Pparγ is Involved in the Transcriptional Regulation of Liver LC-PUFA Biosynthesis by Targeting the Δ6Δ5 Fatty Acyl Desaturase Gene in the Marine Teleost *Siganus canaliculatus*

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Keywords:
LC-PUFA, fatty acyl desaturase, transcriptional regulation, *Siganus canaliculatus*

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Abbreviations

ALA, α-linolenic acid (18:3n-3)
ARA, arachidonic acid (20:4n-6)
CPT-1, carnitine palmitoyl transferase-1
DHA, docosahexaenoic acid (22:6n-3)
EFA, essential fatty acid
EPA, eicosapentaenoic acid (20:5n-3)
Fads, fatty acyl desaturases
FABP, fatty acid binding protein
FO, fish oil
HEK 293T cel, human embryonic kidney 293T cell
HNF4α, hepatocyte nuclear factor 4α
LC-PUFA, long-chain polyunsaturated fatty acids
LPL, lipoprotein lipase
LXR, liver X receptor
Pparγ, peroxisome proliferator activated receptor γ
PUFA, polyunsaturated fatty acids
Q-PCR, quantitative polymerase chain reaction
SCHL, Siganus canaliculatus hepatocyte line
Srebp, sterol regulatory element binding protein
TF, transcription factor
TSS, transcription start site
VO, vegetable oil
Abstract

As the first marine teleost demonstrated to have the ability of long-chain polyunsaturated fatty acids (LC-PUFA) biosynthesis from C\textsubscript{18} PUFA precursors, the rabbitfish *Siganus canaliculatus* provides us a unique model for clarifying the regulatory mechanisms of LC-PUFA biosynthesis in teleosts aiming at the replacement of dietary fish oil (rich in LC-PUFA) with vegetable oils (rich in C\textsubscript{18} PUFA precursors but devoid of LC-PUFA). In the study of transcription regulation of gene encoding the Δ6Δ5 fatty acyl desaturase (Δ6Δ5 Fads), a rate-limiting enzyme catalyzing the first step of LC-PUFA biosynthesis in rabbitfish, a binding site for the transcription factor (TF), peroxisome proliferator-activated receptor γ (Pparγ), was predicted in Δ6Δ5 fads2 promoter by bioinformatics analysis, and thus the present study focused on the regulatory roles of Pparγ on Δ6Δ5 fads2. First, the activity of the Δ6Δ5 fads2 promoter was proved to be down-regulated by *pparγ* overexpression and up-regulated by treatment of Pparγ antagonist (GW9662), respectively, in HEK 293T cells with the dual luciferase reporter assay. Pparγ was further confirmed to interact with the promoter by electrophoretic mobility shift assay. Moreover, in *S. canaliculatus* hepatocyte line (SCHL) cells, GW9662 decreased the expression of *pparγ* together with increase of Δ6Δ5 fads2 mRNA. Besides, Δ6Δ5 fads2 expression was increased by *pparγ* RNAi knock-down and reduced by its mRNA overexpression. Furthermore, knock-down of *pparγ* induced a high conversion of 18:3n−3 to 18:4n−3 and 18:2n−6 to 18:3n−6, while *pparγ* mRNA overexpression led to a lower conversion of that, and finally a significant decrease of 20:4n-6(ARA), 20:5n-3(EPA) and 22:6n-3(DHA) production. The results indicate that Pparγ is involved in the transcriptional regulation of liver LC-PUFA biosynthesis by targeting Δ6Δ5 fads2 in rabbitfish, which is the first report of Pparγ involvement in the regulation of LC-PUFA biosynthesis in teleosts.
Introduction

Long-chain (≥C20) polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) are physiologically essential fatty acids (EFA) for vertebrates, playing important roles in gene regulation, signal transduction, lipogenesis and cell membrane fluidity (Benitezsantana et al., 2007, Schmitz and Ecker, 2008, Uauy et al., 2001, Galli et al., 1994). From a human nutrition standpoint, the so-called “omega-3” (n-3) LC-PUFA such as EPA and DHA have beneficial roles in a variety of human pathologies and disorders (Lorente-Cebrián et al., 2013). Fish, especially marine species, are major sources of n-3 LC-PUFA in the human diets. And, with over half of the production of fish now deriving from aquaculture, research aiming to understand LC-PUFA metabolism of fish farmed species has been driven to ensure the production of high quality (high n-3 LC-PUFA) products (Tocher, 2003). Traditionally, inclusion of high levels of marine ingredients such as fishmeal, but particularly fish oil (FO), in aquafeeds has delivered high n-3 LC-PUFA contents in farmed fish. However, there currently exists a tendency in the farming industry of some species such as the Atlantic salmon showing a reduction of n-3 LC-PUFA in the final product (Bell et al., 2001). This is mostly due to the replacement of dietary FO by alternative oil sources such as vegetable oils (VO) since the latter are devoid of LC-PUFA and rather contain high levels of shorter-chain fatty acids (FA) such as the C18 polyunsaturated fatty acids (PUFA) linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) (Sargent et al., 2002). This problem can aggravate in fish species with limited capacity to convert C18 PUFA contained in dietary VO into LC-PUFA, thus compromising not only its nutritional value for the human consumer but also the dietary provision of essential nutrients for normal growth and development in captivity (Tocher, 2015).

The ability of a certain vertebrate species to convert C18 PUFA into C20-22 LC-PUFA varies among species and depends upon the complement and function of two key enzymes, namely fatty acyl desaturases (Fads) and elongation of very long-chain fatty acid (Elovl) proteins (Castro et al., 2016). Unlike mammals, teleost fish appear to possess only one type of fads-like genes in their genome, this being fads2 (Castro et al., 2012). Consistent with the mammalian FADS2, Fads2 studied from many teleosts are also Δ6 desaturases but, interestingly, many teleost Fads2 have acquired other desaturase capabilities such as the bifunctional Δ6Δ5 desaturases, monofunctional Δ5 desaturases and Δ4 desaturases (Castro et al., 2016). Moreover, Elovl encoding genes with
relevant roles in the biosynthesis of LC-PUFA in teleosts include Elovl2, Elovl4 and Elovl5, of which Elovl4 and Elovl5 are present in virtually all teleosts (Castro et al., 2016). Regulatory mechanisms controlling the expression of teleost fads and elovl have been investigated in order to understand the mechanisms underlying the response of fish to dietary components. With regards to fads2, transcriptional regulation by transcription factors (TF) such as sterol regulatory element binding proteins (Srebp), nuclear factor Y (NF-Y), liver X receptor (Lxr) and specificity protein 1 (Sp1) have been establish for a range of species (Carmona-Antoñanzas et al., 2014, Geay et al., 2012, Xu et al., 2014, Zheng et al., 2009). Particular progress on elucidating the mechanisms involved in fads2 regulation of teleosts has been made in rabbitfish S. canaliculatus, the first marine teleost demonstrated to have the ability of bioconverting C18 PUFA to LC-PUFA (Li et al., 2010, Li et al., 2008, Monroig et al., 2012) and possessing two distinct Fads2 with Δ4 and Δ6Δ5 desaturase capabilities (Li et al., 2010). Thus, the expression of the rabbitfish fads2 is controlled at transcriptional level by Lxr and Srebp1, regulating both Δ4 and Δ6Δ5 fads2 in rabbitfish liver (Zhang et al., 2016a), and by hepatocyte nuclear factor alpha (HNF4α) (Dong et al., 2016), specifically targeting Δ4 fads2 expression. Moreover, unique post-transcriptional regulatory mechanisms have been further established, with miR-17 exhibiting its action directly on Δ4 fads2 (Zhang et al., 2014), and miR-33, whose more indirect action involves regulation of srebp1 (Zhang et al., 2016b).

Peroxisome proliferator-activated receptors (Ppar) play important roles in lipid metabolism (Desvergne et al., 2006, Poulsen et al., 2012, Tontonoz and Spiegelman, 2008) and studies in fish have confirmed that Ppar regulate genes of fatty acid oxidation and deposition among other pathways (Leaver et al., 2008). In general, PPAR can be activated by natural or artificial ligands, then heterodimerized with retinoid X receptor (RXR), and subsequently bound to PPAR response element (PPRE) in target genes (Adeghate et al., 2011). Ppar protein family consists of three isotypes including PPARα, PPARβ (δ) and PPARγ. In mammals, PPARα is involved in stimulating fatty acid oxidation, PPARβ plays a role in the regulation of lipoprotein transport system (Desvergne et al., 2006), while PPARγ regulates glucose and lipid homeostasis, and induces lipid accumulation (Gurnell, 2005, Tontonoz and Spiegelman, 2008). With regards to specific actions within the LC-PUFA biosynthesis, PPARα, along with SREBP-1c, was demonstrated as an important TF in the feedback regulation of murine Fads2 (Matsuzaka et al., 2002), whereas
PPARγ was inferred as a possible target for Lxr (Zhang et al., 2016a). Nevertheless, the mechanisms of transcriptional regulation by Pparγ on LC-PUFA biosynthesis in teleosts remain mostly unclear.

Recently, three Ppar genes including ppara, pparβ (δ) and pparγ were cloned from rabbitfish, and the relatively high expression of ppara and pparγ in liver and intestine, major sites for LC-PUFA biosynthesis in fish (You et al., 2017), suggested potential regulatory roles in these pathways. Supplementation of the Ppar agonist fenofibrate to rabbitfish primary hepatocytes increased the expression of pparγ with depressed expression of Δ6Δ5 fads2, which indicated that Pparγ may have a regulation role on Δ6Δ5 fads2 (You et al., 2017). Moreover, preliminary investigations allowed us to identify a potential Pparγ binding site on Δ6Δ5 fads2 promoter. To further clarify the roles of Pparγ in the regulation of genes encoding pivotal enzymes within rabbitfish LC-PUFA biosynthesis, the present study investigated the transcription regulation of pparγ on Δ6Δ5 fads2 expression. First, the promoter activity of Δ6Δ5 fads2 was studied by overexpression pparγand supplementation of Pparγ antagonist (GW9662) in HEK 293T cells. Second, the gene expression pattern of pparγ and Δ6Δ5 fads2 was measured in S. canaliculatus hepatocyte line (SCHL) by mRNA overexpression or inhibition of pparγ. Furthermore, the capability of SCHL cells in bioconverting C18 PUFA to LC-PUFA was evaluated under pparγ overexpression or siRNA knock-down. These data will increase our knowledge of regulatory mechanisms of LC-PUFA biosynthesis in teleosts, and provide useful information for the successful replacement of VO in feeds for farmed fish.

Materials and methods

Cell lines and cell culture

Human embryonic kidney (HEK 293T) cells (Chinese Type Culture Collection, Shanghai, China) were grown at 37 °C with 5 % CO2 concentration in high glucose Dulbecco's Modified Eagle Medium (DMEM) (GlutaMAX) (Gibco, Life Technologies, USA) supplemented with 10 % fetal bovine serum (FBS, Sijiqing Biological Engineering Material Company, Hangzhou, China). S. canaliculatus hepatocyte line (SCHL) recently established in our lab (Y et al., 2017) was grown at 28 °C using Dulbecco's modified Eagle's medium (DMEM)-F12 medium (Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco) and 0.5 % rainbow trout Oncorhynchus mykiss.
Pparγ overexpression and detection of its influence on the Δ6Δ5 fads2 promoter activity

A potential Pparγ binding site was predicted in the rabbitfish Δ6Δ5 fads2 promoter by using online software TRANSFAC® and JASPAR® (Fig. 1) (Dong et al., 2018). To confirm the effects of Pparγ on the Δ6Δ5 fads2 promoter activity, the overexpression vector pcDNA3.1-pparγ was constructed by cloning the rabbitfish pparγ open reading frame (ORF) (GenBank: JF502072.1) DNA fragment into the pcDNA3.1 vector (Invitrogen).

The rabbitfish Δ6Δ5 fads2 promoter (2044 bp) was cloned from the Δ6Δ5 Fad mRNA of S. canaliculatus (GenBank: EF424276.2) (Dong et al., 2018). The promoter reporter vector was constructed with the Δ6Δ5 fads2 promoter fragment and pGL4.10, and the Δ6Δ5 fads2 Pparγ binding site-directed mutant of the Δ6Δ5 fads2 promoter was constructed with the mutation site in the middle of the primer. The promoter reporter vector contained the Firefly luciferase gene. Subsequently, the overexpression vector pcDNA3.1-pparγ was co-transfected with the Δ6Δ5 fads2 promoter reporter vector into HEK 293T cells seeded in 96-well cell culture plates by Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA), and the content in transfection complex per well was listed as following: pcDNA3.1-pparγ (50 ng), Δ6Δ5 fads2 promoter (100ng) or Δ6Δ5 fads2 Pparγ site-directed mutant, internal control vector pGL4.75 (0.02 ng) and Lipofectamine 2000 Reagent (0.25 μL). The HEK 293T cell is a commercially available cell line commonly used for studying the promoter activity involving dual-luciferase reporter assays. After the cells were grown to about 80% confluency, the transfection assay was conducted. At 48 h post-transfection, the promoter activities were measured by Dual-Glo Luciferase Assay system (Promega, USA) according to manufacturer’s instructions. Specifically, 75 μl of Dual-Glo Luciferase Assay Reagent were added to each well, the plate was incubated at room temperature for 10 min. Then measure firefly luminescence on a Tecon microplate reader (Tecon, Switzerland), followed by the addition of Dual-Glo Stop & Glo Reagent to the plate and incubate at room temperature for 10 min. Finally, chemical luminescence intensity was detected in duplicate readings using a microplate reader (InfiniteM200 Pro, Tecan, Switzerland). The promoter activity was calculated by the ratio of luciferase to renilla intensity in each experiment well, and empty vector pGL4.10 was used as a negative control.
Effect of Pparγ antagonist on the Δ6Δ5 fads2 promoter activity

To detect the effects of a Pparγ antagonist (GW9662) on the Δ6Δ5 fads2 promoter activity, 100 ng of Δ6Δ5 fads2 promoter reporter vector and 0.02 ng of pGL4.75 were co-transfected into HEK 293T cells. The transfection, done as the detailed above for the pparγ overexpression assay, lasted for 24 h, period after which the cell culture medium was replaced with DMEM + 10% FBS containing GW9662 at a final concentration of 20 μM or an equivalent volume of DMSO (concentration did not exceed 0.1 %, v/v). Then, 48 h post-transfection, the promoter activity was measured as the detailed above for the pparγ overexpression assay.

Electrophoretic Mobility Shift Assay (EMSA)

To further confirm the binding between Pparγ and the Δ6Δ5 fads2 promoter, primers were designed according to the Pparγ binding region in the Δ6Δ5 fads2 promoter and 5′-biotin labeled for production of EMSA probes (Table 1). The experimental probe was produced in a 100 μL annealing reaction system including 40 μL nuclease-free water, 40 μL annealing buffer for DNA Oligos (5X) (Beyotime, China), 20 μL primer F for EMSA (50 μM), 20 μL primer R for EMSA (50 μM). The extraction of nuclear protein from the rabbitfish hepatocytes was conducted using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China) according to the manufacturer’s instructions. For the binding reaction, 6 μg of nuclear protein were incubated in a total volume of 10 μL with binding buffer containing 2 μL of biotin labeled probe (BP) (0.1 pM). Competing reaction and cold competing reaction were performed using a 100-fold excess of unlabeled probe (UP) and unlabeled mutant probe (UTP). The reaction was carried out in lane 1 (no proteins, 5′ biotin labeled probe), lane 2 (rabbitfish hepatocyte nucleus proteins, 5′ biotin labeled free probe), lane 3 (rabbitfish hepatocyte nucleus proteins, unlabeled competitor probe, 5′ biotin labeled free probe), lane 4 (rabbitfish hepatocyte nucleus proteins, unlabeled mutant competitor probe, 5′ biotin labeled free probe). Following addition of 2 μL sample buffer, the protein-DNA complexes were resolved on a 4 % non-denaturing polyacrylamide gel in 0.5×TBE buffer at 380 mA for 1 h and then transferred to nylon membrane. Finally, biotin-labeled DNA was detected by chemiluminescence using the Chemiluminescent EMSA Kit (Beyotime, China) according to manufacturer’s protocol.
Effect of Pparγ antagonist on Δ6Δ5 fads2 gene expression in SCHL cells

To determine the effects of Pparγ antagonist on Δ6Δ5 fads2 gene expression, the SCHL cells were seeded in 6-well plates at a density of 2 × 10^6 cells per well and cultured for 24 h. Then the medium was replaced with fresh DMEM + 10% FBS in addition of GW9662 with a final concentration of 20 μM. The control group was treated with the same volume of medium without GW9662. All treatments were run in triplicate wells. After 48 h incubation, the cells were collected and lysed for RNA isolation under the instructions of TriPure RNA Isolation Reagent (Roche).

Effect of siRNA on pparγ and Δ6Δ5 fads2 gene expression in SCHL cells

To further clarify the influence of Pparγ on Δ6Δ5 fads2 regulation, an RNAi fragment targeting the rabbitfish Pparγ was run by transfection into SCHL cells (Lipofectamine 2000, Invitrogen). The siRNA sequence included seq 1 (5’-CCUCCCAACAGUCAGAUUTT-3’) and seq 2 (5’-UUCUCGAACGUGUCACGUTT-3’), seq 1 was 884 bp to ATG and set as experiment group, while seq 2 was negative control. The siRNA (0.2 nmol) was transfected into the SCHL cells after 24 h incubation, after 24 h transfection, the cells were collected and lysed for RNA and lipid extraction. The survival rate of the cells was more than 95 % over the course of the whole operation.

Influence of pparγ mRNA overexpression on Δ6Δ5 fads2 gene expression in SCHL cells

The influence of Pparγ on Δ6Δ5 fads2 expression was further established by running an mRNA overexpression assay performed by transfecting pparγ mature transcripts into SCHL cells. mRNA transcription was performed on a linearized DNA template containing T7 promoter, pparγ ORF (GenBank: JF502072.1) using mMESSAGE mMACHINE T7 Ultra Kit (Ambion, Austin, TX, USA) according to the manufacturer to generate capped mRNA with a poly(A) tail. Plasmid pcDNA3.1-pparγ was used as a template for a linearized DNA template above with T7 promoter primer and antisense primer of pparγ containing termination codon in a pfu-PCR reaction (Table 1). Finally, the mRNA product was purified with MEGA clear TM Kit (Ambion), and stored in -80°C for further transfection into cell line. The method of rabbitfish SCHL cell culture was the
same with antagonist assay above. When the cells were grown to 80% confluence for 24 h, a transfection complex consisting of 2 μg pparγ mRNA and 6.25 μL Lipofectamine™ Messenger-MAX™ Reagent (Invitrogen) were transfected into cells within well. At 48 h after transfection, cells were lysed and harvested for RNA and lipid extraction (see subsequent section for methodological details). The survival rate of the cells was more than 90% over the course of the entire operation.

Quantitative real-time PCR analysis

The expression of pparγ and Δ6Δ5 fads2 from experiments involving SCHL cells was analyzed by quantitative real-time PCR analysis (qPCR). Total RNA was extracted using TRIzol® Reagent (Invitrogen, USA). Total RNA (1 μg) was reverse-transcribed into cDNA with FastKing RT Kit including gDNase treatment (Tiangen, China). Primers used for qPCR were listed in Table 1. The relative gene expression of pparγ and Δ6Δ5 fads2 was normalized with that of 18S rRNA (GenBank: AB276993) with the 2^−ΔΔCt method (Livak and Schmittgen, 2001). Each qPCR (total volume of 20 μL) consisted of 2 μL diluted cDNA (10 ng/μL), 0.5 μM of each primer and 10 μL SYBR Green I Master (Roche), and reactions were carried out on a Lightcycler 480 system (Roche, Switzerland). No template controls (NTC) were run systematically in each plate as negative controls. The qPCR program consisted of an initial activation step at 95 °C for 5 min, followed by 35 cycles of 10 s at 95 °C, 20 s at 60 °C and 20 s at 72 °C. After the amplification, dissociation curves of 0.5 °C increments from 65 °C to 95 °C were carried out to confirm the amplification of a single product in each reaction.

Lipid extraction and fatty acid analysis

The cells were digested with Trypsin-EDTA (Invitrogen) and centrifuged at 4000 × g for 5 min. Fatty acids were extracted from the cells precipitate by steeping in 2 ml chloroform/methanol (2:1 v/v), then 0.5 N methanolic KOH and 14 % boron trifluoride–methanol (Sigma-Aldrich, USA) were used to saponify the FA for fatty acid methyl ester (FAME) (Wijngaarden, 1967) derivative. Finally, FAME were separated and quantified by GC-2010 Plus gas chromatograph (Shimadzu, Japan) as we described before (Li et al., 2008).
Statistical analysis

All data for dual luciferase assays, gene expression and FA contents were presented as mean ± SEM (n=3). The differences among the groups were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or Student's t-test (as indicated) using Origin 7.0. Differences were considered significant at P < 0.05 to all statistical tests performed.

Results

**ppary overexpression decreased the activity of rabbitfish Δ6Δ5 fads2 promoter**

HEK 293T cells co-transfected with the overexpression vector pcDNA3.1-ppary and Δ6Δ5 fads2 promoter showed a significant decrease of Δ6Δ5 fads2 promoter activity, while Δ6Δ5 fads2 promoter with ppre mutant had no response to pparγ overexpression (Fig. 2). No response to the pparγ overexpression was observed in the negative control (pGL4.10). This result was in agreement with our hypothesis that ppre site within the Δ6Δ5 fads2 promoter is the target site for Pparγ.

**Pparγ antagonist GW9662 increased the activity of rabbitfish Δ6Δ5 fads2 promoter**

To further confirm the action of Pparγ on Δ6Δ5 fads2 promoter, the effects of the Pparγ antagonist GW9662 was tested. Thus, HEK 293T cells transfected with the rabbitfish Δ6Δ5 fads2 promoter and treated with Pparγ antagonist GW9662 showed a significant increase of the Δ6Δ5 fads2 promoter activity. On the contrary, the ppre mutant and the control pGL4.10 exhibited no change when treated with GW9662 (Fig. 3). These data further suggested that Pparγ is a negative regulator targeting the rabbitfish Δ6Δ5 fads2 promoter.

**Pparγ interacted with the TF binding site in the Δ6Δ5 fads2 promoter**

Electrophoretic Mobility Shift Assay was performed with rabbitfish hepatocyte nucleus proteins (Fig. 4). No gel shift band was observed in lane 1 (no protein). Nevertheless, a gel shift band was observed in lane 2 containing rabbitfish hepatocyte nucleus proteins and 5’ biotin labeled probe, while unlabeled competitor probe could compete with the binding reaction (lane 3) and unlabeled mutant competitor probe could not compete in the reaction (lane 4). The results confirmed the interaction between Pparγ and the Δ6Δ5 fads2 promoter binding region.
Pparγ antagonist GW9662 down-regulated the expression of pparγ and up-regulated that of Δ6Δ5 fads2

Using SCHL cells, the effect of Pparγ antagonist GW9662 on the expression of pparγ and Δ6Δ5 fads2 was investigated. The results showed that, compared to controls, the mRNA level of pparγ was significantly decreased when SCHL cells were treated with GW9662, while that of Δ6Δ5 fads2 was significantly increased (Fig. 5a). These results indicated a negative regulatory role of Pparγ on gene expression of Δ6Δ5 fads2 in rabbitfish hepatocytes.

siRNA of pparγ induced the expression of Δ6Δ5 fads2 in SCHL cells

An experiment using RNAi of pparγ was conducted to confirm the functional relationship between Pparγ and Δ6Δ5 fads2 at transcription level. The results showed that, compared with the control group, siRNA of pparγ significantly decreased the pparγ mRNA level (70.2 ± 6.8 % decrease). Moreover, the levels of Δ6Δ5 fads2 mRNA were significantly increased (155 ± 14.6 %) (Fig. 5b). These results are consistent with the those with the Pparγ antagonist assay indicating that Pparγ is a negative regulator of rabbitfish Δ6Δ5 fads2.

pparγ mRNA overexpression decreased the mRNA expression of Δ6Δ5 fads2 in SCHL cells

The inhibition of Pparγ (GW9662 treatment and RNAi) was accompanied with an up-regulation of rabbitfish Δ6Δ5 fads2 expression in SCHL cells, and the influence of pparγ overexpression to the possible target gene expression was identified by mRNA transfection into hepatocyte. Compared to the control group, pparγ mRNA in SCHL cells overexpressing pparγ increased between 400 to 500 fold, whereas the Δ6Δ5 fads2 mRNA decreased to 33.1 ± 14.0 % (Fig. 5c). The results clearly show that Δ6Δ5 fads2 transcription is down-regulated under pparγ overexpression, thus proving its role as a negative regulator to Δ6Δ5 fads2.

Overexpression of pparγ decreased while siRNA of pparγ increased the enzymatic activity of Δ6Δ5 Fads in SCHL cells

The impact of Pparγ on the desaturase activity was determined by analyzing the FA profiles of SCHL cells treated with pparγ overexpression (Table 2). FA ratios of desaturation
products/substrates such as 18:3n-6/18:2n-6 and 18:4n-3/18:3n-3 was increased with knock-down of pparγ, but decreased with overexpression of pparγ, indicating a change of Δ6Δ5 Fad desaturation activity. Besides, the level of total LC-PUFA (ΣLC-PUFA) was significantly decreased in cells treated with overexpressing pparγ, and the level of FA precursor 18:3n-3 (ALA) was significantly higher, compared with those in control. Overall, the FA results clearly indicated that Pparγ impacted the LC-PUFA biosynthesis by reducing the desaturase capacity.

Discussion

The present study investigated the role of Pparγ in the regulation of Δ6Δ5 fads2, a gene encoding a fatty acyl desaturase with key roles in the LC-PUFA biosynthesis in rabbitfish. Results from a set of experiments using HEK 293T cells showed that both pparγ overexpression or a Pparγ antagonist (GW9662) treatment affected the activity of the Δ6Δ5 fads2 promoter, and EMSA confirmed that Pparγ interacted with the promoter, suggesting a role for Pparγ in Δ6Δ5 fads2 regulation. Such role of Pparγ as Δ6Δ5 fads2 modulator was further confirmed at transcriptional level in SCHL cells using a varied range of methodological approaches including GW9662 treatment, pparγ targeted siRNA, and pparγ overexpression. Finally, the effects of Pparγ on the LC-PUFA biosynthetic pathways were estimated by determining the changes produced in the FA profiles of SCHL cells overexpressing the rabbitfish pparγ. Overall our data strongly suggested that Pparγ acted as a negative regulator on Δ6Δ5 fads2 expression in rabbitfish.

PPARγ is a key inducer of differentiation, lipogenesis, and insulin sensitivity in white and brown adipocytes and is involved in lipid deposition in many other cell types (Poulsen et al., 2012). The previous study in human skeletal muscle tissue speculated that PPARγ might be a positive regulator for carnitine palmitoyl transferase-1 (CPT-1), lipoprotein lipase (LPL) and fatty acid binding protein (FABP) (Lapsys et al., 2000). PPARγ play an important role in modulating lipid accumulation (Auwerx, 1999), and is activated by many FA (Forman et al., 1996). In Atlantic salmon (Salmo salar), Ppar was demonstrated to be an important factor in mediating enzymatic response to fibrates (Ruyter et al., 1997). A previous study reported that troglitazone (a PPARγ agonist) induced an increase in PPARγ and a decrease in FADS2 (Δ6 desaturase) expression in human skeletal muscle cells, speculating that there is a PPRE element in FADS2 promoter (Wahl et al., 2002). However, another study demonstrated that the PPRE imparted PPARα responsiveness
to the human *FADS2* promoter, but not PPARγ (Tang et al., 2003). These data indicated that the effects of PPARs on human *FADS2* expression in skeletal muscle cells may similar to the situation as demonstrated in rabbitfish.

In rabbitfish, Ppar cDNAs were cloned and characterized their tissue distribution, PPARα was widely expressed in tissues and was particularly abundant in the heart, brain and liver, PPARβ expression is considerably higher in the gills than in the other tissues, PPARγ was predominantly expressed in the intestine, gills and liver (You et al., 2017), indicating that PPARα and PPARγ were potential candidates involved in the regulation of LC-PUFA biosynthesis in rabbitfish. In the present study, a potential Pparγ binding site (ppre located at +51 bp to TSS) was found on the Δ6Δ5 *fads2* promoter. The Δ6Δ5 *fads2* promoter activity increased significantly after treated with GW9662, which was a potent and selective antagonist of PPARγ and showed no effect on transcription on PPARα and PPARσ (Leesnitzer et al., 2002), and decreased significantly after treated with overexpression of *ppary*, while ppre mutant did not response to GW9662 and overexpression of *ppary*. Mutation of ppre resulted in significantly decreased transcriptional activity, which suggested that this TF binding site was important for maintaining Δ6Δ5 *fads2* promoter activity. In rat liver, PPARγ, along with PPARα, interacted with a PPRE located inthe the promoter of lipoprotein lipase (*lpl*) (Schoonjans et al., 1996). In rabbitfish primary hepatocytes, *ppara* expression was depressed in response to supplementation of Pparγ-specific agonist 15-deoxy-D12,14-prostaglandin J2 (15d-J2), in agreement with the depression of rabbitfish Δ6Δ5 *fads2* expression (You et al., 2017). Hence, it was worth considering that whether there was PPARα binding site on the Δ6Δ5 *fads2* promoter, the mechanism for concomitant activation of rabbitfish PPARα and Δ6Δ5 Fads should be further investigated.

The studies in SCHL cells further confirmed that Δ6Δ5 *fads2* is regulated by Pparγ since the expression of Δ6Δ5 *fads2* was regulated by each treatment of Pparγ. Previous study in European seabass (*Dicentrarchus labrax*) demonstrated the concomitant increase of *ppar* and Δ6 *fads2* mRNA levels induced by dietary n-3 LC-UFA deficiency, suggested a potential role of Ppar members in the regulation of Δ6 *fads2* in this species (Vagner et al., 2009). However, when the Atlantic salmon SHK-1 cells were incubated with Ppar agonists (WY14643 and 2-bromopalmitate), there were no significant change in the expression of Ppar encoding genes (Carmona-Antoñanzas et al., 2014). In rabbitfish primary hepatocytes, the PPAR agonist
(Fenofibrate) induced pparγ expression, and meanwhile suppressed the expression of Δ6Δ5 fads2, which suggested a possible regulation of Pparγ on Δ6Δ5 fads2 (You et al., 2017). These results suggested that PPARγ may have different regulatory mechanism in different species. In the present study in SCHL cells, the Pparγ antagonist GW9662 increased the expression of Δ6Δ5 fads2, coinciding with the suppression of Pparγ antagonists, suggesting the role of Δ6Δ5 fads2 as the downstream gene of Pparγ. This was further confirmed by the function assay of Pparγ on Δ6Δ5 fads2 expression such as the pparγ overexpression and knockdown in SCHL cells.

The regulation of Δ6Δ5 fads2 by Pparγ will eventually lead to altered FA profiles, particularly those that are desaturation substrates or products. Previously, a study on human skeletal muscle cells treated with troglitazone exhibited changes in unsaturated FA profiles despite the decrease of Δ6 fads mRNA levels (Wahl et al., 2002). In rabbitfish, functional characterization show that Δ6/Δ5 Fads could efficiently convert 18:3n-3 and 18:2n-6 to 18:4n-3 and 18:3n-6, respectively (Li et al., 2010). The ratio of C18:3n-6/C18:2n-6 could be an index of Δ6 Fads activity (Borkman et al., 1993). In the present study, remarkable changes of the FA profiles were detected in SCHL cells overexpressing and knock-down pparγ mostly associated with decreased and increased levels of the direct Δ6 desaturation products such as 18:4n-3 and 18:3n-6, or further downstream products within the LC-PUFA biosynthetic pathways such as EPA and DHA. We showed that RNAi knockdown of pparγ caused an increase in 18:4n-3/18:3n-3 and overexpression of pparγ caused a decrease in 18:4n-3/18:3n-3, and 18:3n-6/18:2n-6 in rabbitfish hepatocyte cells, which indicates an increase and a decrease in Δ6Δ5 Fads enzymatic activity. And in pparγ mRNA overexpression assay, the ratio of 18:4n-3/18:3n-3 was higher than the ratio of 18:3n-6/18:2n-6 in the control group, suggested that rabbitfish Δ6Δ5 Fads tend to convert n-3 PUFA. This result correspond to the previous study that dietary linoleic (18:2n-6) and α-linolenic acids (18:3n-3) may promote the expression of Δ6 desaturase, the promoting action of α-linolenic acid on Δ6 desaturase gene expression is stronger than that of linoleic acid in S. canaliculatus (Li et al., 2010). Moreover, there were significantly lower contents of EPA, DHA, ARA and higher contents of ALA in pparγ mRNA overexpression group, whereas the contents of LA, showed no significant difference, when compared to control group, indicating that Pparγ has a more significant effect on enzymatic activity of Δ6Δ5 Fads involved in n-3 pathway than that in n-6 pathway. As Pparγ could be activated by fatty acids at physiological concentrations (Gearing et al.,
1994), this could be the underlying molecular mechanism whereby dietary lipids affect LC-PUFA synthesis through Pparγ.

In summary, the present study demonstrated that Pparγ negatively influences the biosynthesis of LC-PUFA by targeting Δ6Δ5 fads in rabbitfish liver, which was the first report of Pparγ involved in regulation of LC-PUFA biosynthesis in teleosts, may contribute to the exploration of enhancing LC-PUFA biosynthesis in fish.

Acknowledgements

This work was financially supported by the Major International Joint Research Project from National Natural Science Foundation of China (No. 31110103913) and China Agriculture Research System (CARS-47).

References


white-spotted spinefoot *Siganus canaliculatus*. *Journal of fish biology*.


### Table 1.
Primers used for gene clone, EMSA, qPCR or vector construction.

<table>
<thead>
<tr>
<th>Aim</th>
<th>Gene/vector name</th>
<th>Primers/oligo nucleotides</th>
<th>Nucleotide sequence</th>
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</thead>
<tbody>
<tr>
<td>Vector reconstruction</td>
<td>pcDNA3.1-ppary</td>
<td>ppary-F</td>
<td>5'-CCCGAATTCCATGTGCCTCCCTGTCGCC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ppary-R</td>
<td>5'-CCCTCTAGATCCCACCTGTTTCCTCTTGCC-3'</td>
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<tr>
<td>pparγ mRNA construction</td>
<td>pparγ</td>
<td>T7 promoter primer</td>
<td>5'-TAATACGACTCACTATAGGG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pparγ-R</td>
<td>5'-CCCTCTAGATCCCACCTGTTTCCTCTTGCC-3'</td>
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<tr>
<td>EMSA probes</td>
<td>pparγ</td>
<td>BPF (5'-biotin labeled)</td>
<td>5'-GGAGCAGCGGTACGCCTAGGAA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BPR (5'-biotin labeled)</td>
<td>5'-TTCTATGGTGACGTACGGTGCTTC-3'</td>
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<tr>
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<td>UPF</td>
<td>5'-GGAGCAGCGGTACGCCTAGGAA-3'</td>
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<td>UPR</td>
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<td>5'-GGAGCAGCTTCACGCGGAAGATAGGAA-3'</td>
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<td>UTPR</td>
<td>5'-TTATTTCCCGTGAAAGCTCGTCC-3'</td>
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<td>qPCR</td>
<td>pparγ</td>
<td>qPCR-ppary-F</td>
<td>5'-CTGCTGGCTGAGTTCTGCTC-3'</td>
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<td>qPCR-ppary-R</td>
<td>5'-ATGACAAAGGCGGTATCTC-3'</td>
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<td>Δ6Δ5 fads2</td>
<td>qPCR-Δ6Δ5 fads2-F</td>
<td>5'-TCATTGGAACCTGCCCCACAT-3'</td>
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<td>qPCR-Δ6Δ5 fads2-R</td>
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<tr>
<td>18S rRNA</td>
<td>qPCR-18S-F</td>
<td>5'-CCCGGAAGAGACGATCAAAC-3'</td>
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<td></td>
<td>qPCR-18S-R</td>
<td>5'-TGATCTTCTCCGAGTTCA-3'</td>
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Table 2.
Fatty acid composition (% total fatty acids) of *Siganus canaliculatus* hepatocyte line (SCHL) cells transfected with *pparγ* siRNA or *pparγ* mRNA.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Knock-down of <em>pparγ</em></th>
<th>Overexpression of <em>pparγ</em></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Negative control</td>
<td><em>pparγ</em> siRNA</td>
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<tr>
<td>14:0</td>
<td>0.43±0.06</td>
<td>0.50±0.02</td>
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<tr>
<td>16:0</td>
<td>16.81±0.77</td>
<td>15.17±0.47</td>
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<tr>
<td>18:0</td>
<td>13.65±0.51</td>
<td>13.57±0.93</td>
</tr>
<tr>
<td>22:0</td>
<td>1.55±0.12</td>
<td>1.54±0.16</td>
</tr>
<tr>
<td>16:1n-7</td>
<td>4.54±0.19</td>
<td>5.08±0.55</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>31.87±0.73</td>
<td>31.38±2.12</td>
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<tr>
<td>20:1n-9</td>
<td>0.72±0.04</td>
<td>0.71±0.04</td>
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<tr>
<td>24:1</td>
<td>0.32±0.13</td>
<td>0.33±0.02</td>
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<tr>
<td>18:2n-6(LA)</td>
<td>3.77±0.11</td>
<td>3.78±0.17</td>
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<tr>
<td>20:2n-6</td>
<td>0.90±0.08</td>
<td>0.82±0.08</td>
</tr>
<tr>
<td>18:3n-6</td>
<td>0.59±0.17</td>
<td>1.53±0.35</td>
</tr>
<tr>
<td>18:3n-3(ALA)</td>
<td>0.62±0.21</td>
<td>0.65±0.04</td>
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<tr>
<td>18:4n-3</td>
<td>0.32±0.05</td>
<td>0.88±0.18</td>
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<tr>
<td>20:4n-6(ARA)</td>
<td>4.84±0.51</td>
<td>4.73±0.73</td>
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<tr>
<td>20:5n-3(EPA)</td>
<td>3.17±0.10</td>
<td>3.22±0.25</td>
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<tr>
<td>22:6n-3(DHA)</td>
<td>16.09±1.33</td>
<td>16.12±1.09</td>
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<tr>
<td>ΣSFA</td>
<td>32.45±0.15</td>
<td>30.78±1.36</td>
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<tr>
<td>ΣMUFA</td>
<td>37.45±0.77</td>
<td>37.49±2.51</td>
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<td>ΣPUFA</td>
<td>30.28±0.69</td>
<td>31.73±1.17</td>
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<tr>
<td>ΣLC-PUFA</td>
<td>24.09±0.79</td>
<td>24.07±1.64</td>
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<tr>
<td>18:3n-6/18:2n-6</td>
<td>0.16±0.04</td>
<td>0.41±0.10</td>
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<tr>
<td>18:4n-3/18:3n-3</td>
<td>0.54±0.16</td>
<td>1.36±0.31</td>
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</table>

Notes: Values are means ± SEM from three treatments (*n* = 3). In the treatment of Knock-down of *pparγ* or overexpression of *pparγ*, different superscripts in the same rows indicate significant difference at *P* < 0.05 by Student's *t*-test. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, fatty acids with 2 or more double bonds; LC-PUFA, sum of ARA, EPA and DHA.
Figure Legends

Fig. 1. The nucleotide sequence and predicted binding sites for Pparγ in the core region of the rabbitfish $\Delta6\Delta5$ fads2 promoter. Numbers are given relative to the first base of the transcription start site (TSS). Potential transcription binding motif for Pparγ is marked in grey (Dong et al., 2018).

Fig. 2. Effects of Siganus canaliculatus pparγ overexpression on activity of $\Delta6\Delta5$ fads2 promoter. HEK 293T cells were co-transfected with the $\Delta6\Delta5$ fads2 promoter vector and the overexpression vector pcDNA3.1-pparγ or the empty vector pcDNA3.1 (control). The negative control (pGL4.10) was an empty vector with no promoter sequence upstream in the reporter gene. $\Delta6\Delta5$ fads2-mutant was $\Delta6\Delta5$ fads2 - Pparγ site-directed mutant. Y-axis is the Firefly/Renilla luciferase ratio, while x-axis stands for different reporter vector. Data are means ± SEM ($n = 3$) and asterisks represent significant differences (Student’s t-test; $P < 0.05$).

Fig. 3. Effects of the Pparγ antagonist GW9662 on activity of the $\Delta6\Delta5$ fads2 promoter. HEK 293T cells transfected with the $\Delta6\Delta5$ fads2 promoter were treated with or without (control) GW9662. The negative control (pGL4.10) was an empty vector with no promoter sequence upstream in the reporter gene. $\Delta6\Delta5$ fads2-mutant was $\Delta6\Delta5$ fads2-Pparγ site-directed mutant. Y-axis is the Firefly/Renilla luciferase ratio, while x-axis stands for different reporter vector. Data are shown as means ± SEM ($n = 3$) and asterisks represent significant differences (Student’s t-test; $P < 0.05$).

Fig. 4. Electrophoretic mobility shift assay (EMSA) of TF binding site in the $\Delta6\Delta5$ fads2 promoter with rabbitfish hepatocytes nucleus proteins. Lane 1, negative control; Lane 2, nucleus proteins reactions; Lane 3, unlabeled probe competing reactions; Lane 4, unlabeled mutant probe competing reactions. BP, biotin labeled probe; UP, unlabeled probe; UTP, unlabeled mutant probe.

Fig. 5. Q-PCR analyses of pparγ and $\Delta6\Delta5$ fads2 expression in Siganus canaliculatus hepatocyte line (SCHL) cells, (a) treated with the Pparγ antagonist GW9662, (b) transfected with pparγ
siRNA, (c) transfected with *ppary* mRNA. Relative expression of the target genes in SCHL cells was quantified for each transcript and was normalized with the expression of *18S rRNA* by $2^{-\Delta\Delta Ct}$ method. Results are means ± SEM ($n=3$) and asterisks indicate significant differences of gene expression between the control and the GW9662 treatment (Student’s *t*-test; $P < 0.05$).
Figures

Fig. 1.

\[ \text{TSS} + 1 \]

-18 TATCAGTGGGTGAATCCGGAACCTATTGAGGAGGATGAGGATGAGGAGGTGA

\[ \text{Ppary} \]

+42 CGAATGTTGGACGGAGCACGGTCAACGTGACCATAGGAACAGACAACGTTTGCAAAT +100
Fig. 2.
Fig. 3.

[Bar chart showing the Firefly/Renilla luciferase ratio for different samples: pGL4.10, Δ6Δ5 fads2, and Δ6Δ5 fads2-mutant, with controls and GW9662 treatments.]
Fig. 4.

<table>
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<tr>
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<th>1</th>
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<tbody>
<tr>
<td>BP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
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<td>UP</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>UTP</td>
<td>–</td>
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<tr>
<td>nuclear protein</td>
<td>–</td>
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</table>
Fig. 5.

(a) Relative RNA expression of control and GW9662 treatment.

(b) Relative RNA expression of control and overexpression of pparγ.

(c) Relative RNA expression of control and siRNA treatment for pparγ and ΔΔ5 fads2.