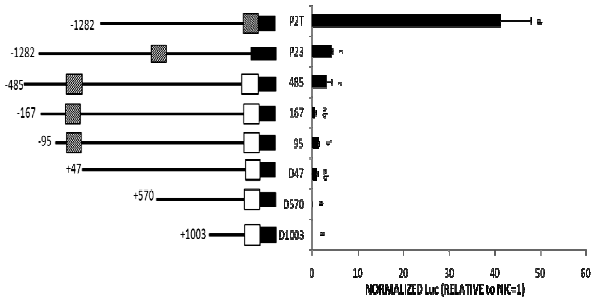

Fig. 2. Structure and deletion analysis of zebrafish *fads2* gene promoter. Deletion constructs are represented on the left. Non-coding Exon I is indicated with shaded box, Exon II (includes ATG) with open boxes while luciferase coding with closed boxes. Sequence is numbered relative to the first base of the transcription start site, assumed to be the first base of the 5' non-coding exon. Promoter activity of constructs are represented on the right with the values indicating normalised activity (firefly luciferase: *Renilla* luciferase) relative to a construct containing no insert. Results are mean ± S.E. (n=3). Mean values were subjected to one-way ANOVA and Tukey's test, $P < 0.05$. Mean values with different alphabets are significantly different. NIC, No insert construct.

Mutagenesis of selected *cis*-elements in the 1.2 kb zebrafish *fads2* promoter

To further explore the significance of the presence of several *cis*-elements occurring in the 1.2 kb *fads2* promoter, these putative binding sites were mutagenized individually by site-directed mutagenesis. Three putative *cis*-elements were chosen for mutagenesis, namely the PPAR (-345), NF-Y (-98) and SREBP (-80) recognition site within the -485 region which account for significant loss of promoter activity upon deletion (**Fig.** 2). The mutagenesis was introduced directly into the core recognition sequences whereby three nucleotides were substituted for each selected *cis*-elements respectively (**Fig.** 3).

Transient transfections into HepG2 cells showed that mutagenesis in the putative NF-Y binding site reduced the luciferase activity from 40-fold to 15-fold of basal level. Significant reduction of promoter activity was also observed when either of putative PPAR or SREBP were mutated which observed the fall of luciferase activity from 40-fold to 6-fold (**Fig.** 3).

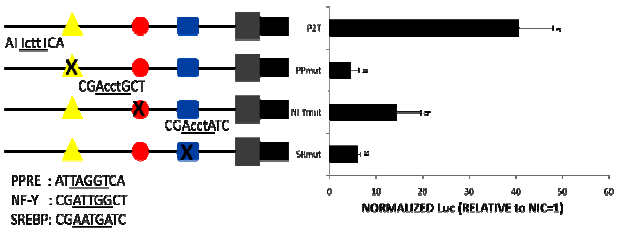

Fig. 3. Effect of mutation on zebrafish *fads2* gene promoter. Various mutations of the zebrafish promoter deletion P2T (-1282 to +1) were generated. Relative positions of putative PPAR (▲), NF-Y (●) and SRE (▬) sites are indicated. Promoter activity of constructs was represented on the right with values indicating normalised activity (firefly luciferase: *Renilla* luciferase) relative to a construct containing no insert. Original sequences of the mutated sites were shown at the lower left hand corner. Results are mean ± S.E. (n=3). Mean values were subjected to oneway ANOVA and Tukey's test, P<0.05. Mean values with different alphabets are significantly different. NIC, No insert control.

Assessment of promoter activity by transient transgenesis in zebrafish embryos

To investigate whether the 1.2 kb fragment was sufficient to direct tissue-specific expression in zebrafish, it was cloned into pZsGreen1-1 reporter vector (Clontech, USA). When the *fads2*-GFP plasmid was microinjected into zebrafish embryos at 1-2 cell stages, the 1.2 kb promoter was able to target GFP expression specifically to the yolk syncytial layer (**Fig.** 4) indicating that the promoter is fully functional in driving gene expression even when introduced *in vivo*. Interestingly, GFP was not detected in earlier stages as reported previously through ISH approach but only occur at 120 hpf.

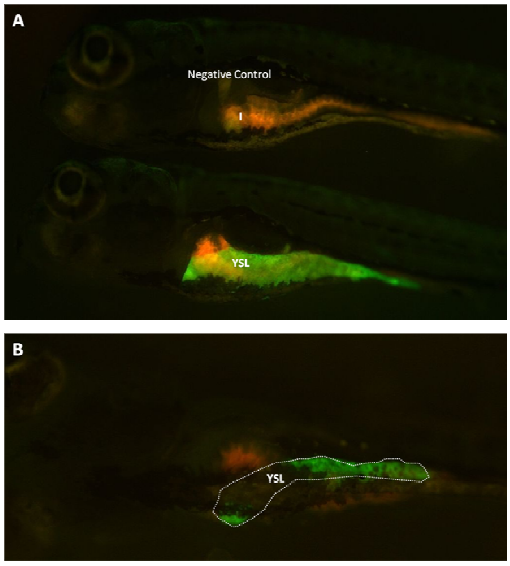

Fig. 4. The 1.2 kb zebrafish *fads2* promoter targets GFP specifically to the yolk syncytial layer of the 120 hpf zebrafish embryos. (A) Dorsal view of both embryos injected with no insert control (upper) and *fads2*-GFP plasmid (lower). (B) Ventral view of 120 hpf embryo with GFP observed in the yolk syncytial layer. Orange colour indicates auto-fluorescence in the intestinal tract. I, intestine; YSL, yolk syncytial layer.

DISCUSSION

This study reports the cloning and functional characterization of a 1.2 kb of putative promoter fragment of zebrafish *fads2* gene. Prior to molecular cloning, bioinformatics analysis revealed conserved modules of *cis*-elements within the *fads2* promoter as compared to the 2.0 kb regulatory sequence to that of other species including salmon, mouse and human (**Fig.** 1). By transient transfection studies, it was shown that this 1.2 kb promoter fragment was capable of directing luciferase activity into HepG2 cell line and that the activity was largely dependent on the proximal 485 bp region. Site directed mutagenesis revealed that *trans*-factors such as SREBP and PPARα were both essential in driving the promoter activity of the P2T fragment. Most importantly, when this promoter fragment was introduced into zebrafish embryos, it could drive tissue specific EGFP expression in a developmentally regulated fashion.

From the previous study of human FADS2 promoter, transcription factors such as NFY, SREBP [10], and PPAR [11] were found to be essentially important in its transcriptional regulation. In the zebrafish *fads2* promoter, bioinformatics analysis showed that these *cis*-elements could be mapped with confidence. Nevertheless, *cis*-elements for factors such as PPAR and SREBP which almost always requires the presence of co-activator like RXR [11] and NF-Y [12] were shown to lie in close proximity to the former. In most cases, SREBP-activated genes require the presence of NF-Y bound to one or more adjacent CCAAT element which is located in close proximity, within 21 bp of the SRE [13-15].

In order to further verify the absence of a second transcription start site which frequently occurs in mammals, RACE-PCR identified only one TSS+1 site which lies within the first exon of *fads2* gene. The presence of multiple transcriptional start sites in the 5' UTR has been a common phenomenon in abundant of genes including the PPARα, PPARγ and PPARβ [16,17]. This observation has often been linked to the absence of a TATA box upstream of the TSS+1 (18). Although it has not been determined whether the mouse FADS2 gene carries any extra TSS, the TATA-less human FADS2 regulatory region was found to possessed two TSS+1 while FADS3 with three TSS+1 and multiple alternative transcripts [11].

In zebrafish, *fads2* expression has also been detected in the brain, yolk syncytial layer [4], intestine and liver [5]. Nevertheless, when introduced into zebrafish embryos by transient transgenesis, our 1.2 kb promoter fragment does not direct EGFP expression into all these tissues except in the yolk syncytial layer (**Fig.** 4). Previously, several genes which appeared in the liver at the larval stage have been implicated with expression in the yolk syncytial layer in its ealier developmental stages. Most of these are lipogenic genes such as the *fabp10* [19], *cas* [20], *tfa* [21], *A2ML* [22], 2008), *Apo14* [23] and *mtp* [24] which has been known for their roles in lipid transport during zebrafish early developmental stage from the yolk. Indeed, upon the organogenesis of liver and intestine at 48 hpf, these lipogenic genes shared expression in both the endodermal organs and yolk syncytial layer. Hence, it may be suggested that the critical expression domain for endodermal organs and brains may fall outside the control of regulatory complex within the cloned promoter and additional *cis*-elements may be required to mimic the whole gene expression domain.

Previously, SREBP-1c has been implicated in engaging in a dual regulation mechanism with PPARα on mouse desaturases

(ie. Δ5- and Δ6- FADS) [1]. In general, PPARα induces genes in the fatty acid catabolism where it regulates genes involved in fatty acid uptake, conversion into acyl-CoA esters, and further degradation via the β-oxidation pathways [25,26], the coupling action of PPARα-SREBP1c can only be observed in the regulation of desaturases expression alone [2,11]. Hence, in agreement with the findings from human FADS2 promoter [2,11], in this study, both PPARα and SREBP-1c were found to be the prominent *trans*-factor necessary for the feedback regulation of *FADS2* transcriptional activation as shown through *in silico* analysis (**Fig.** 1) and mutagenesis analysis (**Fig.** 3).

Although SREBP-1c has been previously known in regulating desaturases, PUFAs were known to represses its transcript maturation of SREBP-1c and subsequent protein level through a negative feedback loop [27,28]. Hence, this places the desaturases in a distinct position among lipogenic genes whereby a diet regime of fasting-refeeding render an unchanged level of desaturases transcript levels. However, once SREBP-1c was taken out of this mechanism through gene knockout, transcript level of both desaturases elevated considerably with a diet lack in PUFAs [27,28]. This thus suggests the presence of a second regulatory system, where the PPARα is capable of sustaining the desaturases expression level even in this PUFAs lacking state [29].

Under PUFAs deficient condition, PPARα target genes induction requires the generation of endogenous ligands other than the essential fatty acid to sustain expression of desaturases. Although the exact identity of such ligand remains to be determined, [30] has suggested the lysophospholipids as a potential candidate. This molecule has previously also been identified as ligand for PPARγ [31]. Under normal conditions, the membrane phospholipids constantly undergo a series of acylation reactions which produce free PUFAs and lysophospholipids and subsequently their re-esterification back into the membrane [32]. The ratio between PUFAs and lysophospholipids establishes an equilibrium function which is basically maintained by the acyltransferases and transacylases enzymes and can be shifted upon dietary PUFAs manipulation [32]. Hence as a PUFAs free diet were fed, the lack of C18 and C20 PUFAs may shift this equilibrium towards which favours the lysophospholipids which in turn act as an endogenous PPARα ligand [30].

Taken together, expression of desaturases could be regulated by two distinct mechanisms *i.e*. PPARα and SREBP-1c. Both *trans*-factors possess opposing activation properties which depends largely on the nutritional status imposed [1] whereby in the fasting state, PPARα constitutively exerts control over basal expression of desaturases while during the refeeding state, SREBP-1c inductively suppresses desaturases expression mediated by PUFAs. This dual regulation mechanism as stated by [1] allows a stable production and maintenance of PUFAs at an optimal level for proper cellular functions.

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