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Title

miR-17 is involved in the regulation of LC-PUFA biosynthesis in vertebrates: effects on liver expression of a fatty acyl desaturase in the marine teleost *Siganus canaliculatus*

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Abstract

Biosynthesis in vertebrates of long-chain polyunsaturated fatty acids (LC-PUFA) such as arachidonic (ARA; 20:4n-6), eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic (DHA; 22:6n-3) acids requires the catalysis by fatty acyl desaturases (Fad). A vertebrate Fad with $\Delta 4$ activity, catalyzing the direct conversion of 22:5n-3 to DHA was discovered in the marine teleost rabbitfish *Siganus canaliculatus*. Recent studies in vertebrates have shown that miRNAs may participate in the regulation of lipid metabolism at post-transcription level. However, their roles in LC-PUFA biosynthesis were not known. In the present study, *in silico* analysis predicts that the rabbitfish $\Delta 4$ Fad may be a target of miR-17 and thus we cloned miR-17, which located to the forepart of miR-17-92 cluster. Dual luciferase reporter assays demonstrated that miR-17 targeted the 3'UTR of $\Delta 4$ Fad directly. Furthermore, the expression level of miR-17 displayed an inverse pattern with that of $\Delta 4$ Fad mRNA in gill, liver and eyes, and also the $\Delta 4$ Fad protein quantity in rabbitfish liver. Incubation of rabbitfish primary hepatocytes with linoleic acid (LA; 18:2n-6), α -linolenic acid (LNA; 18:3n-3), EPA or DHA showed differential effects on miR-17, $\Delta 4$ Fad and $\Delta 6/\Delta 5$ Fad expression. LNA promoted the expression of miR-17 and $\Delta 6/\Delta 5$ Fad, but suppressed the expression of $\Delta 4$ Fad. In contrast, LA and EPA decreased the expression of miR-17 and $\Delta 6/\Delta 5$ Fad, but had no effect on $\Delta 4$ Fad. However, all the above were down-regulated by DHA. These data indicate that miR-17 was involved in the regulation of LC-PUFA biosynthesis in rabbitfish liver by targeting $\Delta 4$ Fad.

1. Introduction

Long-chain polyunsaturated fatty acids (LC-PUFA) such as arachidonic (ARA; 20:4n-6), eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic (DHA; 22:6n-3) acids are highly bioactive and have many critical roles in animal biochemistry and physiology. Furthermore, the n-3 LC-PUFA have been demonstrated to benefit human health by decreasing cardiovascular diseases and enhancing neural development [1-4]. In vertebrates, LC-PUFA can be acquired either from the diet or biosynthesized endogenously from shorter precursors, namely the C18 PUFA linoleic (LA; 18:2n-6) and α -linolenic (LNA; 18:3n-3), through a series of desaturation and elongation reactions [5]. The predominant pathway for biosynthesis of EPA involves $\Delta 6$ desaturation of LNA to produce 18:4n-3 that is elongated to 20:4n-3 followed by $\Delta 5$ desaturation [6]. The same enzymatic activities are required for the biosynthesis of ARA from LA. DHA biosynthesis through the so-called Sprecher pathway requires two further elongations of EPA to produce 24:5n-3, a second $\Delta 6$ desaturation to 24:6n-3 and a peroxisomal chain shortening step [7]. Alternatively, recent findings have demonstrated that some fish including the rabbitfish (*Siganus canaliculatus*) [8] and Senegalese sole (*Solea senegalensis*) [9] have the ability to biosynthesize DHA directly via a $\Delta 4$ desaturation from 22:5n-3.

Due to the limited LC-PUFA biosynthetic capability of humans, there has been increasing interest in understanding the biosynthetic pathways for the production of LC-PUFA in fish, the primary dietary source of the health promoting 'omega-3' LC-PUFA for human consumption. It has been established that fish themselves vary

in their ability to produce LC-PUFA from C18 PUFA. Thus, freshwater and salmonid species have the enzymatic machinery to produce LC-PUFA from C18 PUFA precursors, and thus vegetable oils (VO), devoid of LC-PUFA but rich in LNA and LA, are able to satisfy essential fatty acid (EFA) requirements in these species [10]. In contrast, marine fish have been generally regarded as lacking such an ability due to deficiency of one or more enzymes required for LC-PUFA biosynthesis, and thus require dietary fish oil with preformed EPA and DHA to satisfy EFA requirements [11]. Therefore, the fatty acid specificities of fatty acyl desaturase (Fad) and elongation of very long-chain fatty acid (Elovl) proteins determines the overall ability of LC-PUFA biosynthesis at a molecular level.

In addition, the LC-PUFA biosynthetic pathways in fish are further regulated by environmental (e.g. salinity) and nutritional (e.g. dietary lipid and fatty acid content and composition) factors [12-17]. In liver of Atlantic salmon (*Salmo salar*), $\Delta 6$ Fad expression is higher in parr in freshwater than in smolts in seawater with corresponding changes in LC-PUFA biosynthesis [12]. Our previous results showed that rabbitfish reared at relatively low salinities (10 ppt) had increased expression levels of $\Delta 6/\Delta 5$ Fad and higher DHA content in liver in comparison to fish maintained in seawater (32 ppt) [13]. The LC-PUFA biosynthetic pathway is further regulated by diet and several studies indicate that reduced LC-PUFA in diets result in up-regulation of expression of $\Delta 6$ Fad in salmonids, Atlantic salmon [14, 15] and rainbow trout (*Oncorhynchus mykiss*) [16], and marine fish such as rabbitfish [13] and European sea bass (*Dicentrarchus labrax*) [17]. Minghetti et al. [18] suggested that $\Delta 6$ Fad was

modulated by DHA and EPA (DHA/EPA) at a transcriptional level, which was underpinned by the analysis of Fad promoters from Atlantic salmon, Atlantic cod (*Gadus morhua*) and European sea bass [19, 20]. Moreover, it was hypothesized that post-transcriptional regulation of $\Delta 6$ Fad expression may also take place in European sea bass as suggested by the inconsistent levels of Fad mRNA and protein in liver and intestine [21].

MicroRNAs (miRNAs or miRs) are short non-coding RNA molecules with about 22 nucleotides, which regulate gene expression at post-transcription level [22-24]. Biogenesis of miRNAs in animals is complex. Briefly, the genes of miRNAs are transcribed by RNA polymerase II to produce primary miRNAs followed by cleavage of an endonuclease Drosha to form precursor miRNAs (pre-miRNAs, about 70 nt) in the nucleus. Then pre-miRNAs are processed by Dicer, another endonuclease, to create mature miRNAs (miRNA, about 22 nt) in the cytoplasm [25-27]. Mature miRNAs can partially bind to 3'UTR of target protein-coding mRNA through their "seed region" (six to eight nucleotides at the 5' end of the miRNA) and consequently inhibit the expression of target genes through mRNA destabilization, translation delay or both [22-24]. Recently, accumulating data indicate that miRNAs participate in the regulation of lipid metabolism in mammals [28-31]. It is likely that miRNAs are involved in gene regulation in all vertebrates, however, the functional significance of miRNAs in LC-PUFA biosynthesis of teleosts has not been studied.

Rabbitfish *S. canaliculatus* is a commercially important marine teleost fish widespread along Indo-West Pacific coast, and also known as one of the mainly

harvested wild fish species [13, 32], It is naturally herbivorous consuming algae and seagrass, however they can also feed on compound feed or trash fishes after brief domestication with them [13]. The artificial culture of rabbitfish is developing rapidly in southeastern Asia including China during these years. What should be specially mentioned is that *S. canaliculatus* is the first marine teleost in which all the enzymatic activities of desaturation and elongation required for LC-PUFA biosynthesis have been identified and characterized including mammalian Fads2 orthologs with $\Delta 4$ and $\Delta 6/\Delta 5$ desaturase activities by us [8, 13, 33], and thus the rabbitfish provides an interesting model for studying the regulatory mechanism of LC-PUFA biosynthesis in vertebrates.

In order to investigate the possible role of miRNAs in the regulation of LC-PUFA biosynthesis in rabbitfish, we made a bioinformatic analysis and it was found that there is a conserved complementary site for miR-17 in the 3'UTR of rabbitfish $\Delta 4$ Fad cDNA. This prompted us to investigate the function of miR-17 in the regulation of $\Delta 4$ Fad gene expression and LC-PUFA biosynthesis at post-transcriptional level in rabbitfish. First, the sequence of miR-17-92 cluster was cloned so as to obtain the intact sequence of miR-17, since studies in humans showed that miR-17 belongs to the miR-17-92 cluster, which comprises six miRNAs including miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1, and is transcribed as a typical polycistronic primary transcript that is then processed into multiple individual mature miRNAs [34, 35]. Further *in vivo* and *in vitro* experiments results demonstrated that miR-17 is involved in the regulation of liver LC-PUFA biosynthesis by directly

targeting $\Delta 4$ Fad at a post-transcriptional level. These data increase our understanding of the regulatory mechanisms of LC-PUFA biosynthesis in vertebrates and specifically, fish, which may enable the activity of the LC-PUFA pathway to be optimized in marine teleosts.

2. Materials and methods

2.1. Animals and tissue collection

Rabbitfish juveniles (body mass ~ 13 g, sex indistinguishable visually) were captured in summer 2012 from the coast near Nan Ao Marine Biology Station (NAMBS) of Shantou University, Southern China. Initially, 500 fish were maintained in an indoor seawater pool (32 ppt) for one month. Half the fish were then acclimated from seawater (32 ppt) to brackish water (10ppt) for one month, while the remainder was maintained in seawater. All fish were then acclimated to the laboratory conditions at the two salinities for a further two weeks prior to the initiation of the experiment. Throughout the study, fish were fed a single formulated diet, which contained 32 % crude protein and 8 % crude lipid (a blend of fish oil and vegetable oil) as described previously [36].

The 8-week trial was carried out in an aquarium system at NAMBS. After being starved for 24 h, the fish were anesthetized with 0.01 % 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO, USA), individually weighed and allocated randomly to six cylindrical tanks (90 cm diameter, 100 cm depth), with 20 fish per tank and triplicate tanks at both salinities (10 and 32 ppt). During the trial, oxygen-saturation was maintained by aeration and half the volume was changed twice a day (morning and evening). Temperature was maintained at 22 ± 3 °C and photoperiod was set at a 12 h light: 12 h dark cycle. The fish were fed twice a day (at 9:00 and 16:00 h) at 1-2 % of body weight and the amount of feed recorded daily.

During the acclimation period, liver was sampled from one fish for DNA isolation and cloning of miR-17-92 gene cluster. In addition, several tissues including adipose, brain, dorsal muscle, eyes, gallbladder, gill, heart, intestine, kidney, liver and spleen were sampled from three fish, and used for RNA isolation to investigate the miR-17 and $\Delta 4$ *Fad* tissue-specific expression. At the initiation of the salinity trial, livers were collected from three fish at seawater (32 ppt) as the initial control samples, and latterly used for RNA isolation followed by determination of miR-17 or $\Delta 4$ *Fad* expression by quantitative PCR (qPCR). Similarly, at the end of the trial, livers were collected from two fish per tank (six fish per salinity) for RNA or protein analysis. Tissue samples were immediately frozen in liquid nitrogen after collection and subsequently stored at -70 °C before use.

2.2. Molecular cloning of miR-17-92 cluster and sequence analysis

Studies in human and zebrafish showed that miR-17 was located within the miR-17-92 cluster genomic region [34, 37, 38]. In order to obtain the rabbitfish miR-17, we first cloned part of the miR-17-92 cluster gene by PCR (*Pfu* DNA polymerase, Tiangen Biotech, Beijing, China) using primers (1792-part-F and 1792-part-R) designed in conserved regions of the alignment of the miR-17-92 cluster from human (NG_032702.1), mouse (NC_000080.6), chicken (AY866309.1), frog (GL173376.1) and zebrafish (NC_007112.5). Genomic DNA (gDNA) prepared from rabbitfish liver (DNeasy[®] blood & tissue kit, Qiagen, Hilden, Germany) was used as template. Subsequently, to obtain the full length of pre-miR-17 located at the 5' end

of miR-17-92 gene cluster, the upstream sequence of the acquired miR-17-92 gene cluster was extended by genome walking technology (Takara, Dalian, China) using two gene-specific primers (1792-ups-sp1 and 1792-ups-sp2) and arbitrary primers provided by the kit. Finally, the full-length miR-17-92 cluster and its upstream region were amplified using the specific primers (1792-full-F and 1792-part-R) and using the high fidelity *Pfu* polymerase as above. All primers sequences are shown in Table 1. The putative pre-miR-17 sequence of rabbitfish was compared with its corresponding orthologues from human (accession: MI0000071), mouse (MI0000687), chicken (MI0001184), frog (MI0004803) and zebrafish (MI0001897) in miRBase (<http://www.mirbase.org/>) using ClustalX2 sequence alignment, and secondary structure predicted using RNA Folding Form online (<http://mfold.rna.albany.edu/>).

2.3. Dual luciferase reporter assays

To determine whether $\Delta 4$ Fad was a direct target of miR-17, a dual luciferase assay was performed using human embryonic kidney (HEK 293T) cells (Chinese Type Culture Collection, Shanghai, China), which was a commercial cell line commonly used for gene expression study in dual-luciferase reporter assays.. For heterologous expression of rabbitfish miR-17, a 336 bp DNA fragment encompassing rabbitfish pre-miR-17 was amplified with primer pair PEG-pmiR-17-F/R (Table 1). The PCR product was digested by *EcoRI* and *BamHI* (New England Biolabs, Ipswich, MA, USA) and cloned into pEGFP-C3 plasmid behind an artificial stop codon (pEGFP-miR-17) (Fig. 1A). The pEGFP-C3 plasmid with no insert (pEGFP-empty)

was used as a negative control. The presence of the expression vector pEGFP-miR-17 transfected into HEK 293T cells was verified by qPCR. For construction of dual luciferase reporter vectors, DNA fragments were inserted into pmirGLO dual-luciferase miRNA target expression vector (Promega, Madison, WI, USA) by digestion with *SacI* and *XbaI*. The recombinant vectors were: 1) pmirGLO- $\Delta 4$ fad-3UTR. Partial DNA fragment of rabbitfish $\Delta 4$ Fad (GU594278.1) 3'UTR (1475-1820 bp) was amplified by primers pmirGLO- $\Delta 4$ fad-3UTR-F/R and then inserted into pmirGLO; 2) pmirGLO- $\Delta 4$ fad-3UTR-Mu. By overlap extension PCR (pmirGLO- $\Delta 4$ fad-3UTR-MU-F/R and pmirGLO- $\Delta 4$ fad-3UTR-F/R), the predicted binding site of miR-17 on $\Delta 4$ Fad 3'UTR 5'-CACTTTG-3' (1784-1790 bp) was mutated into 5'-CAGTATC-3' to prevent complementarity of miR-17 and then cloned into pmirGLO; 3) pmirGLO- $\Delta 4$ fad-3UTR-RE. 30 nt oligonucleotide (1766-1795 bp) encompassing the predicted response element (RE) of miR-17 in 3'UTR of $\Delta 4$ Fad was synthesized (Sangon Biotech, Shanghai, China) and then annealed and ligated into pmirGLO vector; 4) pmirGLO-R17. Similarly, 22 nt oligonucleotide containing a 100 % match to miR-17 was constructed and ligated into pmirGLO as positive control. Empty pmirGLO vector (pmirGLO-empty) was used as a negative control. All the PCR reactions were performed using high-fidelity *Pfu* DNA polymerase (Tiangen Biotech) and the insert fragments of recombinant plasmids sequenced by Sangon Biotech (Shanghai, China).

Dual luciferase reporter assays in HEK 293T cells were carried out in triplicate in 96 well plates as described by Allen et al. [39] with Dual-Luciferase[®] reporter assay

system (Promega, Madison, WI, USA). HEK 293T cells were cultured at 37 °C / 5 % CO₂ in high glucose DMEM (GlutaMAX) medium (Gibco, Life Technologies, USA) supplemented with 10 % fetal calf serum (FBS, Sijiqing Biological Engineering Material Company, Hangzhou, China). Twenty-four hours before transfection, cells were seeded in 96-well plates with 2×10^4 cells per well in the same medium so that cells were 70-80 % confluent at time of transfection. HEK 293T cells were co-transfected with either pEGFP-miR-17 (62.5 ng) or pEGFP-empty vector (62.5 ng), and one of the five dual luciferase reporter vectors (62.5 ng) using PolyFect Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Firefly and Renilla luciferase activities were quantified 24 h after transfection by microplate reader (Infinite M200 Pro, Tecan, Switzerland) and firefly luciferase activity was normalized to Renilla luciferase activity.

2.4. Rabbitfish primary hepatocytes isolation and incubation with PUFA

Fatty acid/BSA complexes of PUFA including LA, LNA, EPA and DHA (Cayman Chemical Co., Ann Arbor, USA) at 10 mM concentration were prepared according to Ou et al [40] and stored at -20 °C. Three rabbitfish (~ 35 g) were fasted for 24 h and killed after anaesthesia in 0.01 % 2-phenoxyethanol, followed by immersion in 70 % ethanol for 3 min to sterilize the external surface. Liver tissues were excised aseptically, washed with ice-cold Hank's Balanced Salt Solution (HBSS), immersed in 70 % ethanol for 30 s and immediately washed 3 times with ice-cold HBSS. Liver was chopped and digested with 0.1 % collagenase (Gibco, Life

Technologies, USA) / 0.25 % hyaluronidase (Sigma-Aldrich, St. Louis, USA) for 30 min at room temperature and cell suspensions filtered through a 100 μ m cell strainer (BD Falcon, Franklin Lakes, NJ, USA). Isolated cells were washed in red blood cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) for 2 min at 4 °C. Then the cell viability was evaluated using a haemocytometer under an inverted microscope after the cells were dyed with 0.4% Trypan Blue. Cells with ≥ 98 % viability were plated on 6-well plates coated with 0.1 % gelatin at a density of 2×10^6 cells per well in DMEM/F12 (Gibco, Life technologies, USA) medium containing 20 % FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin, followed by incubation for 24 h at 24 °C / 4 % CO₂. The hepatocytes were incubated for 1 h in FBS-free DMEM/F12 prior to incubation with 100 μ M of LA, LNA, EPA or DHA in triplicate wells. After 6 h incubation, cells were lysed in the wells and harvested for later RNA extraction.

2.5. RNA isolation and quantitative real-time PCR (qPCR) determinations

For tissue expression analysis of miR-17 and $\Delta 4$ Fad in rabbitfish, and quantitative analysis of RNA levels of these two genes in livers of rabbitfish reared in 10 ppt or 32 ppt salinity, total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA). For quantitative analysis of rabbitfish miR-17 in HEK 293T cells or miR-17 (NCBI accession: KF516079) and $\Delta 4$ (GU594278.1) and $\Delta 6/\Delta 5$ *Fads* (EF424276.2) in rabbitfish primary hepatocytes, total RNA was isolated and purified by RNeasy pure cell/bacteria kit (Qiagen Biotech, Beijing, China). The concentration and quality

of total RNA was confirmed by spectrophotometer (Nano-drop 2000, Thermo Scientific, USA). cDNA was synthesized from 1 µg or 500 ng of total RNA from rabbitfish tissues or cells, respectively (miScript II RT Kit, Qiagen, Hilden, Germany).

The expression of miR-17 was determined by miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) with miR-17 specific primer (qPCR-miR-17) and universal primer. For qPCR measurement of $\Delta 4$ or $\Delta 6/\Delta 5$ *Fads*, LightCycler[®] 480 SYBR Green I Master (Roche, Germany) was used with gene-specific primers (Table 1). The relative RNA levels of genes in each sample were normalized with 18S rRNA (AB276993) expression calculated by the comparative threshold cycle (Ct) method [41], where the C_t value of initial samples consisting of three fish was used as control. All reactions were run in LightCycler[®] 480 thermocycler (Roche, Germany) using qPCR programs according to manufacturer's specifications.

2.6. Western blotting of $\Delta 4$ Fad in livers of rabbitfish reared in different salinities

To study the potential relationship between miR-17 and the protein expression level of $\Delta 4$ Fad *in vivo*, Western blotting of rabbitfish $\Delta 4$ Fad was carried out. Total protein from triplicate pooled liver tissue (20 mg each fish) samples from six rabbitfish cultured in the same salinity (10 ppt or 32 ppt) was extracted using tissue or cell total protein extraction kit (Sangon Biotech, Shanghai, China) and concentrations quantified by non-interference protein assay kit (Sangon Biotech, Shanghai, China). Fifty µg protein extract was separated on 12 % SDS/PAGE with 5 µg BSA as a negative control. Separated proteins were transferred onto PVDF membranes and the

membranes probed with primary customized monoclonal antibody against rabbitfish $\Delta 4$ Fad (Abmart Inc., Shanghai, China) at 1:3000, and subsequently secondary goat-anti-mouse antibody (Millipore, Bedford, MA, USA) at 1:5000, both diluted by antibody solution in SignalBoostTM immunoreaction enhancer kit (Merck KGaA, Darmstadt, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for data normalization using specific primary GAPDH antibody (Beyotime Institute of Biotechnology, Haimen, China). Bands were revealed by chemiluminescent detection with Immobilon Western chemiluminescent HRP substrate (Millipore, Bedford, MA, USA), and analyzed by Quantity One software version 4.6.9 (Bio-Rad, Hercules, CA, USA) with the quantity of $\Delta 4$ Fad and GAPDH protein converted into intensity values presented as $\text{Int} \times \text{mm}^2$.

2.7. Statistical analysis

Data were presented as means \pm SEM (n=6 for liver gene expression in rabbitfish reared in 10 ppt or 32 ppt water; n=3 for miR-17 and $\Delta 4$ Fad tissue-specific distribution and gene expression from cell assays) or means \pm SD (n=3) for dual luciferase assay or protein quantification. Differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or Students t-test (as indicated) at a significance level of $P \leq 0.05$ using OriginPro 7.5 software (Originlab Corporation, Northampton, MA, USA).

3. Results

3.1. The structure of rabbitfish miR-17-92 cluster

To obtain the nucleotide sequence of rabbitfish miR-17 and pre-miR-17, a 1958 bp genomic fragment encompassing miR-17-92 cluster was cloned (Fig. 2). It was a polycistron comprised of six miRNAs including miR-17, miR-18(a), miR-19(a), miR-20(a), miR-19(b) and miR-92(a). By comparison with the corresponding miRNAs of zebrafish, the nucleotide (nt) sequence of rabbitfish pre-miR-17 (89 nt) and mature miR-17 (22 nt) were predicted (Fig. 2, 3), and confirmed to belong to the miR-17 family. The rabbitfish pre-miR-17 showed 92 % sequence identity to zebrafish pre-miR-17 and 82-85 % identity to that of human, mouse, chicken and frog. At the 5' end of miR-17, there was a 7 nt (AAAGUGC) "seed sequence", critical for binding site recognition of miRNA and highly conserved among different animal species [42] (Fig. 3). The secondary structure of pre-miR-17 was revealed by mfold (<http://mfold.rna.albany.edu/>), and showed typical and stable stem-loop structure with $\Delta G = -36.5$ (Fig. 4).

3.2. Tissue distribution of rabbitfish miR-17 and $\Delta 4$ Fad

Tissue-specific expression of rabbitfish miR-17 and $\Delta 4$ Fad was determined by qPCR (Fig. 5). Among the eleven tissues examined, miR-17 expression was significantly higher in gill than that in adipose tissue, dorsal muscle, eyes, gallbladder, heart, liver and spleen ($P \leq 0.05$). Intermediate expression levels were detected in brain, intestine and kidney (Fig. 5A). The expression of $\Delta 4$ Fad was highest in brain

followed by intestine, eyes and liver. Low $\Delta 4$ Fad expression was detected in other tissues including adipose tissue, dorsal muscle, gallbladder, gill, heart, kidney and spleen ($P \leq 0.05$). (Fig. 5B).

3.3. Rabbitfish $\Delta 4$ Fad 3'UTR is a target of miR-17

A dual luciferase assay was carried out to determine the responsiveness of rabbitfish $\Delta 4$ Fad 3'UTR for miR-17. Results from qPCR detection showed that HEK 293T cells transfected with pEGFP-miR-17 vector had a high level of rabbitfish miR-17 expression around 120-fold higher than the endogenous background of miR-17 ($P \leq 0.01$) (Fig. 1B). The results of the dual luciferase reporter assay showed that: i) in negative controls, there was no difference between lane 1 and lane 2 treatments (Fig. 6B) in normalized Luc activity which was around 100; ii) in positive control, pEGFP-miR-17/pmirGLO-R17 (Fig. 6B: lane 4) cotransfected group showed significant lower expression than that of pEGFP-empty/pmirGLO-R-17 group (Fig. 6B: lane 3) ($P \leq 0.01$) even though the latter also dropped to 40 in Luc activity; iii) as experimental groups (Fig. 6B: lane 5 - 10), it displayed that heterologous expression of miR-17 in 293T cells resulted in around 40 % lower normalized Luc activity of $\Delta 4$ Fad 3'UTR ($P \leq 0.01$) (Fig. 6B: lane 5 - 6) and around 20 % lower in normalized Luc activity of RE fragment of $\Delta 4$ Fad 3'UTR ($P \leq 0.05$) (Fig. 6B: lane 9 - 10). However, mutation in the predicted binding sites of miR-17 at 3'UTR of $\Delta 4$ Fad abolished its response to miR-17 overexpression (Fig. 6B: lane 7 - 8).

3.4. *miR-17 displayed an inverse pattern of expression with $\Delta 4$ Fad transcript and protein quantity in livers of rabbitfish reared in different salinities*

In livers of rabbitfish reared in 10 ppt or 32 ppt water, the expression level of miR-17 displayed an inverse pattern of expression with $\Delta 4$ Fad at transcript and protein levels. The expression level of miR-17 in livers of rabbitfish reared in 32 ppt was significantly higher than that in 10 ppt ($P \leq 0.05$) (Fig. 7A). In contrast, the expression of $\Delta 4$ Fad at both transcript (Fig. 7B) and protein (Fig. 7C-D) levels was significantly lower at 32 ppt compared to 10 ppt ($P \leq 0.05$).

3.5. *miR-17, $\Delta 4$ Fad and $\Delta 6/\Delta 5$ Fad showed different responses to PUFA supplement*

The relative expression of miR-17, $\Delta 4$ Fad and $\Delta 6/\Delta 5$ Fad in rabbitfish hepatocytes incubated with different PUFA was determined by qPCR. The expression levels of miR-17 and $\Delta 6/\Delta 5$ Fad were significantly lower ($P \leq 0.05$) in hepatocytes supplemented with LA, EPA and DHA, but significantly higher ($P \leq 0.05$) in cells incubated with LNA (Fig. 8A, C). The expression levels of $\Delta 4$ Fad were significantly ($P \leq 0.05$) lower in hepatocytes incubated with LNA or DHA, but showed no changes when incubated with either LA or EPA (Fig. 8B). It should be noted that miR-17 had an inverse trend in expression with $\Delta 4$ Fad with LNA supplementation.

4. Discussion

It has been established that some miRNAs are crucial post-transcriptional regulators of lipid metabolism in vertebrates. In mammals, several miRNAs such as miR-33a/b and miR-27a/b are known to be involved in the regulation of lipid metabolism including cholesterol transport, fatty acid oxidation and adipocyte differentiation [28-31, 43]. Besides its role on tumorigenesis, miR-17, a component of miR-17-92 cluster, is necessary for normal development of tissues such as lung, heart and lymphocytes [44, 45]. Additionally, a role for miR-17-92 cluster in acceleration of adipocyte differentiation has been reported [46]. These data suggest that miRNAs of miR-17-92 cluster such as miR-17 may have more extensive regulatory roles in metabolism and physiology. However, little was known about the actions of miRNAs in the regulation of LC-PUFA biosynthesis in teleosts. A study in sea bass showed that the amount of $\Delta 6$ Fad transcript in liver was inconsistent with its protein expression [21], which suggested that post-transcriptional regulation may exist in fish LC-PUFA biosynthesis. On the basis of our bioinformatic analysis indicating a conserved complementary site for miR-17 in the 3'UTR of rabbitfish $\Delta 4$ Fad, the present study was conducted to demonstrate the role of miR-17 in the regulation of LC-PUFA biosynthesis by directly targeting Fads in rabbitfish liver.

First, the sequence of miR-17-92 cluster was cloned in rabbitfish. The same organization of the six members was found for the rabbitfish miR-17-92 cluster as in human (chromosome 13) and zebrafish (chromosome 1), indicating high conservation through vertebrates and potentially similar roles in metabolic regulation. In zebrafish,

miR-17 is also located in another miRNA cluster consisting of miR-18b, miR-92a-2 at chromosome 9, however, in the present study, the miR-17 sequence was only cloned from the miR-17-92 cluster [37]. This can be attributed to piscine varied and “plastic” genomes, which are prone to gene duplication and polyploidy [47], although other locations of miR-17 cannot be discounted due to a lack of genomic data in rabbitfish. miRNAs recognize targets depending on complementarity between the seed sequence and 3'UTR of target genes, so sequence changes are always detected at the 3' end of miRNAs, but rarely at the 5' seed sequence. The same situation has also been observed in miRNA transcriptomes of teleost [48, 49]. Likewise, the seed sequence of rabbitfish miR-17 is highly consistent with that of other species, which suggests that miR-17 may exert similar regulatory functions in rabbitfish. In addition, the predicted secondary structure of pre-miR-17 shows that the predicted sequence folds to form the typical stem-loop structure necessary for recognition and cleavage by Drosha and Dicer enzymes, and regulatory function [50, 51].

Tissue distribution points to the functional importance of miRNAs [52]. In the present study, miR-17 was expressed ubiquitously among the examined rabbitfish tissues, which was in agreement with the spatial expression pattern of miR-17 in zebrafish embryos that showed miR-17 presented widespread expression in head, spinal cord, gut, outline somites and neuromasts [53]. Generally, miRNAs regulate target genes by repressing their functional expression after transcription [22, 24]. The expression level of $\Delta 4$ Fad was high in brain, eyes, liver and intestine, which were considered to be tissues for LC-PUFA biosynthesis in rabbitfish [13, 33]. In addition,

miR-17 showed relatively low abundance in these tissues, whereas the amount of miR-17 was highest in gill where $\Delta 4$ Fad gene was barely expressed. These data suggest that miR-17 might be involved in the regulation of LC-PUFA biosynthesis in some rabbitfish tissues.

In order to confirm the possible role of miR-17 in the regulation of LC-PUFA biosynthesis of rabbitfish, we tested interaction between miR-17 and the $\Delta 4$ Fad 3'UTR with the dual luciferase reporter assay in HEK 293T cells. The results indicated that miR-17 had potent repression on the 3'UTR of $\Delta 4$ Fad, which provided evidence that $\Delta 4$ Fad was likely a direct target of miR-17 (Fig. 6). This suggested that miR-17 may participate in the regulation of LC-PUFA biosynthesis by interaction with $\Delta 4$ Fad. However, a more significant suppression of 3'UTR than at the known response element for miR-17 was observed, which suggested that the inhibitory effect of miR-17 on $\Delta 4$ Fad 3'UTR may be enhanced by other miRNA paralogs, such as the miR-106b-25 cluster, which function in cooperation with the miR-17-92 cluster in mammals [54].

The regulatory effect of miRNA on its target gene should be investigated in a physiological context as heterologous systems may result in non-physiological interactions due to altered cofactor environments [55]. To further validate the *bona fide* relationship between miR-17 and $\Delta 4$ Fad in the regulatory background of LC-PUFA biosynthesis *in vivo*, the expression pattern of miR-17 was analyzed in combination with quantitative expression of $\Delta 4$ Fad mRNA and protein in livers of rabbitfish reared in different salinity, which is an important environmental factor

affecting gene expression and activity of Fads, as well as LC-PUFA biosynthesis metabolism, in marine teleost [12, 13, 56, 57]. The results showed that the liver expression level of miR-17 was lower at 10 ppt than that at 32 ppt, which showed inverse trends compared to $\Delta 4$ Fad transcript expression and protein quantity. miRNA inhibits the functional expression of target genes through mRNA destabilization and/or translation delay. In the present study, miR-17 may act as a repressor of $\Delta 4$ Fad, inducing the degradation of $\Delta 4$ Fad mRNA and/or repressing $\Delta 4$ Fad translation, resulting in reduced $\Delta 4$ Fad protein. Taken together, the results from miR-17 tissue distribution, luciferase assay and *in vivo* expression support our hypothesis that miR-17 is likely to be involved in regulation of LC-PUFA biosynthesis *in vivo* by targeting the 3'UTR of $\Delta 4$ Fad.

It was reported previously that PUFA are important factors modulating Fad expression and LC-PUFA biosynthesis [12, 58]. In order to further confirm an *in vivo* role of miR-17, *in vitro* experiments were designed to elucidate the role of miR-17 in LC-PUFA biosynthesis. Thus, the gene expression of miR-17 and Fads were measured in rabbitfish primary hepatocytes after incubation with different PUFA. The results showed that incubation with LNA induced the expression of miR-17, but down-regulated expression of $\Delta 4$ Fad mRNA whereas, in contrast, the expression of miR-17 was reduced in cells incubated EPA and LA supplementation, which abolished the inhibitory effect on $\Delta 4$ Fad. The inverse expression pattern of miR-17 and $\Delta 4$ Fad between LNA and LA/EPA treatment was consistent with the *in vivo* results. These data further supported the conclusion that in the LC-PUFA biosynthesis pathway, $\Delta 4$

Fad was regulated by miR-17 at a post-transcriptional level, probably beginning with the degradation of $\Delta 4$ Fad mRNA.

However, it is illogical that $\Delta 4$ Fad was down-regulated by LNA treatment but not LA, even though the up-regulation of miR-17 can account for this. Previously we showed that rabbitfish fed a diet with an LA/LNA ratio of 3.1 or 1.2 (without fish oil) biosynthesized more DHA and EPA in liver than fish fed diet with an LA/LNA ratio of 55.6 [13]. In this regard, we further estimated the expression of $\Delta 6$ Fad in different PUFA treatments. Intriguingly, the expression of $\Delta 6$ Fad showed a complementary pattern to that of $\Delta 4$ Fad in LNA and LA/EPA treatment. As there are two possible pathways for DHA biosynthesis in rabbitfish, the “ $\Delta 4$ Fad” pathway dominated by $\Delta 4$ Fad and the “Sprecher” pathway including “ $\Delta 6/\Delta 5$ Fad”, the complementary expression pattern of $\Delta 4$ Fad and $\Delta 6/\Delta 5$ Fad indicated that there may be a coordinated mechanism between the pathways. So that PUFA may influence the expression of $\Delta 4$ Fad via miR-17 which plays a switch-like role for the “ $\Delta 4$ Fad” pathway whereas it remains to be clarified why $\Delta 6$ Fad is expressed complementarily in LNA and LA/EPA treatments.

In agreement with previous studies in fish that dietary DHA commonly down-regulated the gene expression of hepatic Fads *in vivo* [13, 15, 21, 59, 60], DHA significantly inhibited the expression of both Fads in rabbitfish primary hepatocytes. However, DHA simultaneously decreased the expression of miR-17, which indicated that, different from LNA treatment, the reduction of $\Delta 4$ Fad transcripts in DHA supplement was not a result of miR-17, but likely to be controlled mainly at a

transcriptional level by SREBPs or LXRs [18-20, 61]. However, in humans, the expression of miR-17 was also activated by c-Myc, a transcription factor down-regulated by a DHA/EPA diet [62, 63]. Thus, in the present study, decreased expression of miR-17 may also be attributed to transcriptional control. Furthermore, due to the absence of a rabbitfish liver cell line, the direct regulatory effect of miR-17 on LC-PUFA synthesis in rabbitfish cannot be solely inferred by gain-of-function and loss-of-function trials. Therefore, the extent of the role of miR-17 in the regulation of the LC-PUFA biosynthesis pathway remains to be fully clarified.

In summary, the present study indicates that miR-17 is involved in the regulation of LC-PUFA biosynthesis in rabbitfish by targeting $\Delta 4$ Fad. Moreover, based on the results from PUFA supplementation assays, it suggested that miR-17 may act as a switch to orchestrate the “ $\Delta 4$ Fad” pathway in response to PUFA supplement in rabbitfish liver. To our knowledge, this is for the first time to demonstrate the function of miRNA in LC-PUFA biosynthesis of vertebrates, and also the first study on the regulatory mechanism of LC-PUFA biosynthesis at post-transcriptional level in fish.

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Figure Legends

Fig. 1. miR-17 of rabbitfish is over-expressed in HEK 293T cells transfected with pEGFP-miR-17 expression vector. (A) Construction of expression vectors with miR-17 or recombinant dual luciferase reporter vectors, (B) The expression level of miR-17 determined by qPCR in HEK 293T cells transfected with either pEGFP-empty or pEGFP-miR-17 vectors using PolyFect Transfection Reagent (Qiagen, Hilden, Germany). Data are means \pm SEM (n=3), bars with different superscript letter represent higher significance to each other ($P \leq 0.01$; T-test).

Fig. 2. The genomic organization of rabbitfish miR-17-92 cluster. A 1958 bp gene fragment encompassing miR-17-92 cluster has been obtained. By blasting with zebrafish miR-17-92 cluster, six mature miRNAs are predicted (grey boxes). Sequences between mature miRNAs including non-coding regions are indicated by black lines and nucleotide sequence in front of miR-17 (1130 bp) is indicated by broken line. The sequence encoding pre-miR-17 has been noted by horizontal arrows. Besides, vertical dotted lines with numbers indicate positions of each element at the 1958 bp gene fragment.

Fig. 3. Clustal X2 multiple alignment of the nucleotide sequences of rabbitfish (*Siganus canaliculatus*, sca) pre-miR-17 with its orthologues in human (*Homo sapiens*, hsa, MI0000071), mouse (*Mus musculus*, mmu, MI0000687), chicken (*Gallus gallus*, gga, MI0001184), frog (*Xenopus tropicalis*, xtr, MI0004803), zebrafish (*Danio rerio*,

dre, MI0001897). Sequence data were obtained from miRBase (<http://www.mirbase.org/>). Nucleotide numbers are shown on the right. Identical residues are shaded black and similar residues are shaded grey. The putative miR-17 mature sequence in rabbitfish is dot underlined and seed sequence (AAAGUGC) is framed out.

Fig. 4. Predicted secondary structure of rabbitfish pre-miR-17. The secondary structures were retrieved by mfold (<http://mfold.rna.albany.edu/>) with default parameter and lowest ΔG value= -36.5. Mature sequence of miR-17 is highlighted in grey.

Fig. 5. Relative tissue distribution profile of miR-17 (A) and $\Delta 4$ Fad (B) in rabbitfish determined by qPCR. 18S rRNA was used as an internal control. Values are means \pm SEM (n=3) as fold change from liver and bars without sharing a common superscript letter indicate significantly difference ($P \leq 0.05$; ANOVA, Tukey's test).

Fig. 6. miR-17 targets the 3'UTR of $\Delta 4$ Fad. (A) predicted base pair of mature miR-17 in the 3'UTR of $\Delta 4$ Fad. Asterisks denote site-directed mutant. (B) luciferase activity in HEK 293T cells co-transfected pEGFP-miR-17 or pEGFP-empty with different recombinant dual luciferase reporter vectors: i) pmirGLO-empty as negative control (lane 1 - 2); ii) pmirGLO-R17 as positive control (lane 3 - 4); iii) pmirGLO- $\Delta 4$ fad-3UTR containing 3'UTR of $\Delta 4$ Fad (lane 5 - 6); iv) pmirGLO- $\Delta 4$

fad-3UTR-MU with 3 nt site-directed mutation in 3'UTR of $\Delta 4$ Fad (lane 7 - 8); v) pmirGLO- $\Delta 4$ fad-3UTR-RE only containing predicted miR-17 binding site in $\Delta 4$ Fad 3'UTR (lane 9 - 10). The Renilla luciferase activity was used to normalize that of firefly luciferase. Data are shown as means \pm SD (n=3) and asterisks represent significant differences (* $P \leq 0.05$; ** $P \leq 0.01$; T-test).

Fig. 7. The expression of miR-17 displayed inverse pattern compared to $\Delta 4$ Fad mRNA level and protein quantity in livers of rabbitfish reared in 10 ppt and 32 ppt. Expression levels of (A) miR-17 and (B) $\Delta 4$ Fad mRNA determined by qPCR, where 18S rRNA was used as an internal control, and the mean CT value of initial samples was employed for normalization between 10 ppt and 32 ppt. (C) Western blotting of $\Delta 4$ Fad in livers. The blot was immunoprobed with monoclonal antibody specified for rabbitfish $\Delta 4$ Fad at 1:3000 dilution and second antibody 1:5000 dilution with three replicate lanes from one pool of six fish livers. BSA was used as a negative control. (D) $\Delta 4$ Fad protein level of (C) which was quantified by Quantity One (Bio-rad) and then normalized by GAPDH. Data are means \pm SEM (n=6) for qPCR detection of miR-17 and $\Delta 4$ Fad, or means \pm SD (n=3) for protein quantity. Different superscripts indicate significant differences ($P \leq 0.05$; T-test).

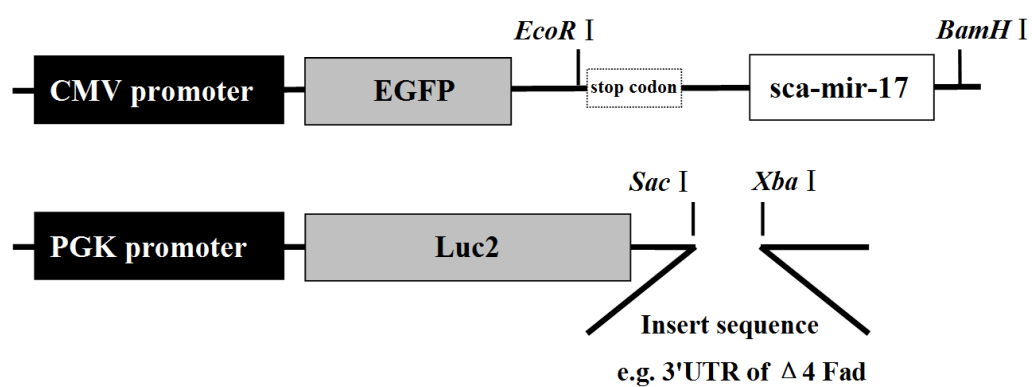
Fig. 8. Effects of PUFA on miR-17, $\Delta 4$ Fad and $\Delta 6/\Delta 5$ Fad gene expression in rabbitfish primary hepatocytes. Isolated hepatocytes were incubated with LA, LNA, EPA or DHA at 100 μ M, or 0.1% BSA (control) for 6h. The relative mRNA levels of

miR-17 (A), $\Delta 4$ Fad (B) and $\Delta 6/\Delta 5$ Fad (C) were assessed by qPCR, where 18S rRNA was used as an internal control. Results were presented as the fold change from control in means \pm SEM (n=3). Statistical analysis was performed using ANOVA followed by Tukey's test among different treatment groups, and black columns with different superscript letters indicated significant difference ($P \leq 0.05$). Difference between each fatty acid treatment and control was analyzed using T-test and asterisks represent significant differences ($P \leq 0.05$).

Table 1**Primers or oligonucleotides used for gene clone, qPCR or vector reconstruction**

Aim	Gene/Vectors name	Primers/oligonucleotides	Nucleotide sequence
Partial gene clone	miR-17-92 cluster	1792-part-F 1792-part-R	5'- GCCGTGTCAAAGTGCTTACAGTGC-3' 5'- CAGGCCGGGACAAGTGCAATA-3'
Genome walking PCR	miR-17-92 cluster	1792-ups-sp1 1792-ups-sp2	5'- CTGAAACTGGGACAGGGCGACAAC-3' 5'- CTAGATGCACCTTAGCACAAAGACAG-3'
Full length PCR	miR-17-92 cluster miR-17 $\Delta 4$ <i>fad</i>	1792-full-F qPCR-miR-17 qPCR- $\Delta 4$ <i>fad</i> -F qPCR- $\Delta 4$ <i>fad</i> -R	5'- AGGCCGCGCGTTTTAGCTTAG-3' 5'- CAAAGTGCTTACAGTGCAGGTA-3' 5'- TGCACCTAACAGGAGTCACGTT-3' 5'- GCTCAGCACAGGATTGAGTACG-3'
qPCR	$\Delta 6/\Delta 5$ <i>fad</i> 18S rRNA	qPCR- $\Delta 6/\Delta 5$ <i>fad</i> -F qPCR- $\Delta 6/\Delta 5$ <i>fad</i> -R qPCR-18S-F qPCR-18S-R	5'- TCACTGGAACCTGCCCACAT-3' 5'- TTCATTCTCAGACAGTGCAAACAG-3' 5'- CGCCGAGAAGACGATCAAAC-3' 5'- TGATCCTTCCGCAGGTTTAC-3'
Construction of expression vector pEGFP-miR-17	pEGFP-miR-17	pEG-pmiR-17-F pEG-pmiR-17-R	5'-CCGGAATTCCTAGATAACTGATCATATTCATTGTCTTAATAAGCTGTCA-3' 5'- CGCGGATCCTCTGCACTAGATGCACCTTAGCA-3'
Construction of reporter vectors	pmirGLO- $\Delta 4$ <i>fad</i> -3UTR	pmirGLO- $\Delta 4$ <i>fad</i> -3UTR-F pmirGLO- $\Delta 4$ <i>fad</i> -3UTR-R	5'- CAGGAGCTCCTGTTTTGGCCTCAGAGCT-3' 5'- CGCTCTAGAGTTTTTATTGTATTGTCTGGAACA-3'
	pmirGLO- $\Delta 4$ <i>fad</i> -3UTR-MU	pmirGLO- $\Delta 4$ <i>fad</i> -3UTR-MU-F pmirGLO- $\Delta 4$ <i>fad</i> -3UTR-MU-R	5'- GAGCATCTTCAGTATCAACTTCTGTTC-3' 5'- CAGAAGTTGATACTGAAGATGCTCAG-3'
	pmirGLO- $\Delta 4$ <i>fad</i> -3UTR-RE	pmirGLO- $\Delta 4$ <i>fad</i> -3UTR-RE-F pmirGLO- $\Delta 4$ <i>fad</i> -3UTR-RE-R	5'- CTCCTGTGCTGAGCATCTTCACTTTGAACCTT-3' 5'-CTAGAAAGTTCAAAGTGAAGATGCTCAGCACAGGAGAGCT-3'
	pmirGLO-R17	pmirGLO-R17-F pmirGLO-R17-R	5'- CTACCTGCACTGTAAGCACTTTGT-3' 5'- CTAGACAAAGTGCTTACAGTGCAGGTAGAGCT-3'

Restriction sites are underlined for *Eco*R I (pEG-pmiR-17-F) / *Bam*H I (pEG-pmiR-17-R) and *Sac* I (pmirGLO-Δ4fad-3UTR-F) / *Xba* I (pmirGLO-Δ4fad-3UTR-R).

**Figure 1a**

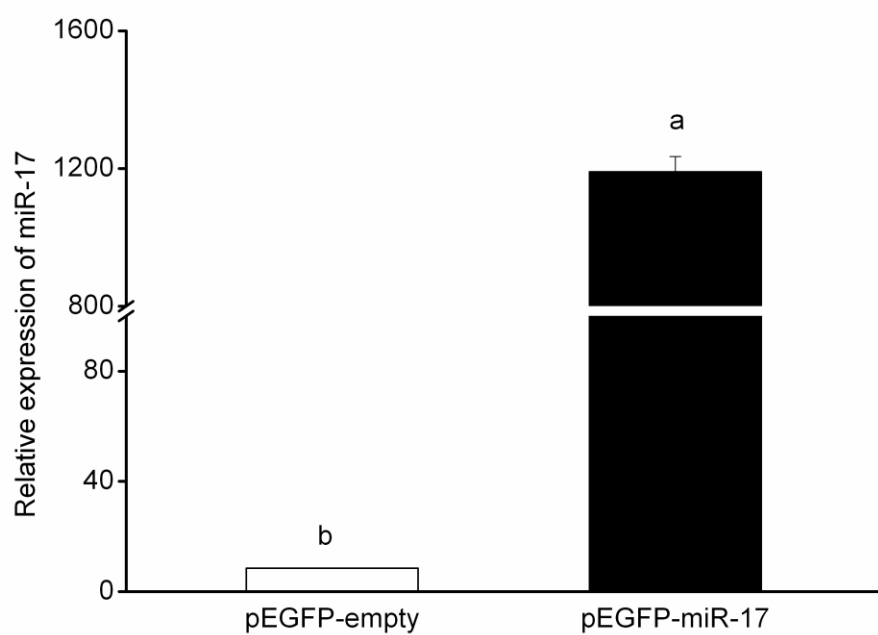
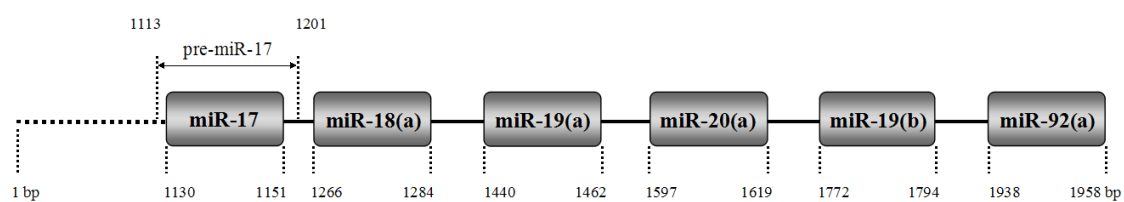
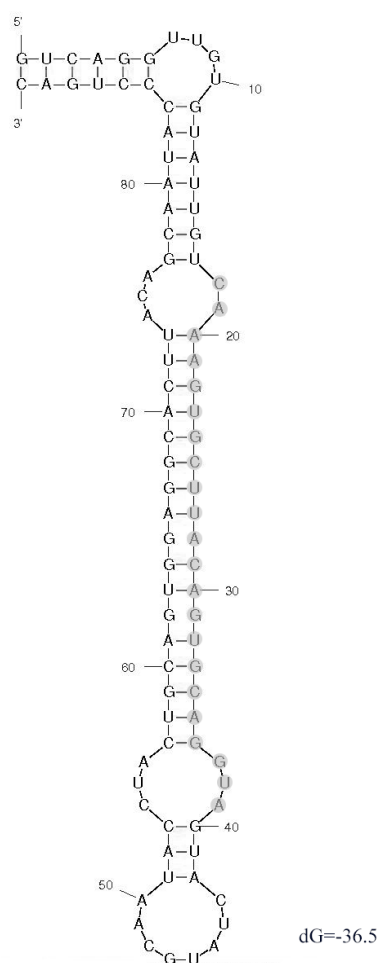


Figure 1b

**Figure 2**

hsa-miR-17GUCA...GAAUAAUGUCAAAAGUGCUUACAGUGCAGGUAGUGAU	42
mmu-miR-17GUCA...GAAUAAUGUCAAAAGUGCUUACAGUGCAGGUAGUGAU	42
gga-miR-17GUCA...GAGUAAUGUCAAAAGUGCUUACAGUGCAGGUAGUGAU	42
xtr-miR-17GUCAA..GAGUAAUGUCAAAAGUGCUUACAGUGCAGGUAGUGAU	43
dre-miR-17a-1	GGACUUUCUUGAGUGGACUUGGUUGGUCA...AUGUAUUGUCAAAAGUGCUUACAGUGCAGGUAGUAU	69
Sca-miR-17GUCAG..GUU...GUGUAUUGUCAAAAGUGCUUACAGUGCAGGUAGUAU	46
.....		
hsa-miR-17	G.U.GCAUCUACUGCAGUGAAGGCACUUGUAGCAUUAU..GGUGAC.....	84
mmu-miR-17	G.U.GCAUCUACUGCAGUGAGGGCACUUGUAGCAUUAU..GCUGAC.....	84
gga-miR-17	A.UAGAACCUCUGCAGUGAAGGCACUUGUAGCAUUAU..GUUGAC.....	85
xtr-miR-17	AACAGAACCUACUGCAGUGAAGGCACUUGUAGCAUUAU..AUUGAC.....	87
dre-miR-17a-1	G.GAAUAUCUACUGCAGUGCAGGCACUUCUAGCAUACACUUGACCAUUUUUAAACCUUCCUCCAGGCAUC	137
Sca-miR-17	G.CAAUACCUCUGCAGUGAAGGCACUUCAGCAUUAU..CCUGAC.....	89

Figure 3

**Figure 4**

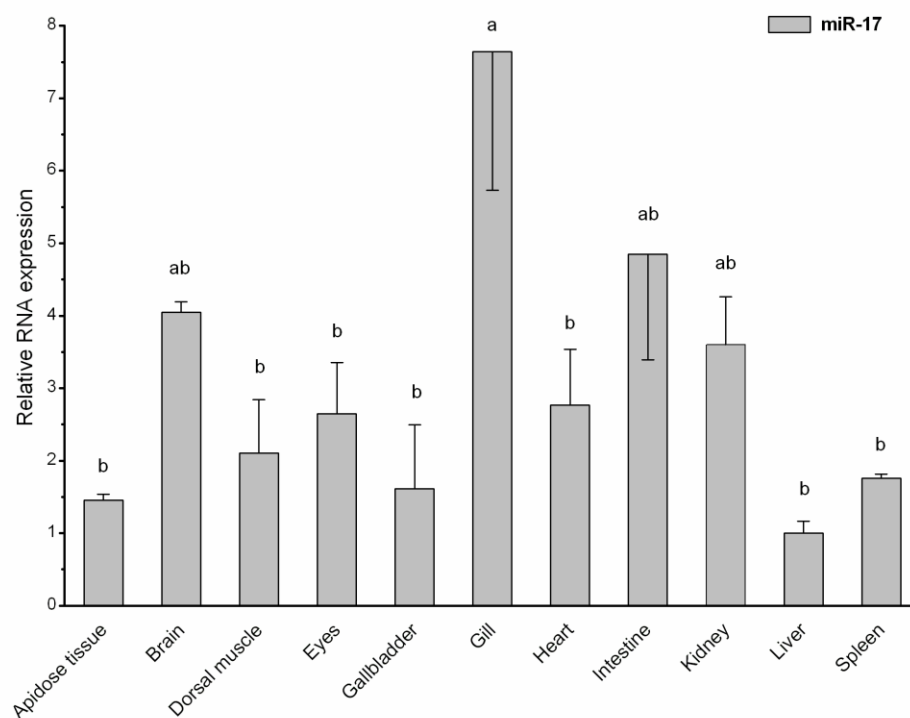
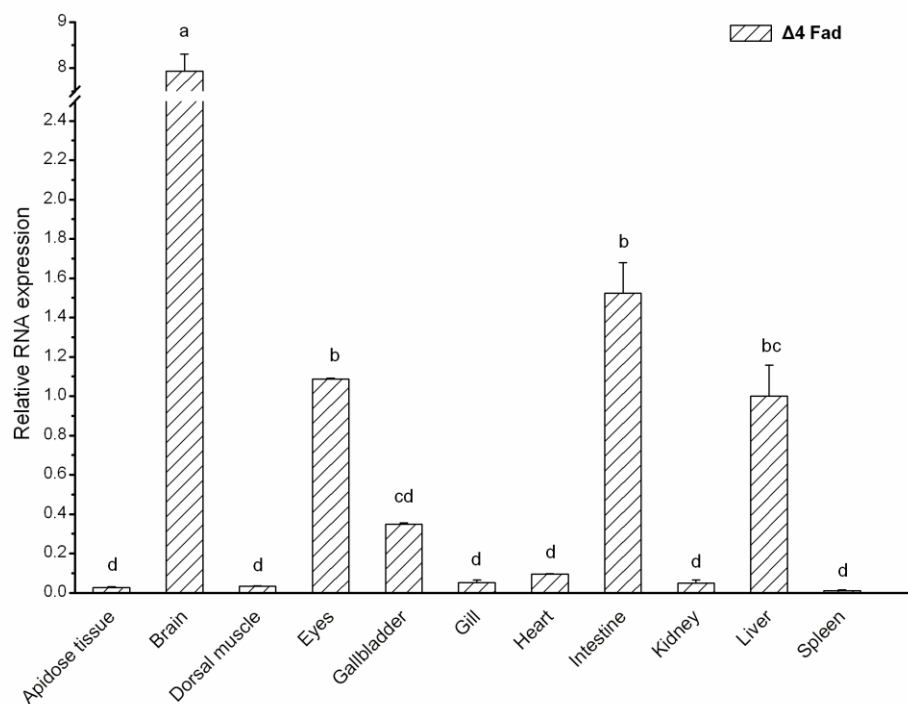


Figure 5a

**Figure 5b**

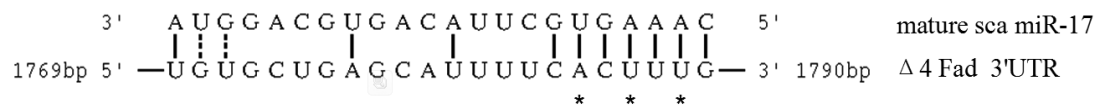


Figure 6a

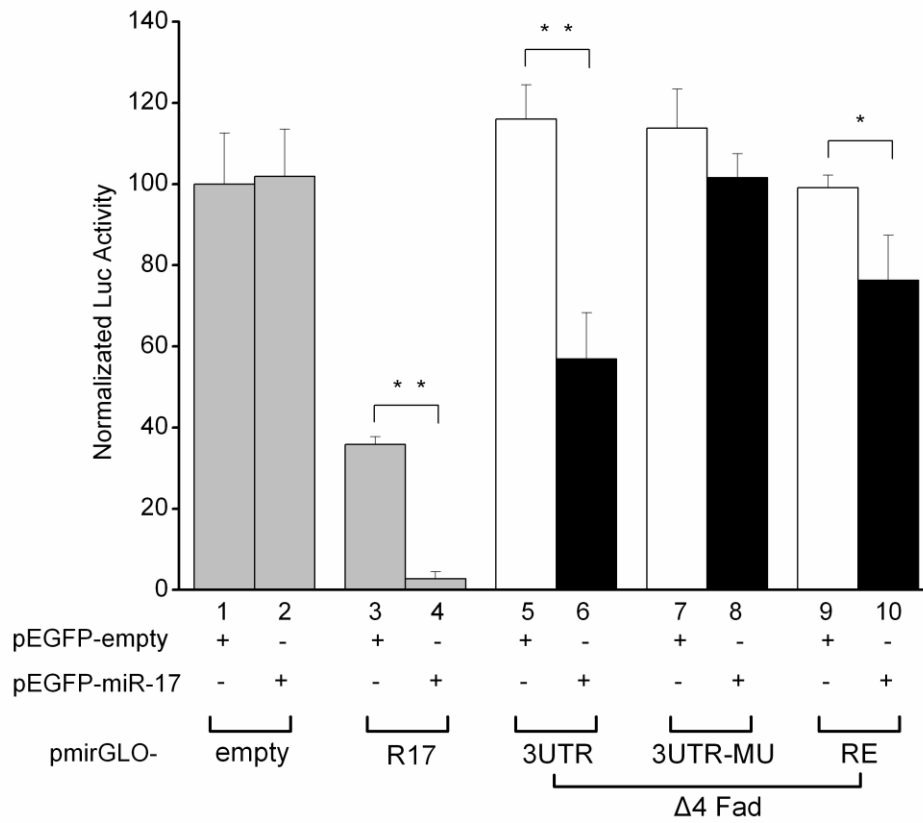


Figure 6b

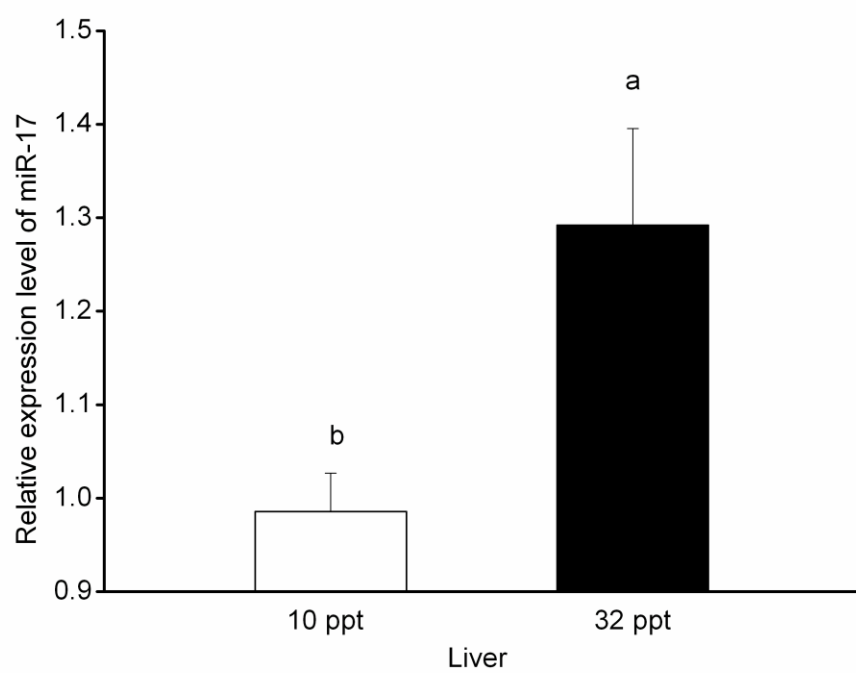


Figure 7a

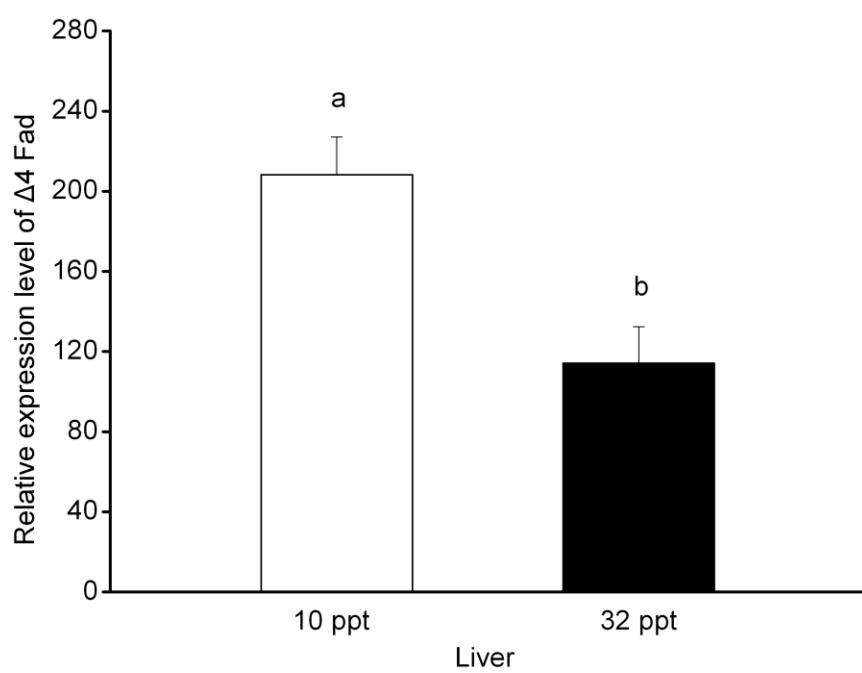
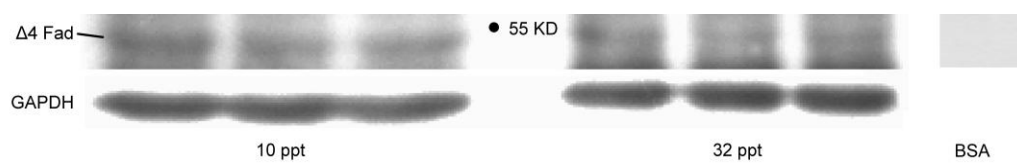


Figure 7b

**Figure 7c**

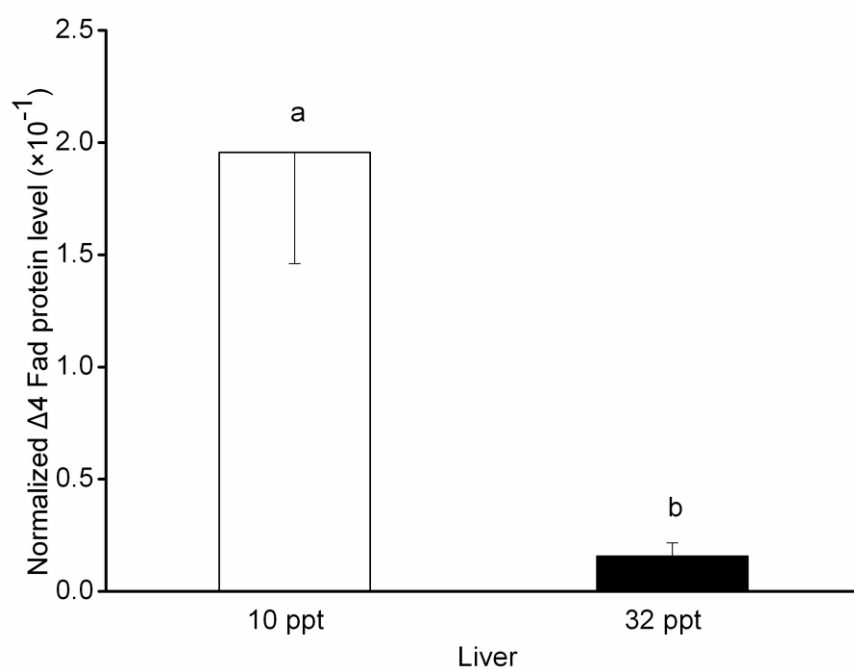


Figure 7d

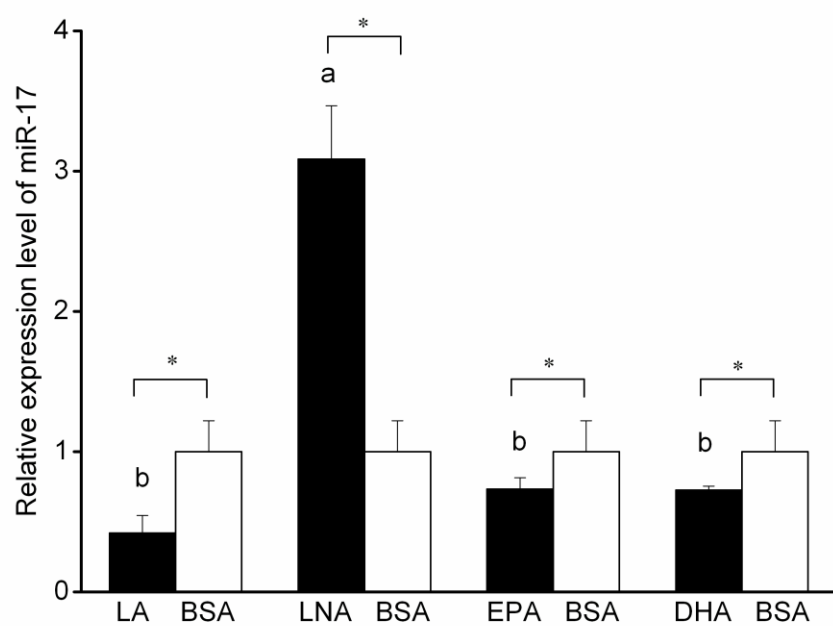


Figure 8a

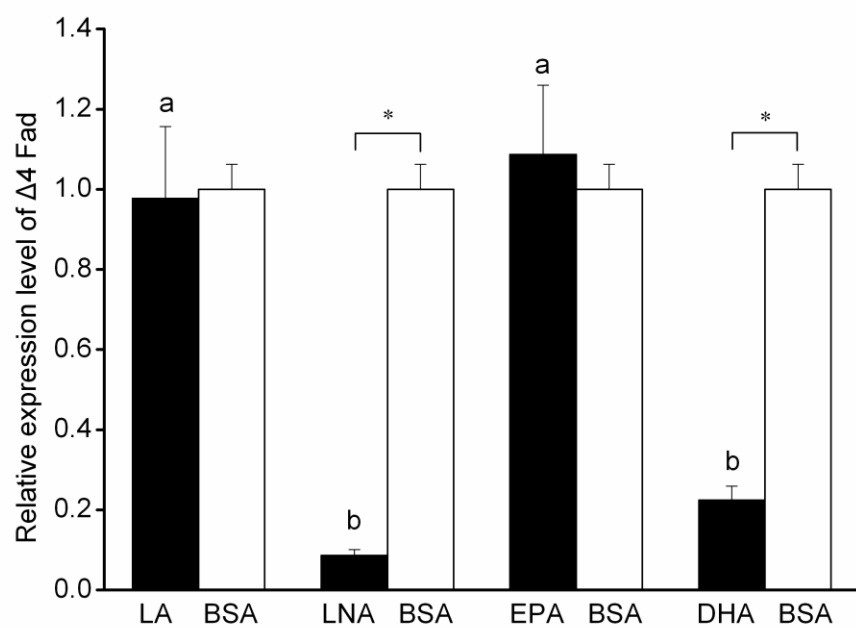


Figure 8b

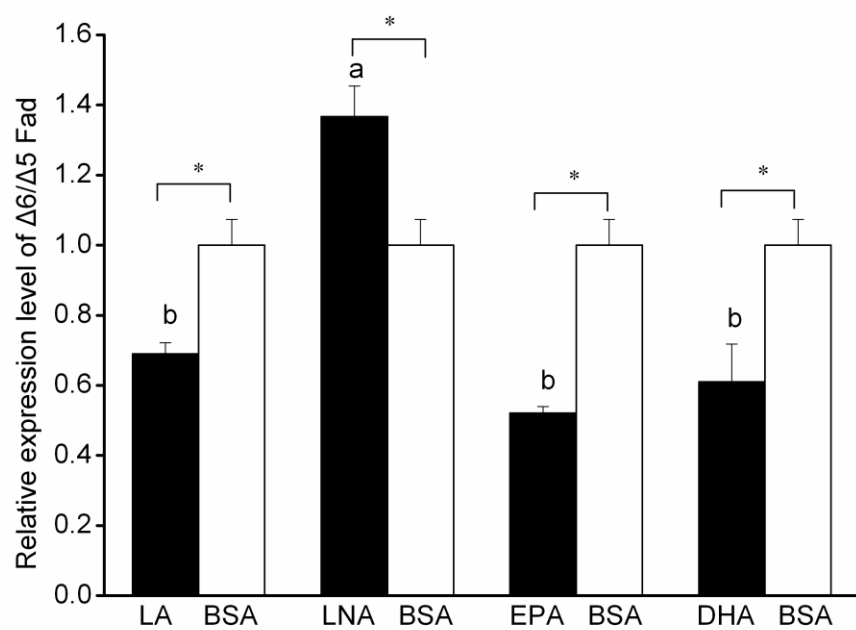


Figure 8c

Highlights

- miR-17 and pre-miR-17 of *Siganus canaliculatus* (rabbitfish) was cloned, locating in the miR-17-92 gene cluster.
- miR-17 is involved in the regulation of LC-PUFA biosynthesis in rabbitfish by targeting $\Delta 4$ Fad, which is for the first time to demonstrate the action of miRNA in LC-PUFA biosynthesis of vertebrates, and also the first study on the regulatory mechanism of LC-PUFA biosynthesis at post-transcriptional level in fish.
- miR-17 may act as a switch to orchestrate the “ $\Delta 4$ Fad” pathway in response to PUFA supplement in rabbitfish liver.
- There is potentially coordinated mechanism of LC-PUFA synthesis between “ $\Delta 4$ Fad” pathway and “Sprecher” pathway in rabbitfish liver.