Hnf4α is involved in the regulation of vertebrate LC-PUFA biosynthesis: insights into the regulatory role of Hnf4α on expression of liver fatty acyl desaturases in the marine teleost *Siganus canaliculatus*

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ABSTRACT

Long chain polyunsaturated fatty acid (LC-PUFA) biosynthesis is an important metabolic pathway in vertebrates, especially fish, considering they are the major source of n-3 LC-PUFA in the human diet. However, most fish have only limited capability for biosynthesis of LC-PUFA. The rabbitfish (*Siganus canaliculatus*) is able to synthesize LC-PUFA as it has all the key enzyme activities required including Δ6Δ5 Fads2, Δ4 Fads2, Elovl5 and Elovl4. We previously reported a direct interaction between the transcription factor Hnf4α and the promoter regions of Δ4 and Δ6Δ5 Fads2, which suggested that Hnf4α was involved in the transcriptional regulation of *fads2* in rabbitfish. For further functionally investigating it, a full-length cDNA of 1736 bp encoding rabbitfish Hnf4α with 454 amino acids was cloned, which was highly expressed in intestine, followed by liver and eyes. Similar to the expression characteristics of its target genes *Δ4* and *Δ6Δ5 fads2*, levels of *hnf4α* mRNA in liver and eyes were higher in fish reared at low salinity than those reared in high salinity. After the rabbitfish primary hepatocytes were respectively incubated with Alverine, Benfluorex or BI6015, which were anticipated agonists or antagonist for Hnf4α, the mRNA level of *Δ6Δ5* and *Δ4 fads2* displayed a similar change tendency with that of *hnf4α* mRNA. Furthermore, when the mRNA level of *hhf4α* was knocked down using siRNA, the expression of *Δ6Δ5* and *Δ4 fads2* also decreased. Together, these data suggest that Hnf4α is involved in the transcriptional regulation of LC-PUFA biosynthesis, specifically, by targeting *Δ4* and *Δ6Δ5 fads2* in rabbitfish.

Key words: fatty acyl desaturase; LC-PUFA biosynthesis; Hnf4α; transcriptional regulation mechanism; rabbitfish *Siganus canaliculatus*

1. INTRODUCTION

Long-chain (≥ C_{20}) polyunsaturated fatty acids (LC-PUFA), particularly the so-called "omega-3" (or n-3) LC-PUFA eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), are vital for human health and have beneficial effects in a range of pathologies including cardiovascular and inflammatory diseases, with DHA also playing important roles in neural development (Calder, 2013; Campoy et al., 2012; Delgado-Lista et al., 2012; Gil et al., 2012). The essential nature of certain LC-PUFA, along with fish and seafood representing unique and rich sources of n-3 LC-PUFA in the human diet (Tur et al., 2012), has prompted increasing interest in understanding the biosynthesis and regulation of LC-PUFA in fish. This is particularly the case in farmed species for which current aquafeed formulations with increased levels vegetable oil devoid of LC-PUFA might compromise the essential fatty acid (EFA) requirements in some species (Tocher, 2015).

It has been historically recognized that freshwater and salmonid species have the capability to biosynthesize LC-PUFA from C_{18} fatty acid precursors such as α-linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6) (Castro *et al.*, 2016). Therefore, dietary vegetable oils (VO) containing ALA and LA can satisfy the EFA requirements for these species (Sargent et al., 2002). In contrast, marine fish have generally limited capability for the biosynthesis of LC-PUFA from C_{18} precursors, due to a lack of some key biosynthetic enzymes (Castro *et al.*, 2016), and hence they require preformed LC-PUFA in the diet to satisfy EFA requirements (Tocher, 2010). This dichotomy is currently regarded as somewhat simplistic and factors such as trophic level and the evolutionary history of each species have been identified as major drivers determining the LC-PUFA biosynthetic capability in teleost fish (Fonseca-Madrigal *et al.*, 2014; Castro *et al.*, 2016). For instance, the rabbitfish *Siganus canaliculatus*, a marine herbivorous teleost, has been shown to have two fatty acyl desaturase 2 (Fads2) enzymes (Δ6Δ5 Fads2 and Δ4 Fads2) and two elongation of very long-chain fatty acid (Elovl) proteins (Elovl4 and Elovl5) that enable this species to perform all reactions required to biosynthesize LC-PUFA from C_{18} dietary precursors (Li et al., 2008; Li et al., 2010; Xu et al., 2012). Interestingly, rabbitfish *S. canaliculatus* can inhabit brackish waters and it was recently demonstrated that the expression and activity of *Δ6Δ5, Δ4 fads2* and *elovl5*

enzymes increased in fish maintained under low-salinity conditions compared with fish reared at seawater salinity (Xie et al., 2015). Overall, this makes rabbitfish *S. canaliculatus* an interesting model to investigate the regulatory mechanisms that environmental factors such as salinity exert on the LC-PUFA biosynthetic pathways in fish.

Previously, we cloned the promoters of *Δ6Δ5* and *Δ4 fads2* (Dong et al., 2016), and studies involving isolation and characterization of proteins interacting with the promoter regions allowed us to identify Hnf4α as a potential factor regulating *fads2* expression in rabbitfish. HNF4α is an important transcription factor involved in the regulation of several physiological processes including lipid, carbohydrate and amino acid metabolism (Martinez-Jimenez et al., 2010; Yin et al., 2011). However, the role of Hnf4α in the regulation of LC-PUFA biosynthesis in teleosts is still unclear. The primary aim of the present study was to investigate the potential role of Hnf4α in LC-PUFA biosynthesis in rabbitfish in order to gain further insight into the regulation of LC-PUFA biosynthesis in teleost fish. To achieve this aim we analyzed tissue expression of Hnf4α in rabbitfish reared at low- or high-salinity conditions, as well as in response to the presence of possible Hnf4α agonists or antagonists. In addition, the expression of *Δ6Δ5* and *Δ4 fads2* was analyzed to determine the effects of Hnf4α activation or suppression on *S. canaliculatus* LC-PUFA biosynthesis, to ultimately elucidate the role of Hnf4α in this pathway. The data obtained have increased our understanding of the transcriptional mechanisms regulating LC-PUFA biosynthesis in teleost fish and vertebrates in general, which may enable the development of practical strategies to enhance the activity of the LC-PUFA biosynthetic pathway in farmed fish.

2. Materials and methods

2.1 *Animals*

Wild rabbitfish specimens (body weight ~100 g) collected off the coast near the Nan Ao Marine Biology Station (NAMBS) of Shantou University, Southern China, were maintained in an indoor seawater tank for one month, during which time they were fed a diet containing fish oil as the major lipid source as described in detail previously (Xu et al., 2012). These fish were used for gene cloning and mRNA tissue distribution analyses. Ten individuals, sacrificed after being anaesthetized with an overdose of 3-amino-benzoate methane sulphonate (Sigma,

USA), were dissected and tissues including heart, spleen, brain, gill, muscle, eye, intestine, visceral adipose tissue and liver were collected, immediately frozen in liquid nitrogen, and subsequently stored at -80℃ until RNA extraction.

For the salinity adaptation experiment, 200 rabbitfish juveniles (body mass ~20 g, sex visually indistinguishable) were collected off the Southern China coast near NAMBS and reared in seawater (32 ppt) for one month with the same diet containing fish oil as above (Xu *et al*., 2012). Half the fish were acclimated from seawater to brackish water (10 ppt) over five days, while the other half was maintained in seawater (32 ppt). After two-weeks acclimation period, the fish were distributed into six 500 L cylindrical tanks (25 fish per tank) and reared in either brackish (10 ppt) or seawater (32 ppt) in triplicate tanks for two months. During the experimental period, oxygen saturation was maintained through aeration, temperature ranged between 22 and 28 ºC, and photoperiod was natural (give an approximate value 12L:12D). Tissue samples including liver, visceral fat depot, eye and intestine from six fish per treatment (2 fish per replicate tank) were collected and stored at -80 ºC as described above.

2.2 Molecular cloning of rabbitfish hnf4α

Total RNA (1 µg) was extracted (Trizol Reagent, Thermo Fisher, USA) from liver of a wild-caught rabbitfish individual and used to prepare cDNA using M-MLV Reverse Transcriptase (Thermo Fisher). Conserved regions from the alignments of the *HNF4α* sequences from *Homo sapiens* (NM_004133.4), *Mus musculus* (NM_008261.2), *Rattus norvegicus* (NM_022180.1), and *Oreochromis mossambicus* (DQ453816.1) (www.ncbi.nlm.nih.gov) enabled the design of the degenerate primers HaF1 (5'-ATCCAGCTATGAGGACAGCA-3', forward) and HaR1 (5'-TTTGCCAGGTGATGCTCTG-3', reverse). The 5' and 3' ends of the *S. canaliculatus hnf4α* were amplified by Rapid Amplification of cDNA Ends (RACE) polymerase chain reaction (PCR) (GeneRacer™ kit, Thermo Fisher). Gene specific primers for 3′ RACE were HaF2 (5'-GGATGCGATACCAGGTCCAGGTCAGTC-3') and HaF3 (5'-GGTAGCGGTGAGAATCCTGGACGAG-3'), whereas gene specific primers for 5′ RACE were HaR2 (5'-CTGATGTCGCCGTTGAGAATGGGAG-3') and HaR3 (5'-GTCATCCAAGGGCAGGTCACAGAAGG-3').

2.3 *Phylogenetic analysis of rabbitfish hnf4α*

A phylogenetic tree comparing the deduced amino acid (aa) sequence of the newly cloned rabbitfish *hnf4α* cDNA with those of orthologues from *Sparus aurata* (ACO56245.1), *Oreochromis niloticus* (XP_003457099), *Tetraodon nigroviridis* (XP_011617337), *Salmo salar* (XP_013991458), *Danio rerio* (NP_919349.1), *Homo sapiens* (CAA61133.1), *Mus musculus* (NP_032287.2) and *Gallus gallus* (NP_001026026.1), and *hnf4β* sequences from *D. rerio* (NP_991109.1), *S. salar* (XP_014031145.1), *Larimichthys crocea* (KKF19684.1) and *Astyanax mexicanus* (XP_007256450.1), as well as *HNF4γ* sequences from *H. sapiens* (AF207953), *M. musculus* (NP_038948.1), *D. rerio* (AAI24294.1), *S. salar* (XP_014012623.1) and *Oncorhynchus mykiss* (XP_021418003) was constructed using the neighbor joining method using Mega7 (Kumar et al., 2016). Confidence in the resulting tree branch topology was measured by bootstrapping through 1,000 iterations.

2.4 Rabbitfish primary hepatocyte culture and agonists/antagonists assays

Potential Hnfα agonists including alverine citrate and benfluorex (Sigma-Aldrich, USA), and the antagonist BI6015 (Millipore, Germany) were tested using primary hepatocytes obtained from wild rabbitfish (body weight ~100 g) collected off the Southern China coast at NAMBS. Stock working solutions in DMSO were prepared at 20 mM for alverine citrate and benfluorex, and 5 mM for BI6015. Isolation and culture of rabbitfish primary hepatocytes was performed according to methodology described by Zhang *et al*. (Zhang et al., 2014a). Briefly, sterile liver tissue was excised and washed three times with ice-cold Hank's Balanced Salt Solution (HBSS, Thermo Fisher). The tissue was chopped and digested with 0.1 % collagenase (Gibco, Life Technologies, USA) / 0.25 % hyaluronidase (Sigma-Aldrich, USA) for 30 min at room temperature. Dissociated cells were obtained by filtering through a 100 μm cell strainer (BD Falcon, USA) and washed in red blood cell lysis buffer (Beyotime Institute of Biotechnology, China) for 2 min at 4 ºC. Cell viability was evaluated using trypan blue staining to ensure the percentage of live cells was > 98 %. The isolated hepatocytes were seeded at a density of 2×10^6 cells per well on 6-well plates coated with 2.5 ml of 0.1 % gelatin in DMEM/F12 medium (Gibco, Life Technologies) containing 20 % fetal bovine

serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, and incubated at 24 ºC, 4 % CO_2 for 24 h. The hepatocytes were then incubated with 10 and 20 μM alverine or benfluorex for 3 days, and 2.5 and 5.0 μM BI6015 for 2 days in triplicate wells. Further triplicate wells were incubated with DMSO as control. After incubation, cells were lysed in the wells and RNA harvested using RNeasyPlus Mini Kit (Qiagen, Germany).

2.5 *RNAi assay*

Double-strand siRNA (21-mer) targeting the *S. canaliculatus hnf4α* were designed and synthesized by Suzhou GenePharma Co., Ltd (GenePharma, http://www.genepharma.com/). The corresponding target mRNA sequence for the siRNA was 5'-GCAGAUCCAGUUUGUCAAATT-3' (si-Hnf4α). The start nucleotide (nt) of si-Hnf4α corresponded to 1138 nt in the target *hnf4α* mRNA sequence (JF502073.1) and a non-target siRNA, 5'-UUCUCCGAACGUGUCACGUTT-3' was used as a mock control. A negative control consisting of untreated cells was also run. Rabbitfish primary hepatocytes were transfected immediately after isolation (Lipofectamine® RNAiMAX Reagent, Thermo Fisher). Briefly, 1 μl of 30 μM RNAi duplex was diluted in 500 μl Opti-MEM I Medium without serum (Thermo Fisher) and mixed with 7.5 μl RNAiMAX (Thermo Fisher). After 15 min incubation at room temperature, the complex was added to each well of the 6-well tissue culture plate in a final volume of 3 ml DMEM/F12 medium containing 20 % FBS, 100 U/ml penicillin and 100 μg/ml streptomycin followed by incubation at 24 ºC / 4 % CO_2 for 6 h. Effects of the RNAi assay were evaluated by analyzing expression of candidate genes including *hnf4α*, *Δ6Δ5* and *Δ4 fads2* by quantitative real-time PCR (qPCR) in primary hepatocytes collected 48 h post-transfection.

2.6 Quantitative real-time PCR analyses

For tissue distribution of *hnf4α* mRNA, total RNA was extracted from tissues by Trizol reagent (Thermo Fisher). The concentration and quality of total RNA were confirmed by spectrophotometry (NanoDrop 2000, Thermo Scientific, USA). The synthesis of cDNA from RNA extracted from rabbitfish tissues was performed using the Fast Quant RT Kit (with gDNase) (Tiangen Biotech, Beijing, China) for RT-PCR. Details of primer sequences used for

qPCR are listed in Table 1. The relative mRNA levels of candidate genes in tissues were normalized with 18S RNA (AB276993). For gene expression analysis of the candidate genes, including *hnf4α*, *Δ6Δ5* and *Δ4 fads2*, in primary hepatocytes, total RNA was isolated and purified by RNeasy Kits (Qiagen). The cDNA was obtained by using SuperScript® III First-Strand Synthesis System. The relative mRNA levels of RNAi sample were normalized with 18S RNA (AB276993). All qPCR reactions were run in a LightCycler®480 thermocycler (Roche, Germany) in a total volume of 20μl containing 10μl and the qPCR programs consisted of initial DNA denaturation at 94 ºC for 5 min and 45 cycles of 94 ºC for 10 s, 60 ºC for 20 s, 72 ºC for 20 s, followed by a melting curve to confirm the amplification of a single product in each reaction. The relative RNA level of genes was calculated by the comparative threshold cycle (Ct) method (Livak and Schmittgen, 2001).

2.7 Statistical analysis

All data were presented as means ± SEM, with $n \geq 3$ as indicated in each experiment above. Data were analyzed by one-way analysis of variance to determine significance of differences using Origin 7.0. A significance of $P < 0.05$ was applied to all statistical tests performed.

3. Results

3.1 Sequence and phylogenetic analyses of S. canaliculatus hnf4α

The *S. canaliculatus hnf4α* consists of a full-length cDNA sequence of 1736 base pairs (bp) that was deposited in the GenBank database under the accession number JF502073. It includes an open reading frame (ORF) of 1365 bp encoding a putative protein of 454 amino acids (aa). The deduced rabbitfish Hnf4α polypeptide has high sequence identify (92 - 98 %) with Hnf4α proteins from other teleost fish including *S. aurata* (ACO56245.1), *O. niloticus* (XP_003457099), *T. nigroviridis* (XP_011617337), *S. salar* (XP_013991458) and *D. rerio* (NP_919349.1), and relatively lower identifies (81 - 84 %) when compared to orthologues of other vertebrates including *H. sapiens* (CAA61133.1), *M. musculus* (NP_032287.2) and *G. gallus* (NP_001026026.1). Such sequence identities were consistent with phylogenetic analysis showing that rabbitfish Hnf4α clustered closely with Hnf4α from teleost fish and

non-teleost vertebrates, and located in different stem branch of Hnf4γ from teleost fish or mammalian, and more distantly from a group containing Hnf4β proteins (Fig. 1).

3.2 Tissue distribution of rabbitfish hnf4α mRNA

The distribution of r*hnf4α* in rabbitfish tissues was determined by qPCR. Among the nine tissues analyzed, the highest expression of *hnf4α* was detected in intestine, followed by liver, eye and visceral adipose tissue, while expression of the *hnf4α* in heart, spleen, muscle and gill was generally low (Fig. 2).

3.3 Effect of salinity on hnf4α expression in tissues of rabbitfish

Tissues with the highest expression levels of *hnf4α*, namely intestine, liver, eye and visceral adipose tissue were selected to assess the effects of salinity on *hnf4α* expression. The expression levels of *hnf4α* mRNA in liver and eyes were significantly higher in rabbitfish reared at a salinity of 10 ppt for 2 months compared to fish reared at a salinity of 32 ppt (Fig. 3). No effects of salinity on the expression of *hnf4α* were observed in intestine or visceral fat (Fig. 3).

3.4 Effects of agonists/antagonists and small RNAi on expression of hnf4α and its potential target genes Δ4 and Δ6Δ5 fads2 in rabbitfish primary hepatocytes

Rabbitfish primary hepatocytes were incubated with potential *hnf4α* agonists (alverine and benfluorex) and antagonist BI6015 to elucidate the role of Hnf4α in LC-PUFA biosynthesis. All three reagents up-regulated expression of *hnf4α* although the level varied according to the agonist/antagonist concentration (Fig. 4). In rabbitfish primary hepatocytes, 10 μM alverine, 20 μM benfluorex and, unexpectedly, 5 μM BI6015 all significantly up-regulated the level of *hnf4α* mRNA by 2-, 1.8- and 3.7-fold, respectively, compared to controls. Importantly, concomitantly with the increased expression of *hnf4α* mRNA, treatment of rabbitfish hepatocytes with the above agents also resulted in increased expression of *Δ4 fads2* and *Δ6Δ5 fads2* (Fig. 4).

The expression of *hnf4α* was effectively knocked-down by the RNAi approach. After transfection of rabbitfish hepatocytes with siRNA, the expression level of *hnf4α* mRNA

decreased 26 % and 24 % compared to mock and negative controls, respectively. Along with the reduced *hnf4α* expression there was a concomitant significant decrease in expression levels of *Δ4* and *Δ6Δ5 fads2* mRNA in comparison to controls (Fig. 4D).

4. Discussion

In order to gain insight into the regulatory mechanisms of LC-PUFA biosynthesis in marine teleosts, we previously conducted a series of studies investigating the regulation of *fads2* in rabbitfish. These showed that miRNA regulated *fads2* expression directly, specifically that miR-17 inhibited *Δ4 fads2* expression (Zhang et al., 2014b). Promoter regions of *Δ6Δ5 fads2* and *Δ4 fads2* genes were cloned and characterized, and several potential transcription factor binding site motifs were identified, including those for Hnf4α within the promoter regions of both *fads2* genes. Hnf4α is a member of the steroid hormone receptor superfamily (Sladek et al., 1990a), which plays a key role in regulating the expression of genes involved in hepatocyte differentiation and lipid homeostasis (Hayhurst et al., 2001; Li et al., 2000; Yin et al., 2011). However, the role of Hnf4α in LC-PUFA biosynthesis had not been studied and so it was not known whether Hnf4α directly regulated *fads2* expression in vertebrates. Therefore, the present study focused on the expression of *hnf4α* and *Δ6Δ5 fads2* and *Δ4 fads2* in in order to determine associations between the expression levels of the transcription factor and those of the fatty acyl desaturases involved in LC-PUFA biosynthesis.

The rabbitfish Hnf4αwas homologous to Hnf4α from other teleost fish and mammals, but not to Hnf4β. Rabbitfish Hnf4α was most closely clustered to *S. aurata* in the phylogenetic tree. Interestingly, its tissue distributions were also similar between these two fish, with highest expression in intestine and liver (Salgado et al., 2012). A relatively high level of expression of *hnf4α* mRNA was observed in rabbitfish eyes, which is another tissue showing significant LC-PUFA biosynthesis. However, in this respect it was surprising that brain, another important tissue for LC-PUFA biosynthesis, showed only very low expression of *hnf4α* mRNA. This result was however consistent with data from humans and mice, which suggested other transcription factors were involved in the regulation of LC-PUFA biosynthesis in brain (Sladek et al., 1990b; Taraviras et al., 1994b).

The LC-PUFA biosynthetic ability in low salinity was higher than that in high salinity in

rabbitfish (Li et al., 2008).Coincidently, the mRNA expression level of Δ4 and Δ6Δ5 *fads2* were up-regulated in low salinity compared to that at full seawater (Li et al., 2008; Xie et al., 2015). In order to demonstrate whether *hnf4α* was involved in the regulation of Δ4 and Δ6Δ5 *fads2* expression in response to salinity, we compared rabbitfish *hnf4α* expression levels in fish grown at low and high salinity in four tissues including intestine, liver, eyes, and adipose tissue. In fish reared at low salinity, the expression of *hnf4α* mRNA in liver and eyes was increased, similar to the effect of salinity on *fads2* expression, whereas the expression of Hnf4α was unaffected by salinity in intestine and adipose tissue. There are no previous studies investigating the influence of salinity on *hnf4α* expression, however, Hnf4 was demonstrated to be affected by stress (Marcil et al., 2010), with HNF4α and PPARα both up-regulated by restrain stress and playing key roles in lipid homeostasis in mice (Konstandi et al., 2013). Furthermore, Hnf4α is regarded as a nuclear receptor providing a rapid-response to environmental stimuli in mammals, such as hypoxia (Zhang et al., 1999) and fasting (Rhee et al., 2003). The results of the present salinity trial indicated that Hnf4α and potential target genes, Δ4 and Δ6Δ5 *fads2*, were all regulated similarly by salinity. We hypothesized that this was a linear pathway with increased Hnf4α promoting Δ4 and Δ6Δ5 *fads2* expression rather than all these genes being jointly up-regulated by low salinity and, therefore, that Hnf4α was an important transcription factor in the regulation of Δ4 and Δ6Δ5 *fads2* expression. Actually, the rabbitfish *S. canaliculatus* usually live in coral reef although they are euryhaline teleost, and their growth were inhibited under low salinity such as 10 ppt (Xie et al., 2015). Analogous to rabbitfish, the mRNA level of *fads2* was higher at low salinity than that at high salinity in another marine teleost red sea bream *Pagrus major* (Sarker et al., 2011). In the fresh water fish *Chirostoma estor*, however, the capacity of LC-PUFA biosynthesis was higher in high salinity (15 ppt) compared with in low salinity (0 and 5 ppt) (Fonseca-Madrigal et al., 2012). Salinity stress or other environmental stress, such as nutrition stress, might increase the transcriptional level of key enzyme genes involved in LC-PUFA biosynthesis through key transcription factors or nuclear receptors, including Hnf4α, which process might involve the AMPK pathway to our speculates.

A series of experiments in primary hepatocytes were performed to test the above hypothesis and gain some insight into the role of Hnf4α in the regulation of LC-PUFA biosynthesis in

rabbitfish. Specifically, the expression levels of *Δ4* and *Δ6Δ5 fads2* mRNA were determined after Hnf4α activation or suppression and the results indicated that Δ4 and Δ6Δ5 *fads2* expression paralleled *hnf4α* expression suggesting that Hnf4α may participate in the regulation of *Δ4* and *Δ6Δ5 fads2*. Consistent with this, a previous study in rabbitfish predicted Hnf4α and Pparγ (PPRE) binding sites in the core promoters of *Δ4* and *Δ6Δ5 fads2*, respectively (Dong et al., 2016). The transcription factor, other than PPARs and their binding partner RXR, known to interact with DR-1 (direct repeat-1) PPRE is HNF4α (Nakshatri and Bhat-Nakshatri, 1998; Nicolas-Frances et al., 2000; Winrow et al., 1993) and, therefore, Hnf4α might interact with the Pparγ binding site in the *Δ6Δ5 FADS2* promoter. A previous study reported that a DR-1 element was present in human *Δ6 FADS2* promoter and that PPARα and/or HNF4α might interact with this element (Tang *et al*., 2003). The WY 14643 activator of PPARα induced human *Δ6 FADS2* promoter activity which depended upon the expression of *PPARα*, however, expressing *HNF4α in vitro* had no effect on *Δ6 FADS2* promoter activity. This suggested that different DR-1 elements may have differential affinity to PPARs or HNF4 depending on the promoter context (Nakshatri and Bhat-Nakshatri, 1998). Based on the results of the present study, the rabbitfish Hnf4α displayed a potential role in *fads2* regulation.

Alverine and benfluorex were activators of human HNF4α and could increase *HNF4α* mRNA level *in vitro*, whereas BI6015 had the opposite effect compared to the above two chemicals and was antagonist to human HNF4α (Kiselyuk et al., 2012b; Lee et al., 2013). Similarly, the expression level of *hnf4α* in rabbitfish primary hepatocytes decreased significantly when incubated with alverine at 20 μM compared with 10 μM, which might indicate a dose-dependent effect. However, incubating rabbitfish primary hepatocytes with 5.0 μM BI6015 for 2 days also increased mRNA expression of *hnf4α* (and *Δ4* and *Δ6Δ5 fads2*), which was opposite to its effect in mammals where BI6015 effectively inhibited the expression of *HNF4α* in a human model (Kiselyuk et al., 2012a). This contradiction may be due to differences aspecific difference between the transcription factor in mammals (human) and fish (rabbitfish) or, possibly, in the autoregulation of HNF4α. Specifically, HNF4α can autoregulate its own transcription through a complex feedback loop between HNF4α and HNF1α, partially involving direct binding of HNF4α to its own promoter (Bailly et al., 2009;

Bailly et al., 2001). In mammals, studies have shown that HNF1 can bind to the promoter of HNF4α and regulate its transcription (Hatzis and Talianidis, 2001; Taraviras et al., 1994a; Zhong et al., 1994), so the existence of a reciprocal cross-regulation between HNF1α and HNF4α in hepatic cells might compensate for increased or reduced *hnf4α* mRNA. It is tempting to speculate that when rabbitfish primary hepatocytes were incubated with BI6015, the *hnf4α* mRNA level gradually decreased until it reached a critical threshold, when the synergistic actions of alternative sets of factors (Hnf1α-Hnf6 or Hnf1β-GATA-6) might activate the transcription of Hnf4α (Hatzis and Talianidis, 2001).

To further confirm the functional relationship between Hnf4α and Fads2, the mRNA expression levels of *Δ4* and *Δ6Δ5 fads2* were analyzed after *hnf4α* was knocked down by siRNA in rabbitfish primary hepatocytes. The results showed that mRNA expression of *Δ6Δ5* and *Δ4 fads2* decreased after *hnf4α* was knocked down indicating that both *Δ6Δ5* and *Δ4 fads2* might be under the regulation of Hnf4α. Previously, a study performed in *Caenorhabditis elegans* showed that inactivation of the *hnf4α* homologue by RNA interference (RNAi) may suppress the expression of stearoyl-CoA desaturase (Brock and Watts, 2006; Liang et al., 2010; Robinson-Rechavi et al., 2005). Taken together, these studies suggest that Hnf4α may be an important regulator of fatty acid desaturases in general, including Fads involved in the biosynthesis of LC-PUFA.

In conclusion, the present study demonstrated a possible role of Hnf4α in the regulation of LC-PUFA biosynthesis in the marine teleost rabbitfish by directly targeting the expression of *Δ4* and *Δ6Δ5 fads2*. Overall, Hnf4α was implicated as a key transcription factor in the regulation of LC-PUFA. The characterization of Hnf4α has contributed to our knowledge of the complex regulation of liver *fads2* that play important roles in LC-PUFA metabolism.

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Table 1. Nucleotide sequences of primers used for quantitative PCR (qPCR)

Target gene	Primers	Sequence（5’-3’）
Δ4 fads2	Δ4 Fad-F	GAACACCATTTGTTCCCGAG
	Δ4 Fad-R	TTCAGTGCCCTGACGACG
Δ6Δ5 fads2	Δ6Δ5 Fad-F	AACACCATTTGTTTCCCACC
	Δ6Δ5 Fad-R	CAGTGACCTGATGATATCAGCG
hnf4α	Hnf4α-F	CCGACTCTACAGAGCATCACCTG
	Hnf4α-R	TCATTAGCAGAACCTCCGAGAAG
18S	18S-F	CGCCGAGAAGACGATCAAAC
	18S-R	TGATCCTTCCGCAGGTTCAC
β-actin	β-actin-F	TGGACTTCGAGCAGGAGATGG
	β-actin-R	CCGAGGAAGGATGGCTGGAA

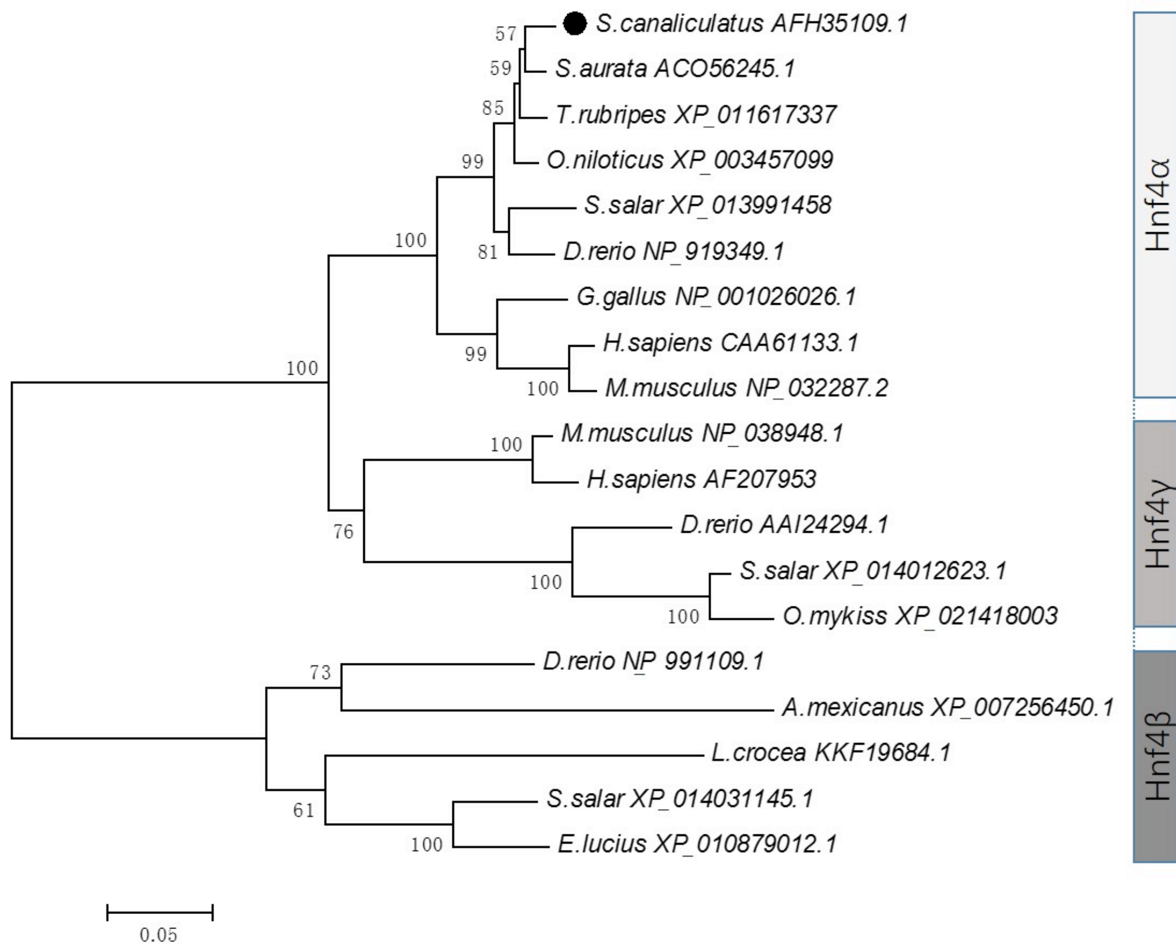

Fig 1. Phylogenetic tree comparing the deduced amino acid sequence of rabbitfish Hnf4α with those Hnf4α, β, γ from other vertebrates. The deduced amino acid sequence of rabbitfish Hnf4α (AFH35109.1) with Hnf4α from *Sparus aurata*, *Oreochromis niloticus*, *Tetraodon nigroviridis*, *Salmo salar*, *Danio rerio*, *Homo sapiens*, *Mus musculus*, *Gallus gallus*, and Hnf4β from *D. rerio*, *S. salar*, *Esox lucius*, *Larimichthys crocea* and *Astyanax mexicanus*, as well as Hnf4γ from *H. sapiens*, *M. musculus*, *D. rerio*, *S. salar* and *Oncorhynchus mykiss*. The tree was constructed using the neighbor-joining method with MEGA4. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The horizontal branch length is proportional to aa substitution rate per site. Evolutionary analyses were conducted in MEGA7(Kumar et al., 2016).

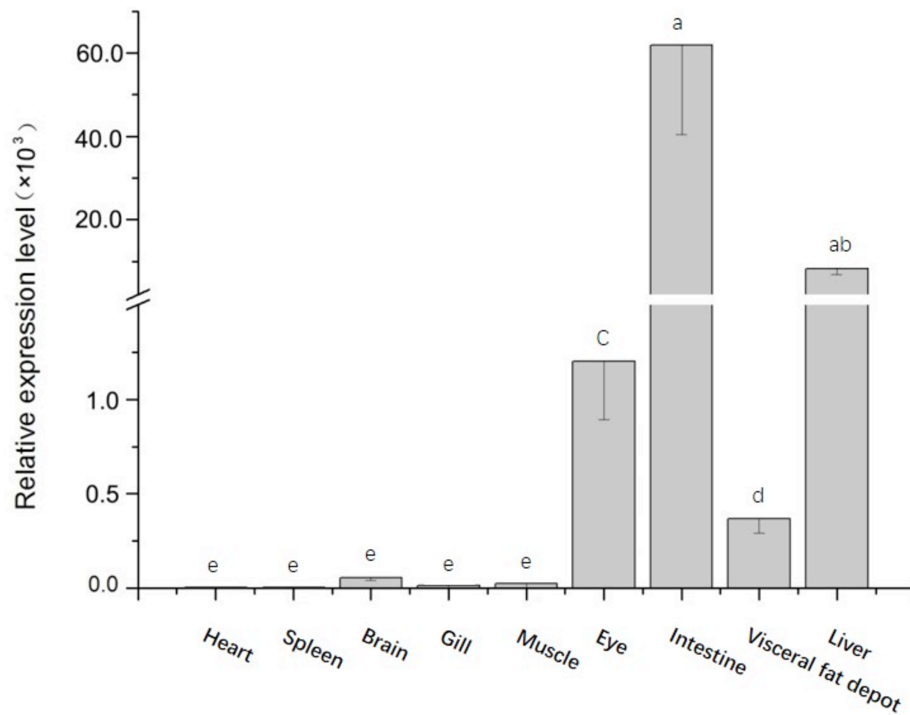

Fig 2. Quantitative distribution of *hnf4α* expression in rabbitfish tissues as determined by qPCR. Relative expression of *hnf4α* was quantified and normalized with 18S rRNA by 2-ΔΔCt method. Results are means ± SEM (n = 4), and different letters show significant differences ($p < 0.05$) among tissues as determined by one way analysis of variance followed by Tukey's multiple comparison test.

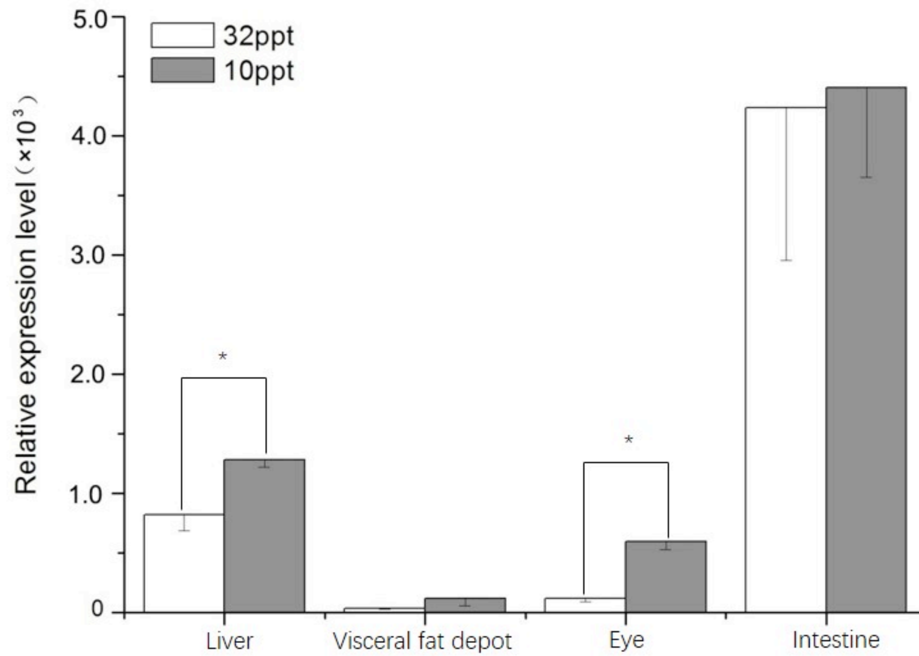

Fig 3. Expression of *hnf4α* mRNA in four tissues of the rabbitfish reared in water of 10 ppt or 32 ppt salinity. Relative expression of *hnf4α* was quantified and normalized with 18S rRNA by 2-ΔΔCt method. Results are means ± SEM (n = 4), analysis was performed using one-way analysis of variance, and asterisks represent significantly different ($P > 0.05$) between two salinity treatments.

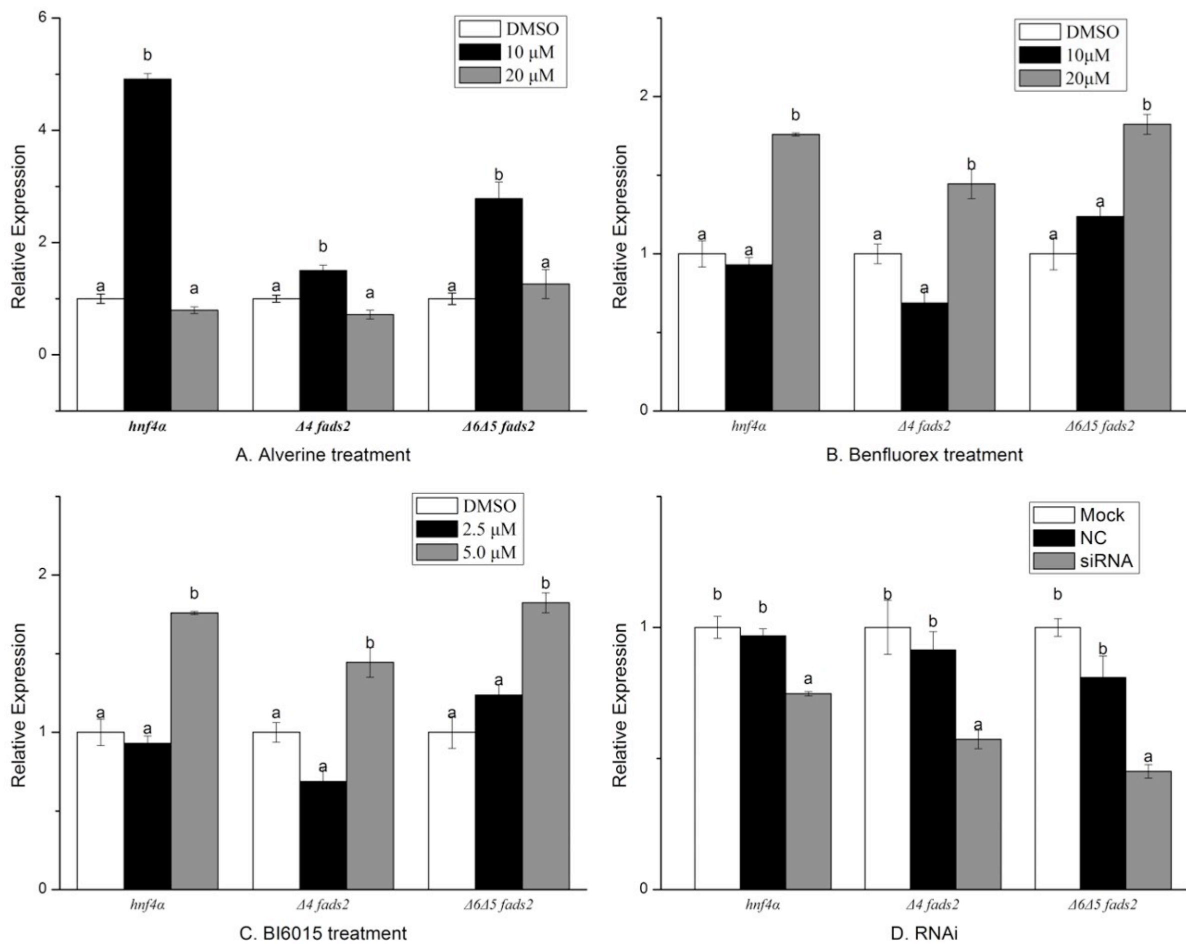

Fig. 4. Effects of gene mRNA levels in rabbitfish primary hepatocytes after incubation with alverine (A), benfluorex (B) and BI6015 (C), or being transfected with siRNA targeting Hnf4α (D). Isolated hepatocytes were incubated with alverine (10 μM and 20 μM) (A) and benfluorex (10 μM and 20 μM) (B) and BI6015 (2.5 μM and 5.0 μM) (C), and target RNAi (D). The expression of *hnf4α*, *Δ4 fads2*, *Δ6Δ5 fads2* was analyzed by qPCR with 18S as reference gene and results expressed relative to a DMSO control (A-C) or relative to a mock control (D), which was a treatment with non-target siRNA, and NC was a negative control without any treatment. Results are presented as means ± SEM (n = 3) and were analyzed by one-way analysis of variance. Bars with different superscripts are significantly different within the same treatment ($P < 0.05$).