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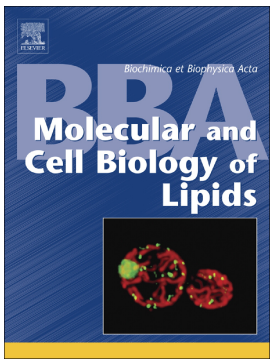

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miR-24 is involved in vertebrate LC-PUFA biosynthesis as demonstrated in marine teleost *Siganus canaliculatus*

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ABSTRACT

Recently, microRNAs (miRNAs) have emerged as crucial regulators of lipid metabolism. However, the miRNA-mediated regulatory mechanism on long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA) biosynthesis in vertebrates remains largely unknown. Here, we address a potentially important role of miRNA-24 (miR-24) in the regulation of LC-PUFA biosynthesis in rabbitfish *Siganus canaliculatus*. miR-24 showed significantly higher abundance in liver of rabbitfish reared in brackish water than in seawater for fish fed vegetable oil diets and in *S. canaliculatus* hepatocyte line (SCHL) cells incubated with alpha-linolenic acid (ALA) than the control group. Similar expression patterns were also observed on the expression of sterol regulatory element-binding protein-1 (s*rebp1*) and LC-PUFA biosynthesis related genes. While opposite results were observed on the expression of insulin-induced gene 1 (*insig1*), an endoplasmic reticulum membrane protein blocking Srebp1 proteolytic activation. Luciferase reporter assays revealed rabbitfish *insig1* as a target of miR-24. Knockdown of miR-24 in SCHL cells resulted in increased Insig1 protein, and subsequently reduced mature Srebp1 protein and expression of genes required for LC-PUFA biosynthesis, and these effects could be attenuated after additional *insig1* knockdown. Opposite results were observed with overexpression of miR-24. Moreover, increasing endogenous *insig1* by knockdown of miR-24 inhibited Srebp1 processing and consequently suppressed LC-PUFA biosynthesis in rabbitfish hepatocytes. These results indicate a potentially critical role for miR-24 in regulating LC-PUFA biosynthesis through the Insig1/Srebp1 pathway by targeting *insig1*. This is the first report of miR-24 involved in LC-PUFA biosynthesis and thus may provide knowledge on the regulatory mechanisms of

LC-PUFA biosynthesis in vertebrates.

Keywords: miR-24, Insig1, Srebp1, LC-PUFA biosynthesis, *Siganus canaliculatus*

1. Introduction

Long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA), such as arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), play numerous physiologically important roles essential to human health [1–3]. In vertebrates, LC-PUFA are generally biosynthesized through sequential desaturation and elongation of precursor C_{18} PUFA, namely alpha-linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6). Although humans have some ability to synthesize LC-PUFA, dietary intake of these essential fatty acids is still required to meet our physiological demands due to our low conversion efficiency [4]. As fish, especially marine fish, are the primary source of n-3 LC-PUFA (e.g. DHA and EPA) in the human diet [2, 5], considerable attention has been focused on understanding the LC-PUFA biosynthetic pathway and its regulatory mechanism in them [6, 7].

In recent years, the pathway of LC-PUFA biosynthesis has been extensively studied in fish species, which have been shown to have multiple fatty acyl desaturase (Fad) proteins including Δ6, Δ5 and/or Δ4 activities, and elongases of very long-chain fatty acids (Elovl) including Elovl2, Elovl4 and Elovl5, necessary for LC-PUFA biosynthesis [6, 8, 9]. Due to the relationship between these enzyme activities and the capacity of a species for endogenous LC-PUFA biosynthesis, much effort has been directed towards to elucidate the regulation of *fad* and *elovl* genes [7, 10]. The expression of these genes and consequent LC-PUFA biosynthesis are shown to be regulated by nutritional (e.g. dietary lipid and fatty acids) [7, 11,

12] and environmental factors, e.g. salinity [13–15]. Several studies demonstrated that *fad* and *elovl* genes were generally up-regulated at the transcriptional level, with corresponding higher LC-PUFA biosynthetic ability, when fish were reared in relatively low salinity or fed with vegetable oil (VO)-based diets devoid of LC-PUFA, but rich in C_{18} PUFA [12, 15, 16]. Furthermore, gene promoter studies have implicated sterol regulatory element binding proteins (Srebps) as important transcriptional regulators of Fad and Elovl enzymes in mammals [17, 18] several fish species, including Atlantic salmon (*Salmo salar*) [19, 20], rainbow trout (*Oncorhynchus mykiss*) and large yellow croaker (*Larimichthys crocea*) [21]. The Srebps family consisted of Srebp1 (Srebp1a and Srebp1c) and Srebp2 proteins that play a crucial role in regulating lipid synthesis [22, 23]. Srebp2 preferentially regulates the expression of genes involved in cholesterol synthesis, while Srebp1 mainly targets entire genes for fatty acid synthesis [24, 25], including those required for LC-PUFA biosynthesis [19–21, 25, 26]. It is well established that Srebps are synthesized as inactive protein precursors that require proteolytic cleavage in Golgi to gain transactivation potency, and this activation process can be blocked by insulin-induced gene 1 (Insig1), an endoplasmic reticulum (ER) membrane protein that facilitates the retention of Srebps precursors in ER [23, 28, 29]. The importance and dependence on Insig1 for the regulation of Srebp1 activity and thereby lipogenic capacity has been demonstrated in mammals [28, 31]. Overexpression of *insig1* inhibited Srebp1c activity and thereby reduced expression of lipogenic genes and lipogenesis in mice, while knockout of *insig1* caused the opposite effects [32, 33]. Although the role of Insig1/Srebp1 pathway in lipogenesis has been studied extensively in mammals, little is known about the regulatory role of this pathway in LC-PUFA biosynthesis in fish

species.

Recently, microRNAs (miRNAs or miR) have been shown to be key regulators of lipid metabolism [34]. miRNAs are a class of short, endogenous and conserved noncoding small RNAs with about 22 nucleotides [35]. We have demonstrated that miR-17 regulates LC-PUFA biosynthesis by down-regulating the expression of Δ4 Fad [36], while miR-33, cooperating with *srebp1*, may be involved in the regulation of LC-PUFA biosynthesis probably by targeting *insig1* in rabbitfish *Siganus canaliculatus* [37]. These new data highlight the important roles of miRNAs in the regulation of LC-PUFA biosynthesis at a post-transcriptional level in vertebrates. It is worth noting that Rabbitfish *S. canaliculatus* is the first marine teleost demonstrated to have the capacity of bioconverting C_{18} PUFA to LC-PUFA with all the key enzymes required for LC-PUFA biosynthesis being functionally characterized as Δ4 Fad, Δ6Δ5 Fad, Elovl4 and Elovl5 [13, 38, 39]. Thus, rabbitfish serves as a good model to investigate the regulatory mechanisms of LC-PUFA biosynthesis in marine teleosts. Moreover, we have recently identified that sterol regulatory elements (SRE) are presented in the core promoters of rabbitfish Δ4 *fad* [40], Δ6Δ5 *fad* [41] and *elovl5* (data not published), suggesting that Srebp1 may also target rabbitfish Δ4 *fad*, Δ6Δ5 *fad* and *elovl5*. Here, in addition to miR-17 and miR-33, we found miR-24 was another miRNA that highly responsive to ambient salinity and precursor ALA, factors affecting LC-PUFA biosynthesis. While *insig1* expression exhibited an opposite pattern with that of miR-24 *in vivo* in liver of fish reared at different salinities for fish fed VO-based diets and *in vitro* in rabbitfish hepatocytes treated with different concentration of ALA, thus leading us to identify rabbitfish *insig1* might also as a target of miR-24. Since inhibition of Insig1 contributes to the

acceleration of Srebp1 processing and thereafter its downstream genes expression, this study aimed to validate and characterize the potential roles of miR-24 on the regulation of LC-PUFA biosynthesis by targeting *insig1* in rabbitfish *S. canaliculatus*.

2. Materials and methods

2.1 Experimental animals and design, and sample collection

All procedures performed on fish were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and approved by the Institutional Animal Care and Use Committee of Shantou University (Guangdong, China). The feeding trial was carried out at Nan Ao Marine Biology Station (NAMBS) of Shantou University, Southern China. 500 rabbitfish juveniles (average body weight around 13 g, sex visually indistinguishable) were captured in May 2012 from the coast near NAMBS. After acclimation in an indoor seawater (32 part per thousand, ppt) tank for one month at NAMBS, half of the fish were then acclimated from seawater to brackish water (10 ppt) and the remaining fish were maintained in seawater (32 ppt) for a further one month prior to the initiation of the experiment. Fish with initial average body weight of 18.04 ± 0.17 g were then randomly distributed into 12 tanks (20 fish per tank), assigning in triplicate tanks for each of the semi-purified experimental diets containing two lipid sources (fish oil and vegetable oil, FO and VO) at two salinities (10 and 32 ppt). The ingredients and proximate composition of the experimental diets were provided previously [14], in which a detailed description of the feeding trial was also included. At the end of the 8-week feeding trial, fish were fasted for 24 h and subsequently anesthetized with 0.01% 2-phenoxyethanol (Sigma-Aldrich, USA) prior to liver tissues excision (six fish per tank). The extracted liver

tissues were immediately immersed in liquid nitrogen and subsequently stored at –80°C for RNA analysis.

2.2 Cell culture

The *S. canaliculatus* hepatocyte line (SCHL) successfully established in our laboratory [42] was cultured in Dulbecco's modified Eagle's medium/nutrient F12 (DMEM/F12, Gibco, USA), supplemented with 20 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulphonic acid (HEPES, Sigma-Aldrich, USA), 10% fetal bovine serum (FBS, Gibco, USA), 0.2% rainbow trout *Oncorhynchus mykiss* serum (Caisson Labs), penicillin (100 U ml^{-1}, Sigma-Aldrich, USA) and streptomycin (100 U ml^{-1}, Sigma-Aldrich, USA), and maintained at 28℃. HEK 293T cells were grown in DMEM (Gibco, USA) supplemented with 10% FBS and maintained at 37℃ with 5% CO_2.

2.3 Fatty acid treatment

Alpha-linolenic acid (ALA, Cayman, USA)-bovine serum albumin (BSA, fatty acid free, Cayman, USA) complexes at 10 mM concentration (10% BSA) were prepared according to Ou et al. [43] and stored at -20℃. The SCHL cells were seeded into six-well plates at a density of 1.0×10^6 cells per well in DMEM/F12 supplemented with only 5% FBS and 0.1% rainbow trout serum. After 24 h, cells were incubated for 2 h in serum-free DMEM/F12 prior to treatment with 0, 50 and 100 μM ALA-BSA complexes in triplicate wells. Each assay was incubated with equal amounts of BSA (final concentration, 0.1%). After incubation for 48 h, the cells were lysed with TRIZOL reagent (Invitrogen, USA) for total RNA isolation.

2.4 RNA isolation and qPCR

Total RNA was isolated with TRIZOL reagent (Invitrogen, USA) following the

manufacture's protocol. The RNA quality was assessed by formaldehyde agarose gel electrophoresis, and the concentration of RNA was quantified by a spectrophotometer (NanoDrop 2000, Thermo Fisher, Germany). After digestion with DNase I (Takara, Japan) at 37ºC for 30 min, 1 μg of high-quality RNA with a ratio of A260/A280 between 1.8 and 2.0 was reverse-transcribed using miScript II RT Kit (Qiagen, Germany). All quantitative real-time PCR (qPCR) runs were conducted in the LightCycler® 480 thermocycler (Roche, Germany) in a total volume of 20 μl with the LightCycler® 480 SYBR Green I Master (Roche, Germany) and miScript SYBR Green PCR kit (Qiagen, Germany) for determination of mRNA and miRNA expression levels, respectively, following the manufacturer's protocol. All amplification reactions were done in triplicate. A non-template control was also included in each run. The relative expression level of mRNA was normalized with that of *β-actin*, whereas miRNA was normalized with 18S rRNA. The primers were designed by Primer 3 software (http://frodo.wi.mit.edu/) based on published sequences and listed in Supporting Table S1.

2.5 miRNA mimics, inhibitors and transient transfection

miR-24 mimics (dsRNA oligonucleotides), miR-24 inhibitor (single-stranded oligonucleotides chemically modified by methylation) and negative control oligonucleotides were obtained from Ribobio (Guangzhou, China). The sequences were as follows: miRNA mimic negative control, sense, 5'-UUUGUACUACACAAAAGUACUG-3'; antisense, 5'-CAGUACUUUUGUGUAGUACAAA-3'; miR-24 mimic, sense, 5'-UGGCUCAGUUCAGGAACAG-3'; antisense, 5'-CUGUUCCUGAACUGAGCCA-3'; miRNA inhibitor negative control, 5'-CAGUACUUUUGUGUAGUACAAA-3'; miR-24

inhibitor, 5'-CUGUUCCUGAACUGAGCCA-3'. Before transient transfection, SCHL cells were seeded into 6-well plates or 60 mm vessels overnight. Cells were subsequently transfected with 10~100 nM of each oligonucleotide in DMEM/F12 containing 5% FBS and 0.1% rainbow trout serum using Lipofectamine 2000^{TM} (Invitrogen, USA). After transfection for 24 or 48 h, cells were harvested for qPCR and Western blotting analysis.

2.6 Plasmid construction

To construct pre-miRNA plasmid, the pre-miR-24 sequence (NCBI accession: MH114077), obtained by genome walking technology as previously described [36], was amplified using PCR and then cloned into pEGFP-C3 vector (Clontech, CA, USA). To generate the wide-type (WT) 3'UTR-luciferase plasmid of *insig1*, the whole 3'UTR of rabbitfish *insig1* (KU598855.1) gene was amplified and inserted into the pmirGLO luciferase reporter vector (Promega, USA) between the *Sac* I and *Xba* I sites. The mutant-type (MT) of *insig1*-3'UTR reporter vector was constructed using Muta-direct^{TM} site-directed mutagenesis kit (SBS Genetech, Beijing, China). All recombinant plasmids used here were extracted using the high pure plasmid isolation kit (Roche, Germany) and confirmed to be correct by Sanger sequencing. Sequences of primers and oligonucleotides used for cloning are provided in Supporting Table S1.

2.7 Dual luciferase reporter assays

For miR-24 target identification, HEK 293T cells were co-transfected with *insig1*-3'UTR WT or MT luciferase reporter vector, along with miR-24 mimics, inhibitors, and negative controls or pre-miR-24 plasmid. Before transient transfection, HEK 293T cells were seeded in 96-well plates with 1×10^4 cells per well for 24 h. Cells were subsequently

transfected with 100 ng of plasmids or 100 nM oligonucleotides using Lipofectamine 3000™ (Invitrogen, USA), according to the manufacturer's instructions. After 48 h, the cells were collected and assayed for reporter activity with a dual-luciferase reporter assay system (Promega, USA) following the manufacturer's instruction, and the *Firefly* luciferase activity was normalized with the *Renilla* luciferase activity. The results shown were done in five wells for each treatment and three independent experiments were conducted.

2.8 RNA interference

Silencing of *insig1* expression was performed using small interfering RNA (siRNA) duplexes obtained from Ribobio (Guangzhou, China) with the following sequences: si-insig1 sense, 5'-CAAAGCUGAAGAAAUGAUdTdT-3'; si-insig1 antisense, 5'-AUCAUUUCUUCAGCUUUGGdTdT-3'. The insig1-specific siRNA (si-insig1) and the negative control siRNA obtained from Ribobio (Guangzhou, China) were performed with Lipofectamine 2000™ (Invitrogen, USA). The cells were seeded into 60 mm vessels overnight and subsequently transfected with 50 nM of each siRNA. The cells were harvested at 48 h post transfection and subsequently subjected to Western blotting analysis.

2.9 Western blotting

After 48 or 72 h post-transfection, total protein from SCHL cells were extracted with the total protein extraction kit (Sangon Biotech, Shanghai, China). The protein concentration was determined by a BCA assay (Sangon Biotech, Shanghai, China). Aliquots of proteins (20~40 μg) were electrophoresed on a 10% sodium dodecyl sulphate-polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA) with a semidry transfer cell (Bio-Rad Trans Blot SD, USA). After incubating for 2 h at room temperature in

blocking buffer TBST (20 mM Tris–HCl [pH 7.4], 150 mM NaCl, 0.1% Tween-20) containing 5% dried skimmed milk powder, membranes were incubated at 4℃ overnight with primary customized mouse monoclonal antibody against rabbitfish Δ4 Fad (1:3000; predicted molecular weight: ~49 kDa) (Abmart Inc., Shanghai, China), rabbit polyclonal antibody against human Insig1 (1:500; predicted molecular weight: ~30 kDa) (Invitrogen, USA), rabbit polyclonal antibody against human mature Srebp1 (1:500; predicted mature Srebp1 molecular weights: ~68 kDa) (WL02093; Wanleibio, Shenyang, China), and mouse monoclonal antibody against β-actin (1:3000; ~42 kDa) (Beyotime, Haimen, China). Then the membranes were washed three times with TBST buffer and incubated for 1 h at room temperature with the secondary antibodies (HRP Goat anti-Rabbit/Mouse IgG, Abcam, USA). Immunoreactive bands were visualized using the Amersham Imager 600 (GE Healthcare, USA). The intensity of each band was analyzed with Image J software (version 1.8.0, NIH, Bethesda, MD, USA). The optical density of each sample run on each blot was normalized to the expression level of β-actin for statistical analysis. The results shown were done in triplicate for each experiment and three independent experiments were conducted.

2.10 Analysis of hepatocyte fatty acid profiles

In order to reduce the amount of LC-PUFA, especially EPA and DHA, in rabbitfish hepatocytes before transfection with miRNA inhibitor and incubation with ALA, SCHL cells were sub-cultured for two passages of in medium containing 5% FBS and only 0.05% rainbow trout serum. After seeded into 100 mm vessels at a density of 5.0×10^6 cells per vessel or 6-well plates at a density of 1.0×10^6 cells per well overnight in DMEM/F12 supplemented with 5% FBS and 0.02% rainbow trout serum, cells in triplicate were

subsequently transfected with 50 nM miR-24 inhibitor or negative control inhibitor (NC inhibitor) using Lipofectamine 2000^{TM} (Invitrogen, USA) for 24 h before incubation with 50 μM ALA-BSA complexes in DMEM/F12 containing with 5% FBS and 0.02% rainbow trout serum. After 48 h incubation, cells were harvested for qPCR, Western blotting and fatty acid composition analysis. At the same time, cells not transfected with any oligonucleotides were also harvested as the mock cells sample for fatty acid composition analysis.

Total fatty acid composition of cell samples was analyzed by gas chromatography after chloroform/methanol extraction, saponification and methylation with boron trifluoride (Sigma-Aldrich, USA) according to the method described previously [38, 44]. Individual fatty acids were identified with known commercial standards (Sigma-Aldrich, USA) and quantified with CLASS-GS10 GC workstation (Shimadzu, Japan). Fatty acid contents were expressed as percentage of total fatty acids.

2.11 Statistical analysis

Data on relative gene expression were obtained using the $2^{-\Delta\Delta CT}$ method, and comparisons were performed by the independent samples *t* test between pairs of groups or one-way analysis of variance (ANOVA) followed by Tukey's test for multiple groups using SPSS version 19.0 (SPSS Inc, Chicago, IL). All data were presented as mean ± SEM. A *P* value < 0.05 was considered to be statistically significant.

3. Results

3.1 The expression profile of miR-24 and insig1 mRNA both in vivo and in vitro

Our previous studies demonstrated that the expression of *srebp1*, *fad* and *elovl* genes and corresponding LC-PUFA biosynthetic ability were higher in the liver of rabbitfish reared at

10 ppt salinity or fed VO-based diets rich in C_{18} PUFA than those reared at 32 ppt salinity or fed FO-based diets rich in LC-PUFA, respectively [13, 14, 27]. The expression of miR-24 showed similarly higher expression pattern with that of *srebp1 in vivo* in liver of rabbitfish reared at 10 ppt compared to those reared at 32 ppt (Fig. 1A). A slight increase of miR-24 was also found in fish fed a FO-based diet when compared to that fish fed a VO-based diet at 10 ppt, but not at 32 ppt. In contrast, the level of *insig1* mRNA was significantly lower in liver of fish reared at 32 ppt than that at 10 ppt for fish fed a VO-based diet ($P < 0.05$). Fish fed a VO-based diet also showed significantly lower *insig1* expression than that of fish fed the FO-based diet at 10 ppt, but not at 32 ppt ($P < 0.05$) (Fig. 1B). However, no significant difference of *insig1* mRNA levels was observed between these two salinities for fish fed a FO-based diet (Fig. 1B). Moreover, *in vitro*, the expression of both miR-24 and *srebp1* were highly up-regulated in rabbitfish SCHL cells incubated with 100 μM ALA-BSA complex than that of cells treated with 50 μM ALA-BSA complex and the control group (BSA incubated cells) (Fig. 2A, B). SCHL cells incubated with ALA also showed significantly higher expression of Δ4 *fad*, Δ6Δ5 *fad* and *elovl5* ($P < 0.05$) (Fig. 2C-E). Conversely, the *insig1* mRNA levels decreased as the treating ALA concentration increased, and a significant decrease of *insig1* mRNA level was occurred in 100 μM ALA-incubated cells when compared to the control group ($P < 0.05$) (Fig. 2F).

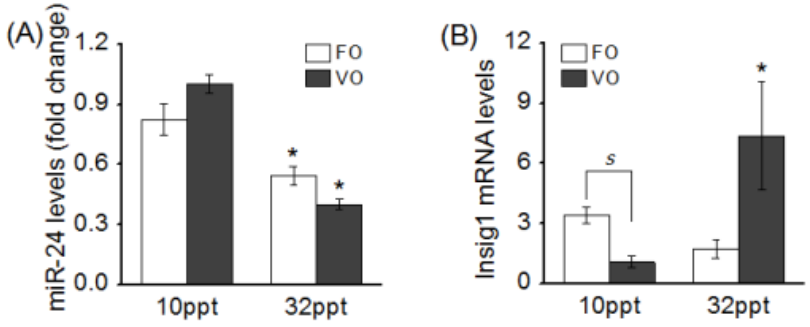

Figure 1. Expression of miR-24 and *insig1* in liver of rabbitfish fed diets with fish oil (FO) or vegetable oil (VO) at two salinities (10 ppt and 32 ppt). The expression of miR-24 (A) and *insig1* (B) were determined by quantitative real-time PCR (qPCR) relative to 18S rRNA or *β-actin* respectively. Values are mean ± SEM as fold change relative to the fish fed diets with VO at 10 ppt water. $^{*}P < 0.05$ *versus* the fish reared in 10 ppt water, *s*, significant ($P < 0.05$).

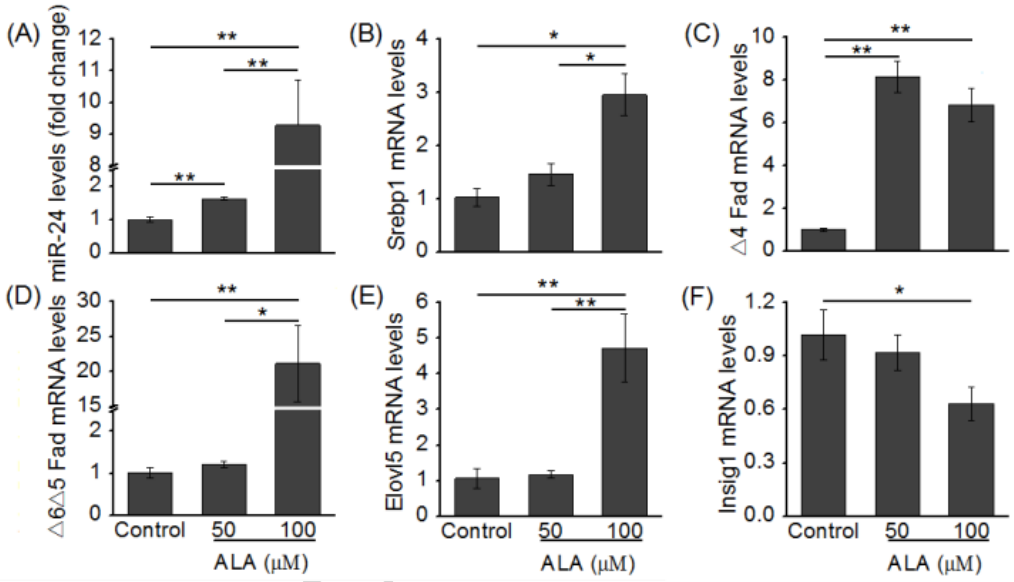

Figure 2. Expression of miR-24, *insig1*, *srebp1*, and LC-PUFA biosynthesis related genes in SCHL cells treated with alpha-linolenic acid (ALA). Rabbitfish *S. canaliculatus* hepatocyte line (SCHL) cells were incubated with ALA-bovine serum albumin (BSA) complex (0 ~ 100 μM) without serum for 48 h. Each assay was treated with equal amounts of BSA (final concentration, 0.1 %). The relative expression of miR-24 (A), *srebp1* (B), Δ4 *fad* (C), Δ6Δ5 *fad* (D), *elovl5* (E) and *insig1* (F) mRNAs was assessed by qPCR as described above. Data were presented as the fold change from control (0.1 % BSA treatment) in mean ± SEM of three independent triplicate experiments. * $P < 0.05$, ** $P < 0.01$.

3.2 Rabbitfish insig1 is a target of miR-24

Further characterizing the interaction between *insig1* and miR-24 involved in LC-PUFA biosynthesis, a putative miR-24 binding site was found in the 3'UTR of rabbitfish *insig1* mRNA using miRNA target prediction programs TargetScan [45] and PicTar [46] (Fig. 3A). To verify the prediction, the 3'UTR region of *insig1* mRNA, as well as the corresponding region in which the seed region had been mutated, was inserted into the pmirGLO luciferase reporter vector (Fig. 3A). The sequence of rabbitfish pre-miR-24 was obtained and cloned into pEGFP-C3 vector to construct the pre-miR-24 plasmid (Fig. 3B). As shown in Fig. 3C, both miR-24 mimic and pre-miR-24 plasmid effectively reduced luciferase activities when co-transfected with wild-type (WT) *insig1* 3'UTR reporter plasmid into HEK 293T cells, but this effect was largely restored for the co-transfected plasmid containing mutated-type (MT) *insig1* 3'UTR region. Additionally, miR-24 mimic and pre-miR-24 plasmid significantly decreased luciferase activity in a dose-dependent manner compared to the negative controls (Fig. 3D-E), and miR-24 inhibitor could antagonize the inhibitory effect of miR-24 mimic on luciferase activity (Fig. 3F). The above results strongly suggest that rabbitfish *insig1* might be a target of miR-24.

3.3 miR-24 inhibits the expression of insig1 at the post-transcriptional level

To further determine miR-24 function in the regulation of Insig1 expression, the intracellular abundance of miR-24 was significantly increased by transfecting miR-24 mimic into SCHL cells in medium containing little ALA and very low LC-PUFA. As shown in Fig. 4A, miR-24 inhibited endogenous Insig1 expression at both the mRNA and protein levels in a dose-dependent manner. In contrast, miR-24 knockdown by transfecting with miR-24

inhibitor into SCHL cells resulted in an increase of mRNA and protein levels of Insig1 (Fig. 4B). These results further indicate that miR-24 might bind the 3'UTR of rabbitfish *insig1* mRNA and down-regulate its expression, most likely operating predominantly at a post-transcriptional level since the effect on protein level was greater than on mRNA.

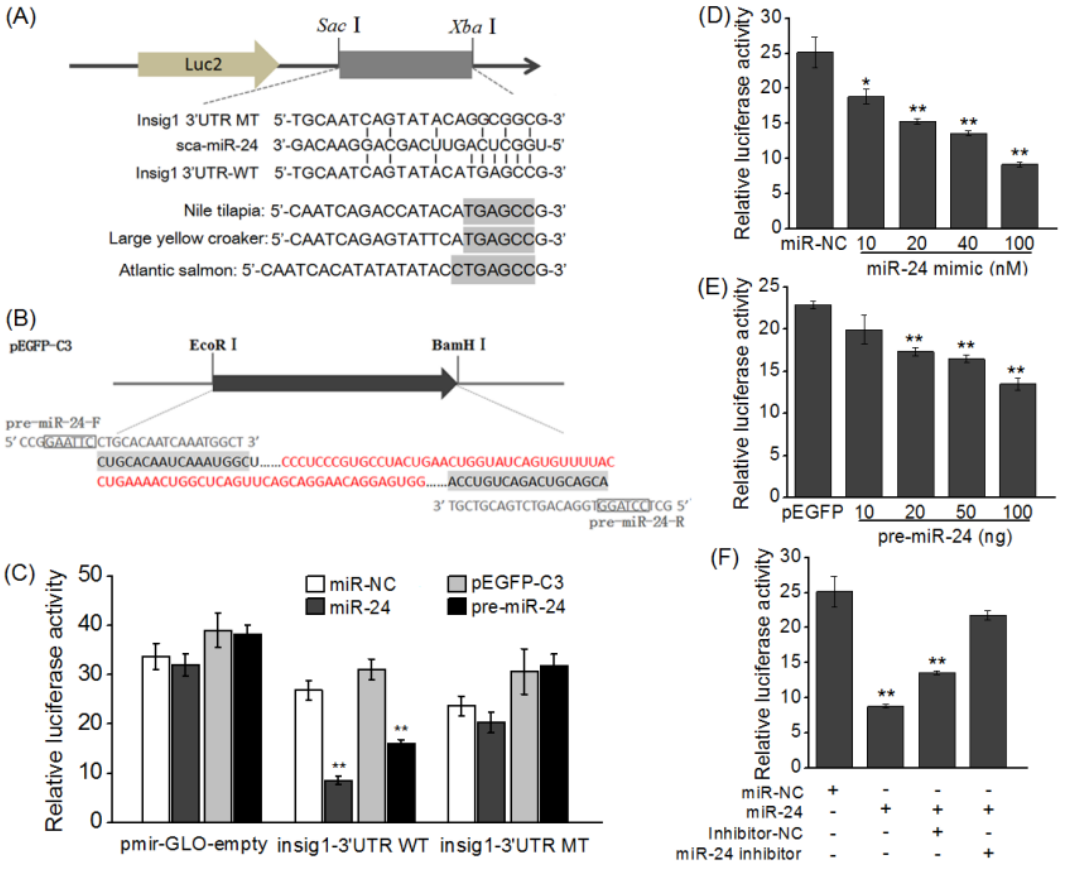

Figure 3. Rabbitfish *insig1* is a target of miR-24. (A, B) Sequence alignment of miR-24 and pre-miR-24, and the construction plasmids. (C) HEK 293T cells were co-transfected with pmirGLO empty plasmid, wild-type *insig1* 3'UTR (WT) and the mutated-type of *insig1* 3'UTR (MT), together with miR-24 mimic or miR-NC and pre-miR-24 plasmid or control plasmid (pEGFP-C3) for 48 h. (D) HEK 293 T cells were co-transfected with *insig1* 3'UTR (WT), together with different concentration of miR-24 (10 ~ 100 nM) or miR-NC for 24 h. Each assay was transfected with equal amounts of oligonucleotides (final concentration, 100

nM). (E) HEK 293 T cells were co-transfected with *insig1* 3'UTR (WT), together with different concentration of pre-miR-24 (10 ~ 100 ng) or pEGFP-C3 for 48 h. Each assay was transfected with equal amounts of plasmids (final concentration, 100 ng). (F) HEK 293 T cells were co-transfected with *insig1* 3'UTR (WT), together with miR-24 or miR-NC and miR-24 inhibitor or inhibitor-NC for 24 h. Each assay was transfected with equal amounts of oligonucleotides (final concentration, 100 nM). The luciferase activity was determined and normalized to *Renilla* luciferase activity. Data are presented as mean ± SEM from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* the controls.

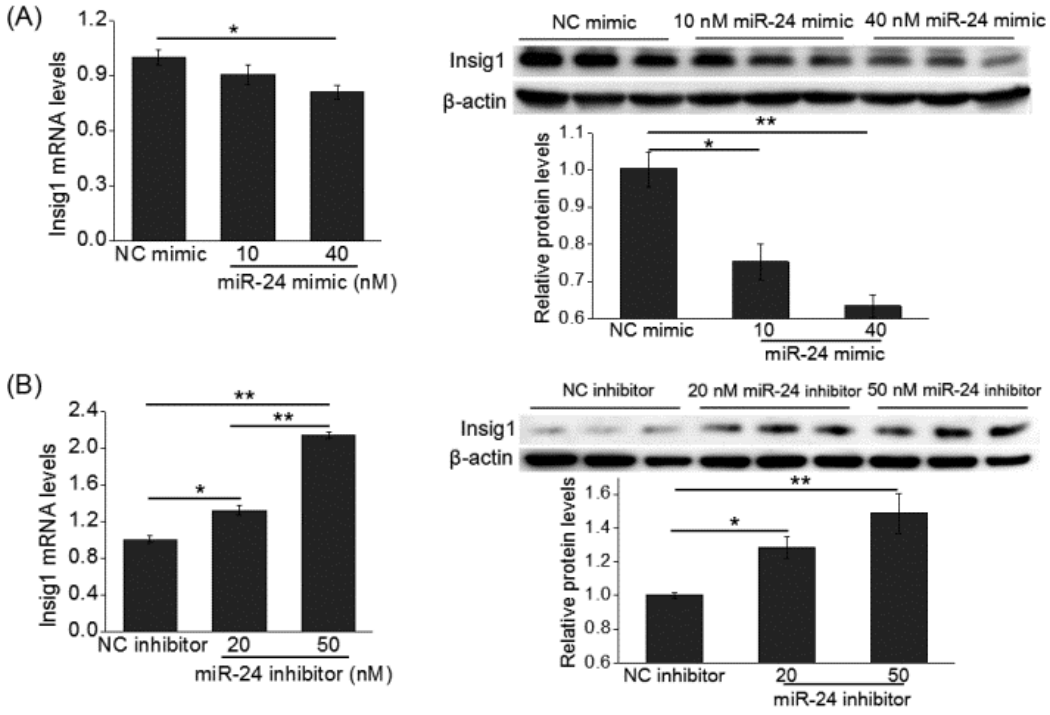

Figure 4. MiR-24 decreases the abundance of insig1 at both mRNA and protein level. (A) Rabbitfish SCHL cells were transfected with miR-24 mimic or NC mimic within the concentration gradient. After 24 h, the expression of *insig1* mRNA (*left*) was determined by qPCR and normalized to *β-actin*. After 48 h, aliquots of proteins from cells were subjected to 10% SDS-PAGE gels and immunoblot analysis of the protein levels of Insig1 (~30 kDa) and

normalized to β-actin (~42 kDa) (*right*) as described in in Materials and Methods. (B) Rabbitfish SCHL cells were transfected with miR-24 inhibitor or its control within the concentration gradient. After 24 h, the expression of *insig1* mRNA (*left*) was determined by qPCR as described above. After 48 h, the Insig1 protein levels were determined by Western blotting and normalized to β-actin (*right*). The Image J software v1.8.0 was used to quantify the intensity of the Western blotting bands. The intensity ratio between Insig1 and β-actin was calculated as an indication of endogenous Insig1 protein expression change. Data are means ± SEM of triplicate treatments as fold change from the controls. $^{*}P < 0.05$, $^{**}P < 0.01$.

3.4 miR-24 down-regulates insig1 expression in rabbitfish SCHL cells resulting in increased mature Srebp1 protein and expression of genes involved in LC-PUFA biosynthesis

Given that miR-24 targets and down-regulates the expression of rabbitfish *insig1*, we examined whether overexpression of miR-24 promotes Srebp1 activation in rabbitfish SCHL cells. As expected, overexpression of miR-24 down-regulated Insig1 and up-regulated protein level of mature Srebp1 in SCHL cells (Fig. 5A), thus suggesting that *insig1* is a potential target in promoting Srebp1 processing by miR-24. To confirm this, miR-24 was inhibited by transfecting miR-24 inhibitor into SCHL cells to induce endogenous *insig1* expression, which was subsequently knocked down using siRNA. We found that miR-24 inhibitor down-regulated the mature Srebp1 protein level, and this was attenuated by subsequent *insig1* knockdown (Fig. 5B). Additionally, qPCR analysis of Srebp1 target genes showed that expression of Δ4 *fad*, Δ6Δ5 *fad* and *elovl5* also increased as *insig1* mRNA decreased by

overexpression of miR-24 in SCHL cells (Fig. 5C). In contrast, the levels of Δ4 *fad*, Δ6Δ5 *fad* and *elovl5* mRNAs were significantly reduced along with that of mature Srebp1 protein in miR-24 knockdown SCHL cells (Fig. 5D). The protein level of Δ4 Fad further revealed that miR-24 modulates LC-PUFA biosynthesis related genes expression through the Insig1/Srebp1 pathway (Fig. 5A, B). The above results indicate that miR-24 facilitates Srebp1 processing, which subsequently induces the expression of genes involved in LC-PUFA biosynthesis by targeting *insig1*.

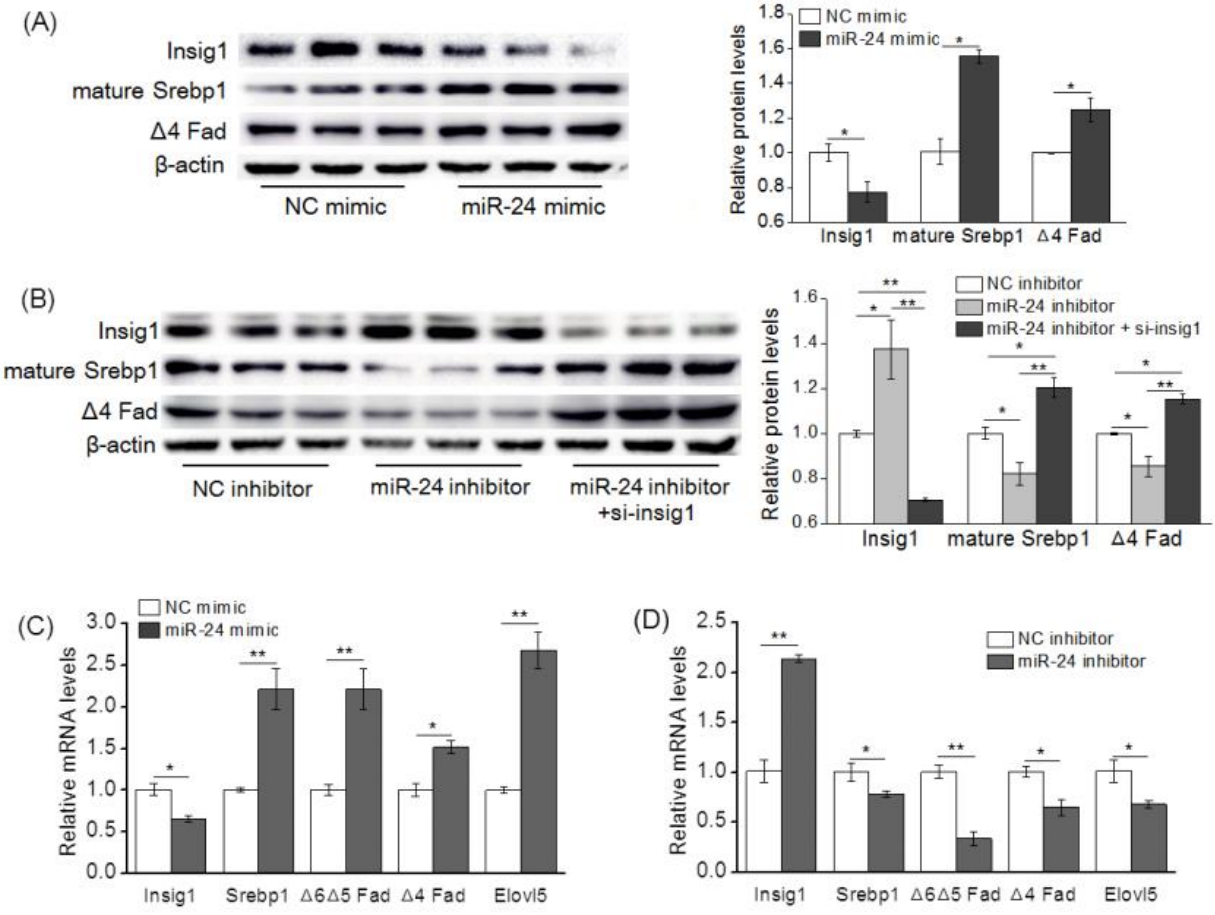

Figure 5. The promotion role of miR-24 on Srebp1 activation and expression of genes responsible for LC-PUFA biosynthesis is mediated by *insig1*. (A) Rabbitfish SCHL cells were transfected with 40 nM miR-24 mimic or NC mimic. After 48 h, aliquots of protein from cells were subjected to 10% SDS-PAGE gels and immunoblot analysis of the protein

levels of Insig1, mature Srebp1 (~68 kDa) and Δ4 Fad (~49 kDa) as above. (B) Rabbitfish SCHL cells were transfected with 50 nM miR-24 inhibitor or NC inhibitor or co-transfected with 50 nM of miR-24 inhibitor and si-insig1. After 48 h, the protein levels of Insig1, mature Srebp1 and Δ4 Fad were determined by Western blotting as above. (C, D) After SCHL cells transfected with 40 nM of miR-24 mimic or NC mimic and 50 nM of miR-24 inhibitor or NC inhibitor for 24 h, the mRNA levels of *insig1*, *srebp1*, Δ4 *fad*, Δ6Δ5 *fad* and *elovl5* were analyzed by qPCR. The Image J software v1.8.0 was used to quantify the intensity of the Western blotting bands. The intensity ratios between Insig1/Srebp1/Δ4 Fad and β-actin were calculated as the indication of endogenous Insig1/Srebp1/Δ4 Fad protein expression changes. Data are means ± SEM of triplicate treatments as fold change from the controls. * $P < 0.05$, $^{**}P < 0.01$.

3.5 Up-regulation of insig1 by knockdown of miR-24 suppresses LC-PUFA biosynthesis in rabbitfish SCHL cells

Next, we assessed whether increasing endogenous *insig1* by knockdown of miR-24 affects LC-PUFA biosynthesis in rabbitfish SCHL cells. To better determine the effects on LC-PUFA profiles of hepatocytes, precursor ALA was supplemented to cells after transfection with miR-24 inhibitor or NC inhibitor. At 48 h post-treatment with ALA, we observed a 20-fold decrease of miR-24 levels in cells receiving miR-24 inhibitor compared to NC inhibitor, along with the increase of insig1 mRNA and protein levels (Fig. 6A, B). Compared to control cells, knockdown of miR-24 in SCHL cells by transfecting miR-24 inhibitor significantly decreased the accumulation of LC-PUFA, including both the products

of the n-3 and n-6 pathway, such as 20:5n-3, 22:5n-3, 22:6n-3, 20:4n-6 and 22:4n-6 (Table 1) ($P < 0.05$). Knockdown of miR-24 also significantly reduced the proportion of MUFA, mainly 18:1n-9 in hepatocytes, and increased SFA, especially 16:0 and 18:0 ($P < 0.05$). These results suggest that increasing the *insig1* expression by knockdown of miR-24 could suppress LC-PUFA and MUFA biosynthesis in rabbitfish SCHL cells.

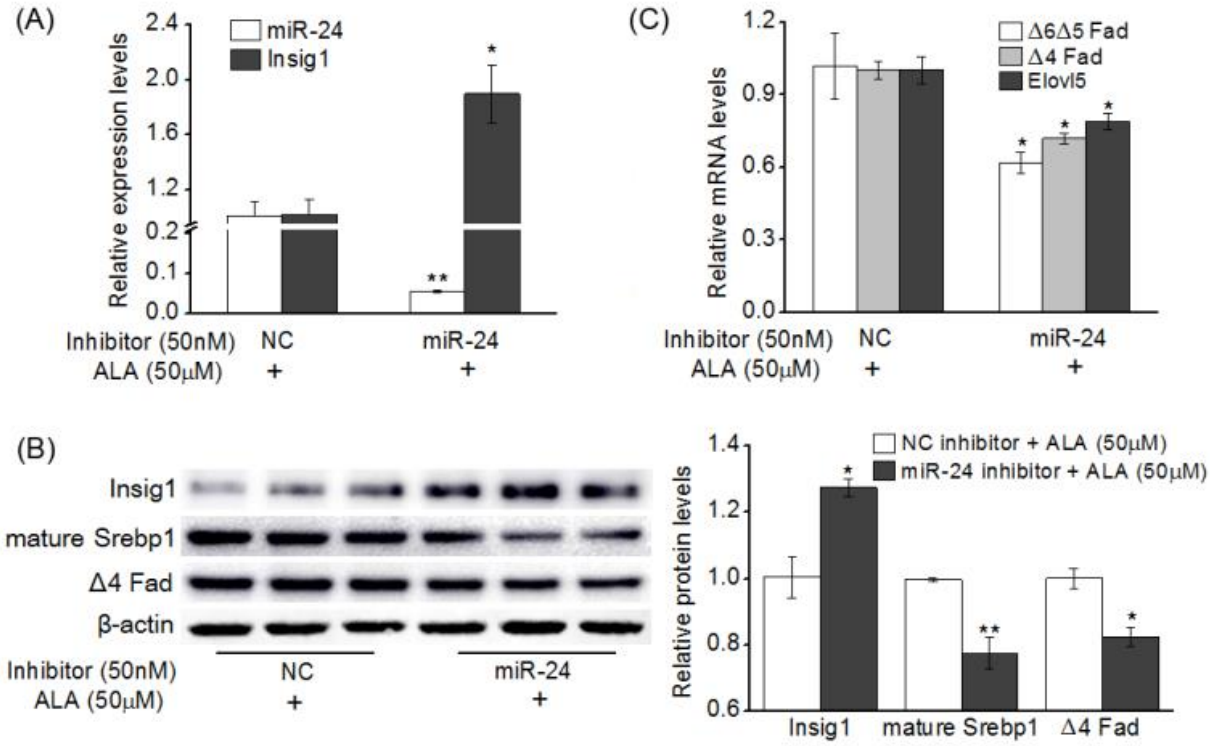

Figure 6. Knockdown of miR-24 suppresses LC-PUFA biosynthesis through inhibiting insig1-dependent Srebp1 activation in rabbitfish hepatocytes. The SCHL cells were transfected with 50 nM of miR-24 inhibitor or NC inhibitor for 24 h, and then treated with 50 μM precursor ALA for another 48 h. (A) The expression of miR-24 and *insig1* mRNA was determined by qPCR as described above. (B) Aliquots of proteins from cells were subjected to 10% SDS-PAGE gels and immunoblot analysis of the protein levels of Insig1, mature Srebp1 and Δ4 Fad as above. (C) The expression of Δ4 *fad*, Δ6Δ5 *fad* and *elovl5* was also analyzed by qPCR as described above. The Image J software v1.8.0 was used to quantify the

intensity of the Western blotting bands. The intensity ratios between Insig1/Srebp1/Δ4 Fad and β-actin were calculated as the indication of endogenous Insig1/Srebp1/Δ4 Fad protein expression changes. Data are means ± SEM of triplicate treatments as fold change from the controls. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* the controls.

3.6 Knockdown of miR-24 inhibits insig1-dependent Srebp1 activation during LC-PUFA biosynthesis in ALA-treated rabbitfish SCHL cells

To further determine whether miR-24 regulating LC-PUFA biosynthesis was mediated through the Insig1/Srebp1 pathway, mature Srebp1 protein level in ALA-treated SCHL cells after receiving miR-24 inhibitor or NC inhibitor were compared. Western blotting showed that miR-24 inhibitor treatment led to increased Insig1 and decreased mature Srebp1 and Δ4 Fad protein in ALA-treated rabbitfish SCHL cells (Fig. 6B). Simultaneously, expression of three target enzyme genes of Srebp1, Δ4 *fad*, Δ6Δ5 *fad* and *elovl5*, was determined in ALA-treated cells after transfection with miR-24 inhibitor or NC inhibitor using qPCR. In the miR-24 inhibitor group, mRNA of the three genes averaged at least 1.45-fold lower than those of control group (Fig. 6C). Together these results indicate that miR-24 involves in LC-PUFA biosynthesis by regulating Insig1-dependent Srebp1 activation and the expression of Srebp1 target enzymes that required for LC-PUFA biosynthesis.

4. Discussion

It is well known that miRNAs have emerged as key post-transcriptional regulators of lipid metabolism in vertebrates. In mammals, several miRNAs such as miR-33a/b, miR-26a/b

and miR-122 are known to be involved in lipid metabolism including cholesterol synthesis and fatty acid metabolism [47–49]. Recently, we have demonstrated that miR-33 and miR-17 are also involved in the regulation of LC-PUFA biosynthesis in marine teleost rabbitfish *S. canaliculatus* [36, 37]. However, the post-transcriptional regulatory mechanism of miRNAs on LC-PUFA biosynthesis remains largely unclear. In the present study, we addressed a potentially important role of miR-24 in LC-PUFA biosynthesis of rabbitfish. We found that miR-24 is another miRNA highly responsive to ambient salinity and especially precursor ALA, factors affecting LC-PUFA biosynthesis. Moreover, the expression of *srebp1* and LC-PUFA biosynthesis related genes Δ4 *fad*, Δ6Δ5 *fad* and *elovl5* were also found to be increased both *in vitro* in rabbitfish SCHL cells incubated with precursor ALA and *in vivo* in liver of rabbitfish reared at 10 ppt salinity and/or fed with VO-based diets rich in C_{18} PUFA than those reared at 32 ppt salinity and/or fed with FO-based diets rich in LC-PUFA [13, 14, 27]. In mammals, miR-24 has been shown to be involved in the regulation of triacylglycerol and cholesterol synthesis by targeting *insig1* [50, 51]. It is well established that Insig1 is an ER membrane protein that blocks Srebp1 activation by facilitating retention of Srebp1 precursor in the ER [29, 32]. Overexpression of *insig1* could inhibit Srebp1c processing and reduce the expression of lipogenic genes and thereby lipogenesis in mice, while knockout of *insig1* caused the opposite effects [32, 33]. Previous studies have demonstrated that Srebp1 is a key transcription factor for the regulation of Fad and Elovl genes responsible for LC-PUFA biosynthesis in mammals [17, 18] and several fish species [19–21]. In the present study, we found that, contrary to the expression of miR-24 and *srebp1*, the expression of *insig1* significantly decreased in liver of rabbitfish fed the VO-based diet both than that fish fed the

FO-based diet at 10 ppt and that fish reared at 32 ppt. An opposite expression pattern of *insig1* with that of miR-24 and *srebp1* was also observed *in vitro* in rabbitfish hepatocytes incubated with ALA, suggesting a potential functional role for the interaction between miR-24 and *insig1*-dependent Srebp1 activation in the regulation of LC-PUFA biosynthesis in rabbitfish. However, no significant salinity effect was detected in *insig1* expression in liver of rabbitfish fed the FO-based diet (Fig.1B). Similar results were also found in LC-PUFA biosynthesis related enzyme genes expression in our previous study [14]. Previous studies indicated that dietary C_{18} PUFA and lower salinity may promote, whereas LC-PUFA and high salinity may suppress the expression of enzyme genes involved in LC-PUFA biosynthesis and thereafter LC-PUFA biosynthetic ability [12, 14-16]. Xie et al. [14] suggested that there might be a combined effect of dietary fatty acids and salinity on the expression of LC-PUFA biosynthesis related enzyme genes in fish. Similar combined effects may be occurred in the expression of *insig1*. That may be one of the reasons why *insig1* not always showed inverse expression pattern with that of miR-24 in the present study. Further studies are needed to clarify the salinity and nutritional regulation mechanism of *insig1* and miR-24 in fish.

As expected, we functionally validated rabbitfish *insig1* as a bona fide target of miR-24. Both insig1 mRNA and protein were reduced by ectopic miR-24 expression in rabbitfish SCHL cells. miR-24 is likely operating predominantly at a post-transcriptional level since the effect on protein levels was greater than on mRNA. Moreover, we demonstrated that overexpression of miR-24 led to down-regulated Insig1 protein level, up-regulated mature Srebp1 protein and expression of Δ4 *fad*, Δ6Δ5 *fad* and *elovl5* mRNAs in rabbitfish SCHL

cells. In contrast, increased in endogenous Insig1 by knockdown of miR-24 could inhibit the expression of mature Srebp1 protein and Δ4 *fad*, Δ6Δ5 *fad* and *elovl5* genes, and these effects could be attenuated by additional *insig1* knockdown. The inverse relationship between Insig1 and the expression of mature Srebp1 in rabbitfish SCHL cells further highlights the importance of Insig1 in the activation of Srebp1 and its target genes in rabbitfish. Our previous studies have shown that Srebp1 is involved in the regulation of LC-PUFA biosynthesis probably by activating the expression of Δ6/Δ5 *fad* and Δ4 *fad* genes in rabbitfish [27]. Cooperating with *srebp1*, miR-33 may be involved in the regulation of LC-PUFA biosynthesis also probably by targeting *insig1* in rabbitfish [37]. And very recently, we have identified an active SRE in the promoter region of Δ4 *fad* [40], Δ6/Δ5 *fad* [41] and *elovl5* (data not published), respectively. Taken together, all these findings suggest that miR-24-induced Insig1-dependent Srebp1 activation might be a candidate pathway that involved in the regulation of LC-PUFA biosynthesis in rabbitfish.

Fatty acid profile analysis performed on rabbitfish SCHL cells with knockdown of miR-24 supports above concept, because the amounts of LC-PUFA, especially n-3 LC-PUFA, were significantly reduced in cells transfected with miR-24 inhibitor than that of control group. As a previous study reported that miR-24 could promote hepatic lipid accumulation and hyperlipidemia by targeting *insig1* in mice fed a high-fat diet [50], the present results revealed that knockdown of miR-24 not only reduced MUFA accumulation but also suppressed LC-PUFA biosynthesis through the Insig1/Srebp1 pathway in rabbitfish. We demonstrated that up-regulation of *insig1* by knockdown of miR-24 inhibited Srebp1 processing and subsequently suppressed Δ4 *fad*, Δ6Δ5 *fad* and *elovl5* expression, and thereby

LC-PUFA biosynthesis in hepatocytes. Additionally, stearoyl-CoA desaturase-1 (Scd1), as a key enzyme involved in the synthesis of MUFA [10], is another preferentially regulated target of Srebp1 [30]. In the present study, significantly lower MUFA accumulation was also observed in hepatocytes treated with miR-24 inhibitor may be due to the expression of Scd1 being reduced by the inhibition of Srebp1 activation as suggested in earlier studies, where increased *insig1* expression inhibited Srebp1 activation and thereby suppressed the expression of Scd1 and MUFA accumulation [30, 32, 50, 52]. Furthermore, it is of interest to find that the accumulation of ALA and LA were also significantly reduced in miR-24 knockdown hepatocytes. Many animal studies showed that most of MUFA, especially 16:1 and 18:1n-9, and C_{18} PUFA, especially LA and ALA, are the preferred substrates for energy production [53-56]. It could be assumed that much more LA and ALA might prefer to be oxidized for energy production rather than accumulated in rabbitfish hepatocytes due to the significant decrease of MUFA contents, especially 18:1n-9. Although the mechanism by which miR-24 regulates LC-PUFA biosynthesis requires further investigation, the present study established an important role of miR-24 in regulating LC-PUFA biosynthesis by targeting *insig1 in vitro*. When developing miR-24 or *insig1* as the potential targets for the treatment of fatty liver disease or hyperlipidemia as suggested previously by Ng et al. [50], it is important to note that the facilitation of miR-24 on LC-PUFA biosynthesis mediated by the Insig1/Srebp1 pathway cannot be ignored because of the key roles of LC-PUFA, especially EPA and DHA, for human health. Additionally, miR-24 also has a conserved binding motif on the 3'UTR of *insig1* mRNA both in Nile tilapia and Atlantic salmon and, since these two fish species possess relatively higher capacity to synthesize LC-PUFA from C_{18} PUFA [7, 56], it

is therefore suggested that miR-24 and its target *insig1* may be used as potential molecular targets for nutritional and/or environmental regulation of LC-PUFA biosynthesis in teleosts.

In summary, we established an important role for miR-24 in the regulation of LC-PUFA biosynthesis in rabbitfish, which provides new insights into the regulatory mechanisms of LC-PUFA biosynthesis in vertebrates. In addition to miR-33, miR-24 is another miRNA highly responsive to ambient salinities and especially precursor ALA that regulates LC-PUFA biosynthesis probably through the Insig1/Srebp1 pathway by targeting *insig1* in rabbitfish.

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Author contributions

C. C., S. W. and Y. L. conceived and designed the experiments; C. C., M. Z., and B. C. performed the experiments and analyzed the results; D. X. carried out the feeding trial; C. C., and Y. L. contributed reagents/materials/analysis tools; C. C. and S. W. wrote the paper; K. W., C. Y., D. R. T. and M. Ó. revised and proofread the paper.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

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FOOTNOTES

The abbreviations used are: LC-PUFA, long-chain polyunsaturated fatty acids; ALA, linolenic acid; LA, alpha-linoleic acid; VO, vegetable oil; FO, fish oil; miRNAs, microRNAs; Fad, fatty acyl desaturase; Elovl, elongase of very long-chain fatty acids; Insig1, insulin-induced gene; Srebp, sterol regulatory element-binding protein; SCAP, Srebps cleavage-activating protein; Scd1, stearoyl-CoA desaturase-1.

Table 1

Fatty acid composition (% total fatty acid) of rabbitfish SCHL treated with 50 μM ALA for another 48 h after transfection with 50 nM NC inhibitor or miR-24 inhibitor for 24h [*].

Fatty acid	Mock cells[#]	NC inhibitor	miR-24 inhibitor	*P*-value
12:0	0.33	0.42 ± 0.19	0.14 ± 0.02	0.227
14:0	0.92	0.80 ± 0.10	0.67 ± 0.04	0.260
16:0	15.30	14.82 ± 0.80	33.04 ± 0.45	0.000
18:0	14.58	12.83 ± 0.23	17.59 ± 0.46	0.001
20:0	0.42	0.67 ± 0.08	0.78 ± 0.04	0.286
22:0	0.16	0.16 ± 0.10	0.32 ± 0.11	0.313
16:1n-7	4.03	3.69 ± 0.29	1.92 ± 0.44	0.004
16:1n-9	2.01	1.78 ± 0.35	0.29 ± 0.07	0.014
18:1n-9	14.25	14.87 ± 0.45	6.42 ± 0.34	0.000
20:1n-9	0.32	0.24 ± 0.13	0.24 ± 0.06	0.995
22:1n-9	0.57	0.52 ± 0.06	0.38 ± 0.05	0.153
18:2n-6	3.44	3.66 ± 0.12	2.20 ± 0.29	0.009
18:3n-6	0.20	0.25 ± 0.14	0.26 ± 0.14	0.938
20:2n-6	0.28	0.27 ± 0.07	0.25 ± 0.08	0.910
20:3n-6	0.76	0.89 ± 0.12	0.77 ± 0.03	0.415
20:4n-6	4.04	4.78 ± 0.36	2.74 ± 0.09	0.005
22:2n-6	1.47	1.20 ± 0.30	0.80 ± 0.04	0.024
22:4n-6	0.26	0.29 ± 0.01	0.16 ± 0.03	0.012
18:3n-3	6.85	7.58 ± 0.35	4.69 ± 0.58	0.013
18:4n-3	0.99	0.68 ± 0.10	0.86 ± 0.06	0.179
20:3n-3	0.57	0.63 ± 0.15	0.51 ± 0.01	0.469
20:4n-3	0.14	0.15 ± 0.08	0.22 ± 0.03	0.454
20:5n-3	1.90	2.03 ± 0.17	0.80 ± 0.01	0.002
22:5n-3	1.03	1.04 ± 0.01	0.45 ± 0.02	0.000
22:6n-3	2.11	1.87 ± 0.04	0.98 ± 0.07	0.000
SFA	31.72	29.69 ± 0.96	52.54 ± 0.70	0.000
MUFA	21.18	21.10 ± 0.76	9.25 ± 0.38	0.000
PUFA	24.03	25.31 ± 0.60	15.68 ± 0.75	0.001
LC-PUFA	10.81	11.68 ± 0.65	6.62 ± 0.20	0.002
n-3 LC-PUFA	5.74	5.72 ± 0.17	2.96 ± 0.08	0.000
n-6 LC-PUFA	5.06	5.96 ± 0.48	3.66 ± 0.13	0.010

[*] Data presented as mean ± SEM (n = 3).

[#] Mock cells: SCHL cells were treated with 50 μM ALA for another 48 h after not transfection with any oligonucleotides for 24 h.

SFA: Saturated fatty acids;

MUFA: Monounsaturated fatty acids;

PUFA: Polyunsaturated fatty acids;

LC-PUFA: Long-chain polyunsaturated fatty acids, included 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, 22:6n-3, 20:3n-6, 20:4n-6, and 22:4n-6 in this table.

Highlights

1. miR-24 is highly responsive to ALA and ambient salinity in rabbitfish

2. miR-24 shows an inverse expression pattern with *insig1* in rabbitfish hepatocytes treated with ALA

3. Rabbitfish *insig1* is a potential target of miR-24

4. up-regulation of *insig1* by knockdown of miR-24 suppresses LC-PUFA biosynthesis

5. miR-24 regulates LC-PUFA biosynthesis through Insig1/Srebp1 pathway by targeting *insig1*