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**miR-26a mediates LC-PUFA biosynthesis by targeting the Lxra-Srebp1 pathway in the marine teleost *Siganus canaliculatus***

Cuiying Chen<sup>1</sup>, Shuqi Wang<sup>1</sup>, Yu Hu<sup>1</sup>, Mei Zhang<sup>1</sup>, Xianda He<sup>1</sup>, Cuihong You<sup>1</sup>, Xiaobo Wen<sup>2</sup>, Óscar Monroig<sup>3</sup>, Douglas R. Tocher<sup>4</sup>, and Yuanyou Li<sup>2\*</sup>

<sup>1</sup> Guangdong Provincial Key Laboratory of Marine Biotechnology & Research Center for Nutrition, Feed and Healthy Breeding of Aquatic Animals of Guangdong Province, Shantou University, Shantou 515063, China. <sup>2</sup> College of Marine Sciences of South China Agricultural University & Guangdong Laboratory for Lingnan Modern Agriculture, Guangzhou 510642, China. <sup>3</sup> Instituto de Acuicultura Torre de la Sal, Consejo Superior de Investigaciones Científicas (IATS-CSIC), 12595 Ribera de Cabanes, Castellón, Spain. <sup>4</sup> Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland, UK.

Running title: *MiR-26a as a key mediator of LC-PUFA biosynthesis*

Present address of the corresponding author: College of Marine Science, South China Agricultural University, 483 Wushan Road, Guangzhou 510642, China

\* Corresponding author, email: yyli16@scau.edu.cn; tel/fax number: +86 20 85283529.

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**ABSTRACT**

MicroRNAs (miRNAs) have been recently shown to be important regulators of lipid metabolism. However, the mechanisms of miRNA-mediated regulation of long-chain polyunsaturated fatty acids (LC-PUFA) biosynthesis in vertebrates remain largely unknown. Herein, we for the first time addressed the role of miR-26a in LC-PUFA biosynthesis in the marine rabbitfish *Siganus canaliculatus*. The results showed that miR-26a was significantly down-regulated in liver of rabbitfish reared in seawater and in *S. canaliculatus* hepatocyte line (SCHL) incubated with the LC-PUFA precursor  $\alpha$ -linolenic acid (ALA), suggesting that miR-26a may be involved in LC-PUFA biosynthesis due to its abundance being regulated by factors affecting LC-PUFA biosynthesis. Opposite patterns were observed in the expression of liver X receptor  $\alpha$  (*lxra*) and sterol regulatory element-binding protein-1 (*srebp1*), as well as the LC-PUFA

biosynthesis related genes ( $\Delta 4$  *fads2*,  $\Delta 6\Delta 5$  *fads2* and *elovl5*) in SCHL cells incubated with ALA. Luciferase reporter assays revealed rabbitfish *lxra* as a target of miR-26a, and overexpression of miR-26a in SCHL cells markedly reduced protein levels of Lxra, Srebp1 and  $\Delta 6\Delta 5$  Fads2 induced by the agonist T0901317. Moreover, increasing endogenous Lxra by knockdown of miR-26a facilitated Srebp1 activation and concomitant increased expression of genes involved in LC-PUFA biosynthesis, and consequently promoted LC-PUFA biosynthesis both *in vitro* and *in vivo*. These results indicate a critical role of miR-26a in regulating LC-PUFA biosynthesis through targeting the Lxra-Srebp1 pathway and provide new insights into the regulatory network controlling LC-PUFA biosynthesis and accumulation in vertebrates.

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Long-chain polyunsaturated fatty acids (LC-PUFA), particularly arachidonic acid (ARA,

20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are major components of complex lipid molecules that are involved in numerous critical biological processes and play physiologically important roles essential to human health (1–3). Since the capacity for fatty acyl desaturation and elongation of the C<sub>18</sub> polyunsaturated fatty acids (PUFA) precursors such as alpha-linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6) to C<sub>20/22</sub> LC-PUFA has previously been reported to be limited in humans, dietary intake of LC-PUFA is required to achieve optimal health (4). It is commonly accepted that fish, especially marine fish, are the main readily available source of n-3 LC-PUFA for human consumption (5, 6) and, with declining wild fisheries, aquaculture supplies an increasing proportion of these essential nutrients in human diets (7). However, the use of large volumes of fish oil (FO), the lipid source traditionally used by the aquafeed industry to produce farmed fish rich LC-PUFA, is increasingly recognized as an environmentally unsustainable and economically unviable practice (8, 9). In this context, significant global attention has focused on finding alternative oils to potentially replace FO in aquafeed formulations. Arguably, vegetable oils (VO) are the most sustainable alternatives to replace FO in aquafeed. However, unlike FO, VO are devoid of C<sub>20/22</sub> LC-PUFA but often rich in monounsaturated fatty acids (MUFA) and C<sub>18</sub> PUFA (10, 11). The extent to which fish can convert C<sub>18</sub> PUFA to C<sub>20/22</sub> LC-PUFA varies with species and is associated with many other factors, including age, sex, gene polymorphisms, among others (8, 12). Therefore, it is essential to understand the regulatory mechanisms of LC-PUFA biosynthesis in order to enable fish to make effective use of dietary VO.

It is well known that C<sub>18</sub> PUFA can be converted to C<sub>20/22</sub> LC-PUFA through a series of carbon chain elongation and desaturation processes in the endoplasmic reticulum (ER), but little is known about how these processes occur and are regulated *in vivo* (13, 14). In recent years, a variety

of fatty acyl desaturases ( $\Delta 6$ ,  $\Delta 5$  and/or  $\Delta 4$  Fads2) and elongases (Elovl2, Elovl4, Elovl5, and Elovl8), critical enzymes in the LC-PUFA biosynthesis pathway, have been cloned and functionally characterized from a range of vertebrates, including freshwater and marine teleosts (15–17). Our previous studies and those of others have shown that many factors are likely to regulate the process of LC-PUFA biosynthesis, among which nutritional (e.g. dietary lipid and fatty acids, especially PUFA) (8, 18, 19) and environmental factors (e.g. salinity) (19–21) have been demonstrated clearly as important ones affecting the capacity of LC-PUFA biosynthesis in fish. Previous studies showed that expression of *fads* and *elovl* genes were generally up-regulated, with corresponding higher activity of the LC-PUFA biosynthesis pathway, when fish were reared in brackish water and/or fed with C<sub>18</sub> PUFA (such as ALA and/or LA) rich diets (i.e. VO-based) compared to fish reared in sea water and/or fed with LC-PUFA rich diets (i.e. FO-based) (18, 19, 22). Moreover, several transcriptional factors, including sterol regulatory element binding protein 1 (Srebp1) (23, 24), nuclear factor Y (NF-Y) (25), hepatic nuclear factor 4 alpha (Hnf4 $\alpha$ ) (26, 27), peroxisome proliferator-activated receptor gamma (Ppar $\gamma$ ) (28) and stimulatory protein 1 (Sp1) (29), have been demonstrated to directly regulate the expression of *fads* and *elovl* genes at a transcriptional level. The liver X receptor (Lxr) is a member of the nuclear hormone receptor superfamily with important roles in the transcriptional control of lipid metabolism (30). There are two Lxr isoforms, Lxr $\alpha$  and Lxr $\beta$ , which can be activated by many endogenous or synthetic ligands, such as T0901317, forming heterodimers with the retinoid X receptor upon ligand binding, and binding to Lxr response elements (LXRE) in the promoters of Lxr target genes (31). Previous studies have shown that Lxr plays a critical role in regulation of LC-PUFA biosynthesis through direct regulation or Srebp1-dependent regulation of *fads* and *elovl* genes (32–34). Recently, we found that microRNAs (abbreviated as miRNAs or miR) also regulate the

expression of *fads* and *elovl* genes in fish (23, 24, 35, 36), suggesting that post-transcriptional regulation by miRNAs may be one of the key regulatory mechanisms of LC-PUFA biosynthesis. However, the mechanisms of the post-transcriptional regulation for LC-PUFA biosynthesis remained largely unclear in teleosts and other vertebrates.

MiRNAs are small non-coding RNAs with approximately 22 nucleotides that regulate gene expression at the post-transcriptional level by binding to specific mRNAs to either inhibit translation or promote mRNA degradation. Multiple studies have established the important roles of certain miRNAs as key regulators of lipid metabolism in mammals (reviewed in 37). Our recent studies in rabbitfish *Siganus canaliculatus* also demonstrated that miR-17 and miR-146a regulate LC-PUFA biosynthesis by negative regulating the liver expression of  $\Delta 4$  *fads2* and *elovl5*, respectively (35-36), while miR-24 and miR-33 can enhance LC-PUFA biosynthesis through activating the Srebp1 pathway by targeting insulin-induced gene protein 1 (Insig1) (23, 24). 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 noteworthy that rabbitfish *S. canaliculatus* was the first marine teleost demonstrated to have the ability to synthesize  $C_{20/22}$  LC-PUFA from  $C_{18}$  PUFA precursors with all the key enzymes required for LC-PUFA biosynthesis (20, 38, 39). Thus, rabbitfish provides a good model to investigate the regulatory mechanisms of LC-PUFA biosynthesis in marine teleosts. Here, in addition to above miRNAs reported in rabbitfish, we found miR-26a was also highly responsive to ambient salinity and precursor ALA, factors affecting LC-PUFA biosynthesis, suggesting miR-26a might be involved in the regulation of LC-PUFA biosynthesis in rabbitfish. In mammals, the miR-26 family (miR-26a/b) has been reported to be involved in adipogenesis and cholesterol metabolism (40–42). However, nothing is currently known about the role of miR-26a in the

regulation of LC-PUFA biosynthesis in any vertebrates. Interestingly, bioinformatic analyses showed that miR-26a potentially targets the 3' untranslated region (3'UTR) of rabbitfish *Lxra* mRNA. Since the activation of *Lxra* can increase expression of *srebp1* and its downstream *fads* and *elovl* genes involved in LC-PUFA biosynthesis (30–32, 43, 44), the present study aimed to validate and characterize the potential roles of miR-26a in the regulation of LC-PUFA biosynthesis by targeting *Lxra* in rabbitfish *S. canaliculatus*.

## Results

### *Expression profiles of miR-26a, lxra and LC-PUFA biosynthesis related genes in vivo and in vitro*

As shown in Fig. 1A, miR-26a showed significantly higher abundance in liver from rabbitfish reared at 32 ppt compared to that of reared at 10 ppt ( $P < 0.05$ ). An increase of miR-26a expression was also found in fish fed FO diets (rich in  $C_{20/22}$  LC-PUFA) when compared to fish fed VO diets (rich in  $C_{18}$  PUFA) (Fig. 1A). Moreover, *in vitro*, the abundance of miR-26a was significantly reduced in rabbitfish SCHL cells incubated with 50~100  $\mu$ M ALA-BSA complex compared to the control group (BSA incubated cells) ( $P < 0.05$ ) (Fig. 1B). These results indicated that miR-26a was responsive to ambient salinity and supply of precursor ALA both *in vivo* and *in vitro*.

Our previous studies reported that both gene expression of *srebp1*, *fads* and *elovl* and enzymatic activity of LC-PUFA biosynthesis were higher in liver of rabbitfish reared at 10 ppt salinity or fed VO diets when compared to fish reared at 32 ppt salinity or fed FO diets, respectively (20-21, 32). Rabbitfish fed a FO diet displayed higher expression of *lxra* in liver than fish fed VO diets, while ambient salinity produced no significant change in the expression of *lxra* (32). However, *in vitro*, the *lxra* mRNA level was significantly increased with increasing ALA concentration increased ( $P < 0.05$ ) (Fig. 1C), which was similar to the expression patterns of *srebp1*,  $\Delta 4$  *fads2*,  $\Delta 6\Delta 5$

*fads2* and *elovl5* previously reported in rabbitfish SCHL cells incubated with ALA (23). In addition, tissue-specific distribution of rabbitfish miR-26a was determined in selected tissues by qPCR. As shown in Fig. 2, miR-26a was highly ( $\Delta Ct < 4$ ) and widely expressed in all examined tissues with higher abundance in brain, heart, intestine, gill and eyes, and lower abundance in spleen, muscle and liver. Taken together, there may be an interaction among miR-26a, *lxra* and *srebpl* probably involved in LC-PUFA biosynthesis.

#### ***Rabbitfish lxra is a target of miR-26a***

To explore the relationships between miR-26a and *lxra*, *srebpl* as well as LC-PUFA biosynthesis related genes, we used bioinformatic tools (TargetScan and PicTar) to predict the potential miRNA targets. Our prediction from *in silico* algorithms showed that there was a conserved complementary site for miR-26a in the 3'UTR of rabbitfish *lxra* mRNA (Fig. 3A). To investigate the interaction between miR-26a and the predicted binding site, the full 3'UTR region of *lxra* 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 rabbitfish pre-miR-26a was obtained by cloning from the introns of the gene encoding for C-terminal domain RNA polymerase polypeptide A small phosphatase 2 (CTDSP2) for secondary structure analysis (Supplemental Fig. S1), and its sequence was cloned into pEGFP-C3 vector to construct the pre-miR-26a plasmid (Fig. 3B). As shown in Fig. 3C, both miR-26a mimic and pre-miR-26a plasmid effectively reduced luciferase activities when co-transfected with wild-type (WT) *lxra* 3'UTR reporter plasmid into HEK 293T cells, but this effect was largely restored for the co-transfected plasmid containing mutated-type (MT) *lxra* 3'UTR region. Consistently, the inhibitory effect of miR-26a mimic on luciferase activity was markedly reversed by miR-26a inhibitor, a synthetic RNA designed to specifically inhibit the function of mature miRNA (Fig. 3D). The above results suggest strongly that rabbitfish *lxra* might be a direct target of miR-26a.

#### ***MiR-26a inhibits the expression of lxra at the post-transcriptional level***

To investigate whether miR-26a was involved in the regulation of Lxra expression, miR-26a was over-expressed and knocked down by transfecting with gradient concentrations of miR-26a mimics and inhibitors into rabbitfish SCHL cells, respectively. As shown in Fig. 4A and B, we found that overexpression and knockdown of miR-26a in SCHL cells produced no significant changes in the level of endogenous *lxra* mRNA. In contrast, endogenous Lxra protein level was markedly inhibited by miR-26a in a dose-dependent manner (Fig. 4A), whereas knockdown of miR-26a in SCHL cells resulted in increased Lxra protein level with increasing miR-26a inhibitor concentration (Fig. 4B). These results indicate that miR-26a might directly bind the 3'UTR of rabbitfish *lxra* mRNA and downregulate its protein expression, likely by inhibiting translation. Furthermore, we examined whether miR-26a could repress the agonist-stimulated Lxra expression and activation. Rabbitfish SCHL cells were transfected with miR-26a mimics, then treated with T0901317. As expected, both mRNA and protein levels of Lxra were successfully upregulated by T0901317 (Fig. 4C). Moreover, the agonist-induced Lxra activation was significantly inhibited by miR-26a mimics (Fig. 4C). Overall, the above results identified *lxra* as a novel target gene of miR-26a in rabbitfish.

#### ***Downregulation of lxra mediated by miR-26a induced repression of Srebpl activation and expression of LC-PUFA biosynthesis related genes***

Our previous study determined that activation of Lxra by the agonist T0901317 in rabbitfish primary hepatocytes could stimulate the expression of *srebpl* and some critical genes involved in LC-PUFA biosynthesis (32). As expected, Lxra expression stimulated by T0901317 in rabbitfish hepatocyte line, SCHL, resulted in significant upregulation of *srebpl*,  $\Delta 4$  *fads2*,  $\Delta 6\Delta 5$  *fads2* and *elovl5* ( $P < 0.05$ ) (Fig. 5A). Moreover, the mature Srebpl and  $\Delta 6\Delta 5$  Fads2 protein levels were also significantly induced after SCHL cells treated with

T0901317, and this effect was markedly inhibited by miR-26a mimics ( $P < 0.05$ ) (Fig. 5B). To further examine whether miR-26a suppressed the key enzyme genes expression through a Srebp1-dependent pathway by targeting Lxra, we inhibited miR-26a by transfecting miR-26a inhibitors into rabbitfish SCHL cells to induce endogenous expression of Lxra, and then knocked down the induced Lxra using siRNA. We found that miR-26a inhibitors markedly increased mature Srebp1 and  $\Delta 6\Delta 5$  Fads2 protein levels and this was attenuated by subsequent Lxra knockdown (Fig. 5C), which established Lxra as a potential key target of miR-26a in suppressing Srebp1 activation and expression of its downstream LC-PUFA biosynthesis related genes. These observations led us to conclude that the crosstalk between miR-26a and Lxra-Srebp1 pathway plays a key role in the regulation of LC-PUFA biosynthesis in rabbitfish.

#### ***Suppression of miR-26a expression promotes LC-PUFA biosynthesis both in vitro and in vivo***

Next, we assessed whether inducing endogenous Lxra by knockdown of miR-26a affects LC-PUFA biosynthesis in rabbitfish SCHL cells *in vitro* and rabbitfish *in vivo*. To better examine the effects on LC-PUFA profiles in SCHL cells, precursor ALA was supplemented to cells after transfection with miR-26a inhibitor or negative control (NC) inhibitor. At 48 h post-treatment with ALA, we observed a 55 % reduction of miR-26a expression in cells that received miR-26a inhibitor compared to NC inhibitor, along with a 3-fold increase of Lxra protein level (Fig. 6A, B). However, the *lxra* mRNA level was marginally decreased. Compared to control cells, knockdown of miR-26a in SCHL cells by transfection with miR-26a inhibitors significantly increased the accumulation of LC-PUFA, including products of both the n-3 and n-6 biosynthetic pathways, such as 20:5n-3, 22:6n-3 and 22:4n-6, while the proportion of saturated fatty acids (SFA), including 16:0 and 18:0, were significantly reduced in cells after knockdown of miR-26a ( $P < 0.05$ ) (Table 1).

In addition, rabbitfish were injected

intraperitoneally with either miR-26a antagomir specifically targeting miR-26a or negative control antagomir for 21 d. We observed an 83 % reduction of hepatic miR-26a expression in rabbitfish that receiving miR-26a antagomir compared to the negative control, and a 1.7-fold increase of Lxra protein level, but no statistical difference was observed in *lxra* and *srebp1* mRNA levels (Fig. 7A, B). Treatment with miR-26a antagomir had no effect on rabbitfish body and liver weight. We then examined the LC-PUFA contents in some tissues that preferentially to accumulate LC-PUFA, such as brain and eyes. Knockdown of miR-26a increased accumulation of total LC-PUFA in liver, muscle, brain and eyes and, in particular, significantly increased DHA accumulation in all examined tissues ( $P < 0.05$ ) (Fig. 7D). Conversely, the contents of precursors ALA and LA showed a corresponding decrease in miR-26a knockdown fish tissues, especially brain and eyes, when compared to the negative control group. Taken together, these results suggest that increasing endogenous Lxra expression by knockdown of miR-26a could promote LC-PUFA biosynthesis in rabbitfish.

#### ***Knockdown of miR-26a facilitates Lxra-dependent Srebp1 activation during LC-PUFA biosynthesis both in vitro ALA-treated hepatocytes and in vivo rabbitfish***

To further determine whether miR-26a regulation of LC-PUFA biosynthesis was mediated through the Lxra-Srebp1 pathway, mature Srebp1 protein level in ALA-treated SCHL cells after receiving miR-26a inhibitor was examined. Western blotting showed that miR-26a inhibitor treatment led to increased Lxra and subsequent mature Srebp1 and  $\Delta 6\Delta 5$  Fads2 protein levels in ALA-treated rabbitfish cells (Fig. 6B). Simultaneously, the expression levels of three Srebp1 targeted enzyme genes,  $\Delta 4$  *fads2*,  $\Delta 6\Delta 5$  *fads2* and *elovl5* were also upregulated in ALA-treated cells after transfection with miR-26a inhibitor as determined by qPCR (Fig. 6C). Moreover, *in vivo*, knockdown of miR-26a also significantly increased the expression of mature

Srebp1 and  $\Delta 6\Delta 5$  Fads2 protein and the transcripts of  $\Delta 6\Delta 5$  *fads2* and *elov15* in liver ( $P < 0.05$ ) (Fig. 7B, C). Together these results indicate that miR-26a is involved in the regulation of LC-PUFA biosynthesis by targeting the Lxr $\alpha$ -Srebp1 pathway.

## Discussion

LC-PUFA research is a thriving field that mainly focused on human health for more than 30 years. Although in some organisms endogenous synthesis of LC-PUFA from C<sub>18</sub> PUFA precursors is possible, the conversions and efficiencies are specific to cell types and species (15). In humans, the capacity of LC-PUFA biosynthesis is rather limited and uptake of n-3 LC-PUFA mainly through consuming marine fish and other seafood, is necessary to satisfy the requirements for these essential nutrients (5–7). However, most marine teleosts have no or very limited ability to convert C<sub>18</sub> PUFA precursors into C<sub>20/22</sub> LC-PUFA due to the absence of certain enzymes activities required in one or more steps of the LC-PUFA biosynthetic pathways, and little is known about how these processes occur *in vivo* and how they are regulated (13, 14). With increasing use of VO sources in feeds used in fish farming, it is critical to understand the regulatory mechanisms of LC-PUFA biosynthesis in order to enable fish to make effective use of dietary C<sub>18</sub> PUFA supplied in the diet to produce LC-PUFA that both satisfies the physiological demands of the fish itself and guarantees a healthy food item for humans.

MiRNAs have emerged as key regulators of lipid metabolism in vertebrates (37), and, recently, we have demonstrated that miRNAs are also involved in the regulation of LC-PUFA biosynthesis in the marine teleost rabbitfish *S. canaliculatus* (23, 24, 35, 36). However, the post-transcriptional regulatory mechanisms of miRNAs on LC-PUFA biosynthesis remain largely unclear. In the present study, for the first time, we identified a potentially important role for miR-26a in LC-PUFA biosynthesis of rabbitfish. We found that miR-26a is highly responsive to ambient salinity *in vivo* and,

especially, precursor ALA *in vitro*, suggesting it may be involved in the regulation of LC-PUFA biosynthesis. In mammals, the miR-26 family (miR-26a/b) has been reported to be involved in adipogenesis (40, 42) and they can control Lxr-dependent cholesterol efflux by targeting Lxr target genes that play critical roles in cholesterol metabolism (41). Based on the expression profiles of LC-PUFA biosynthesis related genes *in vivo* and *in vitro* (32), we found miR-26a showed an inverse expression pattern with *srebp1* in liver of rabbitfish fed two different lipid diets, and with both of *lxra* and *srebp1* in rabbitfish SCHL cells treated with ALA (23). Moreover, since tissue expression of miRNA might, to some extent, reflect the function of miRNA (45), the tissue distribution of miR-26a was examined. It was found that miR-26a was ubiquitously expressed among the examined rabbitfish tissues, with relatively low abundance in liver, where the anabolic reaction of LC-PUFA is well known to be highly occurred in vertebrates. In contrast, the expression level of *lxra* was relatively high in liver (32). These data suggest that there might be an interaction between miR-26a and *lxra* that involved in the regulation of LC-PUFA biosynthesis in rabbitfish. Further *in silico* analyses predicted that, among those genes related to LC-PUFA biosynthesis, miR-26a potentially targeted the *lxra* 3'UTR, and *in vitro* luciferase reporter assays confirmed that rabbitfish *lxra* was a novel target gene of miR-26a. In addition, knockdown of miR-26a upregulated the expression of Lxr $\alpha$ , Srebp1 and key enzymes involved in LC-PUFA biosynthesis and, consequently, increased LC-PUFA contents both *in vitro* in rabbitfish hepatocytes and *in vivo* in rabbitfish. These findings indicate that miR-26a is a novel key regulator of LC-PUFA biosynthesis *via* targeting Lxr $\alpha$  in rabbitfish.

Lxr $\alpha$  is a member of the nuclear hormone receptor superfamily that plays a critical role in the transcriptional regulation of lipid metabolism (46). It was found that Lxr $\alpha$  activation promoted LC-PUFA biosynthesis through direct regulation of

Elovl5 and Srebp1-dependent regulation of key enzymes (Elovl5 and Fads) in human macrophages (34). *Elovl5* is the direct Lxr $\alpha$  target gene in human macrophages (34), while indirect regulation of *elovl5* by Lxr $\alpha$  through a Srebp1-dependent pathway has been reported in mouse liver and salmon head kidney cell line (SHK-1) (33, 47). Similarly, in rabbitfish primary hepatocytes and the hepatocyte cell line SCHL we found that activation of Lxr $\alpha$  by agonist T0901317 can stimulate the expression of *srebp1* and Srebp1 target genes, such as  $\Delta 4$  *fads2*,  $\Delta 6\Delta 5$  *fads2* and *elovl5* (32), and these effects were markedly attenuated by miR-26a mimics. However, in the core promoters (-200bp) of the key enzyme genes we did not find any LXREs using the bioinformatics software TRANSFAC<sup>®</sup> and TF binding<sup>®</sup> (26, 27, 48). To further examine whether miR-26a suppressed the expression of the key enzyme genes through a Srebp1-dependent pathway by targeting Lxr $\alpha$ , we used siRNA to knock down the endogenous expression of Lxr $\alpha$  induced by transfecting miR-26a inhibitors into rabbitfish SCHL cells. The results showed that knockdown of miR-26a markedly increased Lxr $\alpha$ , mature Srebp1 and  $\Delta 6\Delta 5$  Fads2 protein levels and this was attenuated by subsequent Lxr $\alpha$  knockdown, which established that miR-26a may suppress the expression of LC-PUFA biosynthesis related genes through a Srebp1-dependent pathway by targeting Lxr $\alpha$ .

Fatty acid profile analysis performed on rabbitfish SCHL cells *in vitro* and rabbitfish tissues *in vivo* after knockdown of miR-26a supported the above hypothesis, since the amounts of LC-PUFA, especially DHA, were markedly increased in both cells and fish knocked down of miR-26a compared to controls, with increased expression levels of mature Srebp1 protein and enzyme genes. It was important to note that, consistent with our previous study (21), more DHA than EPA and ARA was preferentially deposited in rabbitfish tissues, particularly liver, brain and eyes, where the LC-PUFA biosynthetic activity is particularly high in this species (39). The preferential accumulation

of DHA but not EPA or ARA in these tissues may be due to the higher specificity of the fatty acyl transferase for DHA incorporation into these tissues and the relative lower  $\beta$ -oxidation of DHA than that of EPA and ARA (49, 50). Although the mechanism by which miR-26a controls LC-PUFA biosynthesis and accumulation requires further investigation, our study revealed an important role for the interaction between miR-26a and Lxr $\alpha$ -Srebp1 pathway in rabbitfish *in vivo*.

MiRNAs are small non-coding RNAs that regulate gene expression at the post-transcriptional level by binding, in most instances, to the 3' UTR of target mRNAs to either inhibit translation directly or promote mRNA cleavage (37). In the present study, we found that overexpression of miR-26a significantly reduced the protein level of target Lxr $\alpha$ , but no corresponding decrease of *lxra* mRNA level was observed. This suggested that miR-26a might target the 3'UTR of rabbitfish *lxra* mRNA and downregulate its expression more likely by inhibition of translation rather than by mRNA degradation. Previous studies showed that, in some cases, individual inactivation of single sites among the seed region (2-8 mer) disrupts miRNA-mediated regulation (51, 52), thereby demonstrating that the miRNA will specify cleave the target mRNA if the mRNA 3' UTR has sufficient complementarity to it, or it will repress productive translation if the complementarities are partial (53, 54). There was a mismatch in position 8 between miR-26a seed region and *lxra* 3'UTR, and this may further support the above inference about translation inhibition of *lxra* by miR-26a. Although some interactions between LC-PUFA metabolism and Lxr-mediated pathways have been suggested (55, 56), there are few data on the impact of Lxr on LC-PUFA metabolism. Several LC-PUFA such as ARA, EPA and DHA are known to be potent Lxr antagonists and inhibitors of Srebp1 transcription (56, 57), and LC-PUFA can selectively suppress Srebp1 transcription through proteolytic processing and autoloop regulatory circuit (56). The present study also suggested there may be an autoregulatory



loop in the activation of Lxr $\alpha$ -Srebp1 pathway in rabbitfish SCHL cells, and this may be the reason why knockdown of miR-26a did not increase, but rather slightly decreased the mRNA levels of *lxra* and *srebp1* accompanied by increased LC-PUFA production in rabbitfish hepatocytes *in vitro*. Although this was not the case in rabbitfish liver *in vivo* where marginally higher *lxra* and *srebp1* mRNA levels occurred in fish receiving miR-26a antagomir than that of the NC antagomir group, no statistical differences of *lxra* and *srebp1* mRNA levels were found both *in vitro* and *in vivo*. In addition, such a small discrepancy may be due to the amounts of end products of LC-PUFA biosynthesis, such as DHA and ARA, deposited in fish body at the sampling being not sufficient to trigger the endogenous regulatory mechanism as occurs in SCHL cells. Moreover, in contrast to *srebp1*, *lxra* showed a higher expression level in liver of rabbitfish fed a FO diet than that of fish fed a VO diet. FO is not only rich in LC-PUFA but also cholesterol which is the precursor of oxysterols that are the endogenous ligands for Lxr. As such, Lxr $\alpha$  is not the only physiological regulator for Srebp1 expression in rabbitfish physiologically and the complexity of the molecular mechanisms of Lxr $\alpha$  and Srebp1 in the regulation of LC-PUFA biosynthesis of teleosts requires further investigation.

In summary, we identified miR-26a as a key mediator in the regulation of LC-PUFA biosynthesis in rabbitfish by targeting the Lxr $\alpha$ -Srebp1 pathway, which provides new insights into the regulatory mechanisms of LC-PUFA biosynthesis in vertebrates. Targeting this regulatory network might be crucial for regulating the accumulation of LC-PUFA in farmed fish through nutritional strategies.

## Materials and methods

### Ethics statement

Rabbitfish juveniles (10~20g) for the feeding trial and miRNA antagomir injection study were captured from the coast near Nan Ao Marine

Biology Station (NAMBS) of Shantou University. 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).

### Animals and sample collection

Liver samples of rabbitfish juveniles fed two diets with different lipid sources (fish oil and vegetable oil, FO and VO) and reared at two salinities (10 and 32 ppt) were obtained from the feeding trial which described in detail by Chen et al (23). At the end of the feeding trial, fish were fasted for 24 h and anesthetized with 0.01 % 2-phenoxyethanol (Sigma-Aldrich, USA) prior to liver excision (six fish per tank), with liver samples immediately immersed in liquid nitrogen and subsequently stored at  $-80^{\circ}\text{C}$  until further analysis.

### Reagents, cells and antibodies

The *S. canaliculatus* hepatocyte line (SCHL), initially established in 2017 year (58), were cultured in Dulbecco's modified Eagle's medium/nutrient F12 (DMEM/F12; Gibco, USA) 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), streptomycin (100 U ml $^{-1}$ , Sigma-Aldrich, USA) and penicillin (100 U ml $^{-1}$ , Sigma-Aldrich, USA). Cells were maintained in a normal atmosphere incubator at 28  $^{\circ}\text{C}$ . The HEK 293T cells were cultured in DMEM (Gibco, USA) containing 10 % FBS and maintained at 37  $^{\circ}\text{C}$  with 5 % CO $_2$ . The Lxr ligand T0901317 was obtained from Sigma Chemical Company (Sigma, USA). The mouse monoclonal antibody against rabbitfish  $\Delta 6\Delta 5$  Fads2 (~48 kDa) and rabbit polyclonal antibody against rabbitfish Lxr $\alpha$  (~50 kDa) were customized by Abmart (Shanghai, China) and Wanleibio (Shenyang, China), respectively. The rabbit polyclonal antibody against human mature Srebp1 (1:500; predicted mature Srebp1 molecular weights:

~68 kDa, WL02093) and mouse monoclonal antibody against  $\beta$ -actin (1:3000; ~42 kDa; WL01372) were purchased from Wanleibio (Shenyang, China).

#### **Incubation of rabbitfish SCHL cells with ALA**

The ALA (Cayman, USA) / bovine serum albumin (BSA, fatty acid-free; Cayman, USA) complex at 10 mM concentration (10 % BSA) were prepared according to the method described by Ou et al. (56) and stored at -20°C. After rabbitfish SCHL cells were cultured to 90 % confluence in six-well plates with DMEM/F12 containing only 5 % FBS and 0.1 % rainbow trout serum, cells were incubated for 2 h in serum-free DMEM/F12 prior to treatment with 0 (BSA alone), 50 and 100  $\mu$ M ALA in triplicate wells. After incubation for 48 h, the cells were harvested for total RNA isolation. Each assay was incubated with equal amounts of BSA (final concentration, 0.1 %).

#### **miRNA mimics, inhibitors, siRNA, transient transfection and Lxra agonist treatment**

The miR-26a mimics (dsRNA oligonucleotides), miR-26a inhibitor (single-stranded oligonucleotides chemically modified by methylation) and negative control oligonucleotides were commercially synthesized (Ribobio, Guangzhou, China). Their sequences were as follows: negative control miRNA mimic, sense, 5'-UUUGUACUACACAAAAGUACUG-3'; antisense, 5'-CAGUACUUUUGUGUAGUACAAA-3'; miR-26a mimic, sense, 5'-UUCAAGUAAUCCAGGAUAGGCU-3'; antisense, 5'-AGCCUAUCCUGGAUUACUUGAA-3'; negative control miRNA inhibitor, 5'-CAGUACUUUUGUGUAGUACAAA-3'; miR-26a inhibitor, 5'-AGCCUAUCCUGGAUUACUUGAA-3'. Silencing of rabbitfish *lxra* expression was performed using small interfering RNA (siRNA) duplexes (Hippobio, Huzhou, China) with the following sequences: si-*lxra* sense, 5'-GCAGCUGGACUGCAUGAUUTT-3'; si-*lxra*

antisense, 5'-AAUCAAUGCAGUCCAGCUGCAG-3'. After rabbitfish SCHL cells were cultured to 90% confluence in six-well plates or 90 mm vessels overnight, cells were subsequently transfected for 24 or 48 h with 5-40 nM of each oligonucleotide or 50 nM of each siRNA in DMEM/F12 with 5 % FBS and 0.1 % rainbow trout serum using Lipofectamine 2000<sup>TM</sup> (Invitrogen, USA). After transfection with 10 nM miR-26a or negative control mimics for 24 h, cells were treated with Lxra agonist T0901317 (2  $\mu$ M) for a further 24 h. Cells treated with dimethyl sulfoxide (DMSO; Sigma, USA) was the negative control, while T0901317 was the positive control. After incubation, cells were harvested for qPCR and Western blotting analysis.

#### **Plasmid construction and dual luciferase reporter assays**

The pre-miR-26a sequence (NCBI accession: MN443954) was obtained from an Illumina-based transcriptome sequence database of *S. canaliculatus* prepared in our laboratory (data not published). Primers pre-miR-26a-F1/R1 (Table S1) were designed to validate the sequence, and the product was cloned into pEGFP-C3 vector (Clontech, CA, USA) to construct the pre-miRNA expression plasmid. To generate the wide-type (WT) 3'UTR-luciferase plasmid of *lxra*, the entire 3'UTR of rabbitfish *lxra* (JF502074.1) gene was amplified by PCR and inserted into the pmirGLO luciferase reporter vector (Promega, USA) between the *Sac* I and *Xba* I sites. The mutant-type (MT) of *lxra*-3'UTR reporter vector was generated using *Muta-direct*<sup>TM</sup> site-directed mutagenesis kit (SBS Genetech, Beijing, China). The sequences of primers and oligonucleotides used for cloning are provided in Supporting Table S1.

For miR-26a target identification, HEK 293T cells were co-transfected with *lxra*-3'UTR WT or MT luciferase reporter vector, along with miR-26a mimics, inhibitors, and negative controls or pre-miR-26a plasmid. Before transient transfection, HEK 293T cells were cultured to 80% confluence in 96-well plates overnight. Cells were subsequently

transfected with 100 ng of plasmids or 100 nM oligonucleotides using Lipofectamine 2000™ (Invitrogen, USA), according to the manufacturer's instructions. After 48 h, the cells were collected and assayed for reporter activities with a dual-luciferase reporter assay system (Promega, USA) following the manufacturer's instructions, with the *Firefly* luciferase activities normalized with the *Renilla* luciferase activities. The assays were performed in six wells for each treatment per experiment and three independent experiments were conducted.

#### ***In vivo miR-26a antagomir injection experiment***

After acclimation in an indoor seawater (32 ppt) tank for 2 weeks at NAMBS, rabbitfish juveniles (~15g) were then acclimated from seawater to brackish water (10 ppt) for further 2 weeks. The rabbitfish were subsequently divided into two groups, with eight fish per group. One group was treated with miR-26a antagomirs and the other was treated with the negative control antagomirs. The miRNA antagomirs were chemically modified anti-sense oligonucleotides complementary to the mature miRNAs, which can inhibit the function of target miRNAs and are stable *in vivo* for at least 2 weeks (59). The miR-26a antagomir and the negative control antagomir were commercially synthesized from Hippobio (Huzhou, China). Fish were injected intraperitoneally twice weekly for 3 weeks with 100 µl of total antagomirs diluted in PBS to 50 nmol/ml or with the negative control antagomir. During the *in vivo* injection experiment, fish were fed a commercial diet, with the fatty acid composition of the diet presented in Table S2. Twenty-one days after the first antagomir injection, fish were fasted for 24 h and anesthetized with 0.01 % 2-phenoxyethanol (Sigma-Aldrich, USA), and liver, muscle, brain and eyes samples collected and immediately immersed in liquid nitrogen and stored at -80°C for further analysis.

#### ***RNA isolation and qPCR***

Total RNA was isolated with TRIzol reagent (Invitrogen, USA) following the manufacturer's protocol. After DNase I digestion (Takara, Japan) at 37°C for 30 min, 1 µg of high-quality RNA was

reverse-transcribed using miScript II RT Kit (Qiagen, Germany). All real-time quantitative PCR (qPCR) assays were performed in a LightCycler® 480 thermocycler (Roche, Germany) as described previously (23). The relative expression levels of mRNAs were normalized by  $\beta$ -actin, whereas miRNAs were normalized by 18S rRNA. All amplification reactions were carried out in triplicate using the primers designed by Primer 3 software (<http://frodo.wi.mit.edu/>) and listed in Supporting Table S2.

#### ***Western blotting***

Samples of tissues and cultured cells were lysed in RIPA Buffer (ThermoFisher, USA) and centrifuged at 12000g for 10 min at 4 °C. After determination of protein concentration, aliquots of protein (20 – 40 µg) were separated on 10 % sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to 0.45 µm polyvinylidene fluoride membranes (Roche, Germany). The membranes were blocked for 1 h at room temperature with 5% non-fat milk in Tris-buffered saline (TBS) plus 0.05 % Tween-20 (TBST) followed by an overnight incubation with antibodies diluted in blocking buffer at 4 °C. After three 5 min washes with TBST buffer, the membranes were incubated for 1 h at room temperature with the appropriate secondary antibodies (HRP Goat anti-Rabbit/Mouse IgG; Abcam, USA). Immunoreactive bands were visualized using the Odyssey infrared imaging system (LI-COR, USA), and the intensity of each band was analyzed with Image Studio Software (version 5.2, LI-COR, USA). The optical density of each sample run on each blot was normalized to the expression level of  $\beta$ -actin for statistical analysis.

#### ***Fatty acid composition profiles***

After the SCHL cells were seeded into 90 mm vessels or 6-well plates and cultured overnight in DMEM/F12 supplemented with 5 % FBS and 0.1 % rainbow trout serum, cells in triplicate were subsequently transfected with 20 nM miR-26a inhibitor or negative control inhibitor (NC inhibitor) using Lipofectamine 2000™ (Invitrogen, USA) for

24 h before incubation with 30  $\mu$ M ALA-BSA complexes. After 48 h incubation, cells were harvested for qPCR, Western blotting and fatty acid composition analysis.

Fatty acid composition of cultured cells and tissues samples was analyzed by gas chromatography after extraction of total lipid by chloroform/methanol, saponification and methylation with boron trifluoride (Sigma-Aldrich, USA) all according to the methods described in detail previously (38, 60). Individual fatty acids were identified by retention indices in comparison

with known commercial standards (Sigma-Aldrich, USA) and quantified relative to the internal standard (17:0).

#### **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  $\pm$  SEM. A *P* value  $< 0.05$  was regarded as statistically significant.

#### **Data availability**

All data used to support the findings of this study are contained within the manuscript and the original data can be available from the corresponding author upon request.

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#### **Conflict of interest**

The authors declare no conflict of interests and no permission is required for publication.

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## **FOOTNOTES**

The abbreviations used are: LC-PUFA, long-chain polyunsaturated fatty acids; ALA, linolenic acid; LA, linoleic acid; VO, vegetable oil; FO, fish oil; miRNAs, microRNAs; Fads, fatty acyl desaturase; Elovl, very long-chain fatty acyl elongase; Lxr, liver X receptor; Srebp1, sterol regulatory element-binding protein 1; LXRE, Lxr response elements; 3'UTR, 3' untranslated region;

Table 1

Fatty acid composition (% total fatty acid) of rabbitfish *S. canaliculatus* hepatocyte line (SCHL) treated with 30  $\mu$ M ALA for another 48 h after transfection with 20 nM NC inhibitor or miR-26a inhibitor for 24h \*.

Fatty acid	Mock cells <sup>#</sup>	NC inhibitor	miR-26a inhibitor	P-value
16:0	12.71	13.73 $\pm$ 0.12	11.42 $\pm$ 0.40	0.005
18:0	14.57	14.29 $\pm$ 0.52	12.42 $\pm$ 0.20	0.028
16:1n-7	1.19	1.42 $\pm$ 0.18	1.46 $\pm$ 0.06	0.868
16:1n-9	1.34	1.53 $\pm$ 0.10	1.49 $\pm$ 0.06	0.739
18:1n-9	21.04	19.63 $\pm$ 1.28	18.83 $\pm$ 0.24	0.572
20:1n-9	0.47	0.48 $\pm$ 0.03	0.54 $\pm$ 0.03	0.190
18:2n-6 (LA)	2.52	3.46 $\pm$ 0.64	3.57 $\pm$ 0.32	0.883
18:3n-6	nd	nd	0.12 $\pm$ 0.06	0.116
20:2n-6	0.66	1.14 $\pm$ 0.20	1.56 $\pm$ 0.01	0.101
20:3n-6	1.33	1.33 $\pm$ 0.03	1.35 $\pm$ 0.03	0.749
20:4n-6 (ARA)	6.10	6.69 $\pm$ 0.32	7.07 $\pm$ 0.17	0.358
22:4n-6	0.58	0.58 $\pm$ 0.02	0.81 $\pm$ 0.05	0.015
18:3n-3 (ALA)	1.57	1.86 $\pm$ 0.34	1.76 $\pm$ 0.26	0.827
20:3n-3	0.52	0.93 $\pm$ 0.24	0.85 $\pm$ 0.48	0.886
20:4n-3	0.23	0.19 $\pm$ 0.10	0.39 $\pm$ 0.07	0.166
20:5n-3 (EPA)	2.47	2.29 $\pm$ 0.09	2.76 $\pm$ 0.09	0.021
22:5n-3	2.17	2.21 $\pm$ 0.23	2.77 $\pm$ 0.01	0.071
22:6n-3(DHA)	7.34	7.08 $\pm$ 0.04	8.83 $\pm$ 0.19	0.001
SFA	27.28	28.02 $\pm$ 0.63	23.85 $\pm$ 0.60	0.009
MUFA	24.04	23.06 $\pm$ 1.30	22.31 $\pm$ 0.25	0.604
PUFA	25.46	27.74 $\pm$ 0.91	31.84 $\pm$ 0.49	0.016
LC-PUFA	20.71	21.29 $\pm$ 0.45	24.83 $\pm$ 0.53	0.007
n-6 LC-PUFA	7.98	8.61 $\pm$ 0.33	9.23 $\pm$ 0.16	0.166
n-3 LC-PUFA	12.73	12.69 $\pm$ 0.26	15.61 $\pm$ 0.39	0.003

\* Data presented as mean  $\pm$  SEM (n = 3).

<sup>#</sup> Mock cells: SCHL cells were treated with 30  $\mu$ M ALA for another 48 h after not transfection with any oligonucleotides for 24 h.

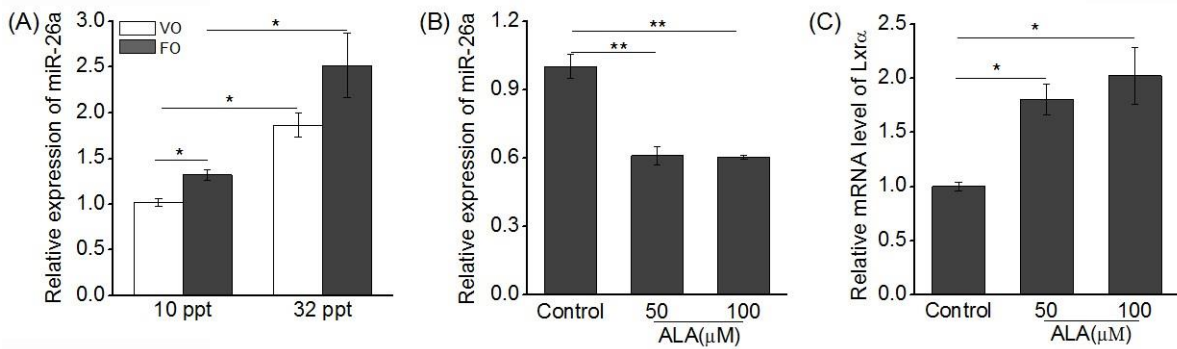
SFA: Saturated fatty acids;

MUFA: Monounsaturated fatty acids;

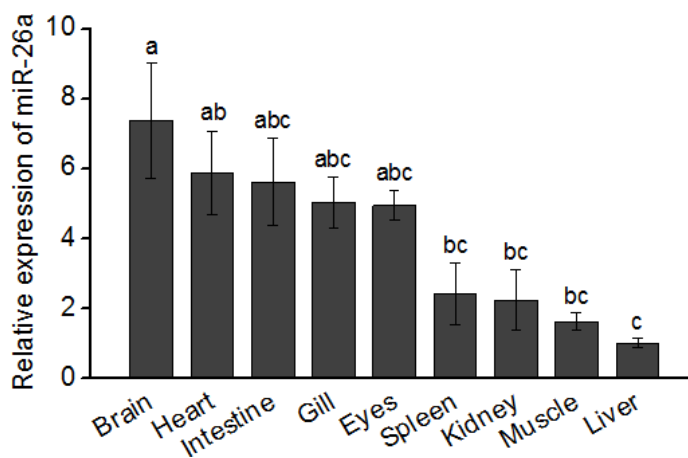
PUFA: Polyunsaturated fatty acids;

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.

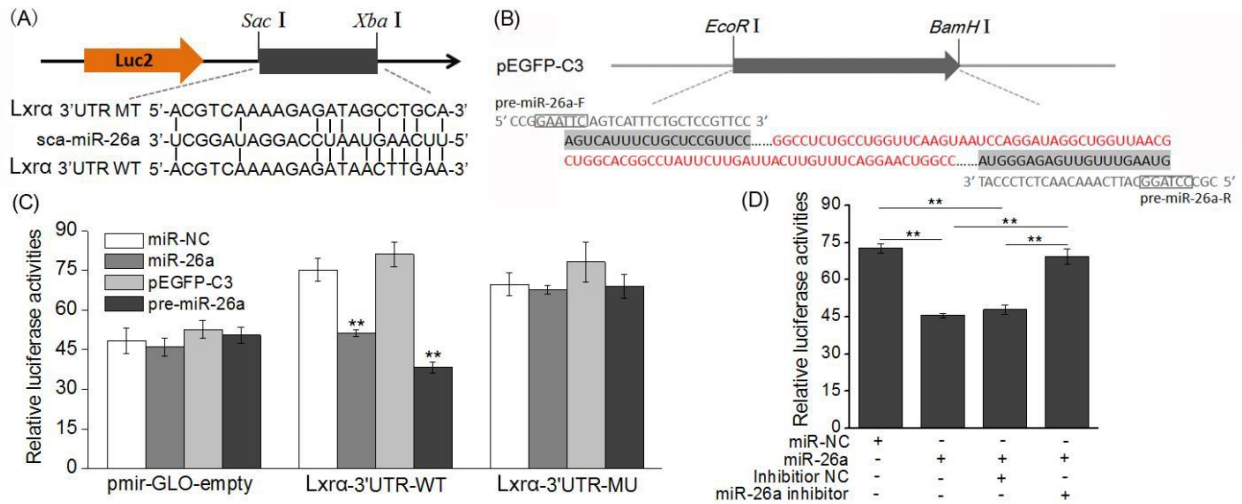
nd: not detected, < 0.01.



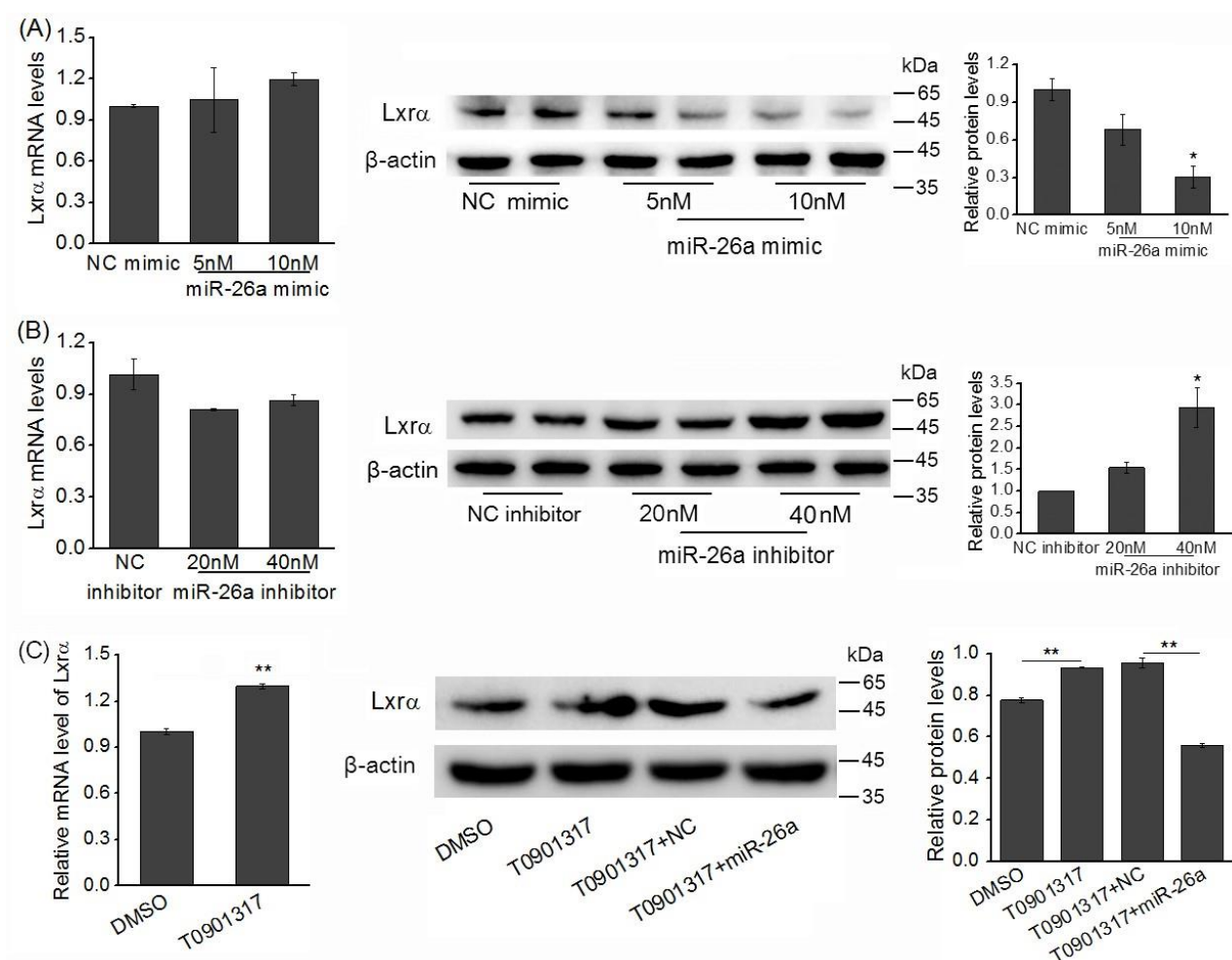
**Figure 1. The expression of miR-26a and *Lxra* both *in vitro* hepatocytes treated with alpha-linolenic acid (ALA) and *in vivo* liver of rabbitfish fed different lipid sources (fish oil and vegetable oil, FO and VO) diets at two salinities (10 ppt and 32 ppt).** The expression of miR-26a (A) was determined by quantitative real-time PCR (qPCR) relative to 18S rRNA. Values are mean  $\pm$  SEM as fold change relative to the fish fed diets with VO at 10 ppt water. Rabbitfish *S. canaliculatus* hepatocyte line (SCHL) cells were incubated with ALA-bovine serum albumin (BSA) complex (0 ~ 100  $\mu$ M) without serum for 48 h. Each assay was treated with equal amounts of BSA (final concentration, 0.1 %). The relative expression of miR-26a (B) and *Lxra* mRNA (C) was assessed by qPCR relative to 18S rRNA or  $\beta$ -actin respectively. Data were presented as the fold change from control (0.1 % BSA treatment) in mean  $\pm$  SEM. \*  $P < 0.05$ , \*\*  $P < 0.01$ .



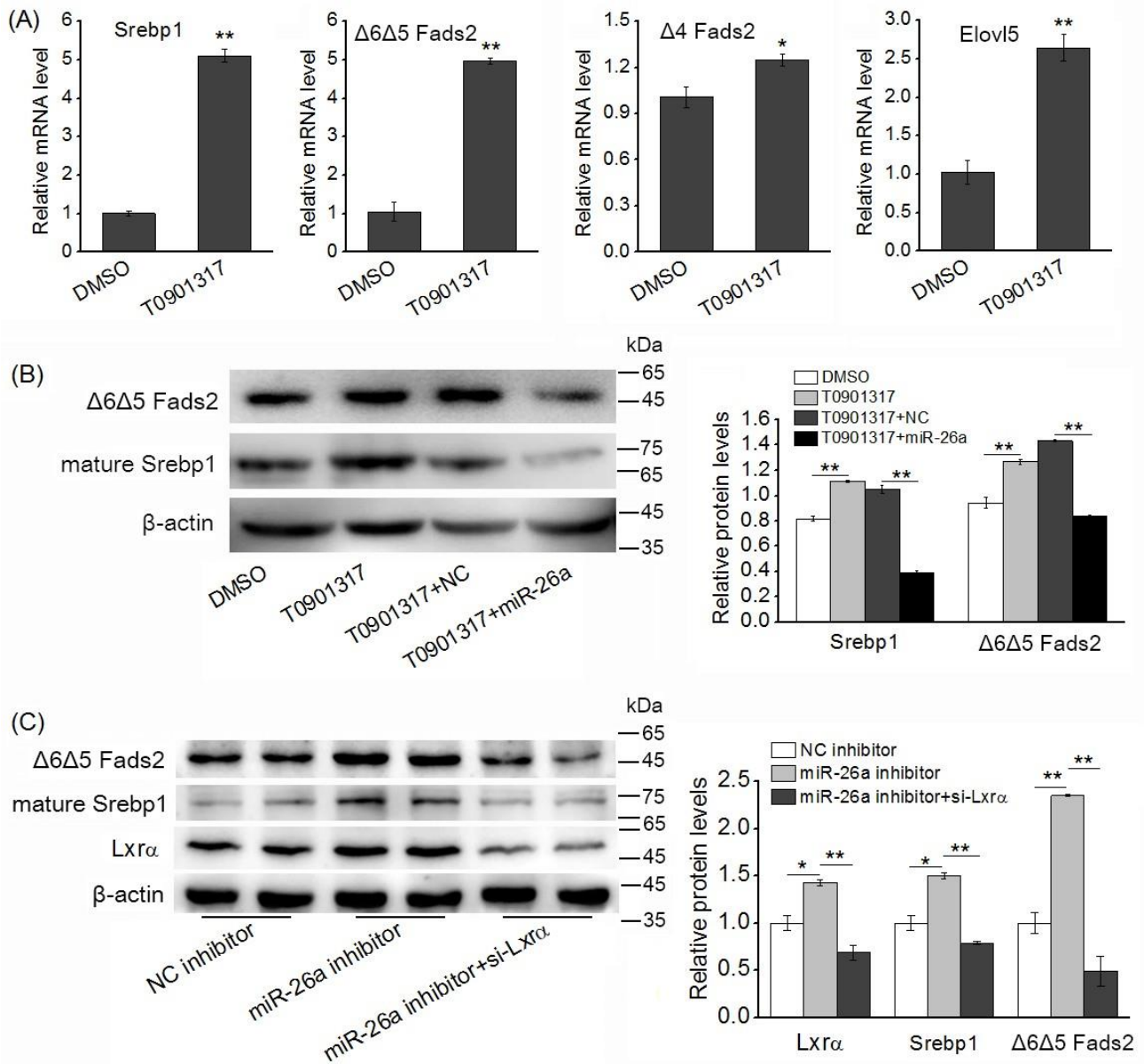
**Figure 2. Relative tissue distribution profile of miR-26a in *S. canaliculatus* by qPCR.** Values are mean  $\pm$  SEM (n=6) as fold change from the liver. Bars not sharing a common superscript letter indicate significant difference among the detected tissues.



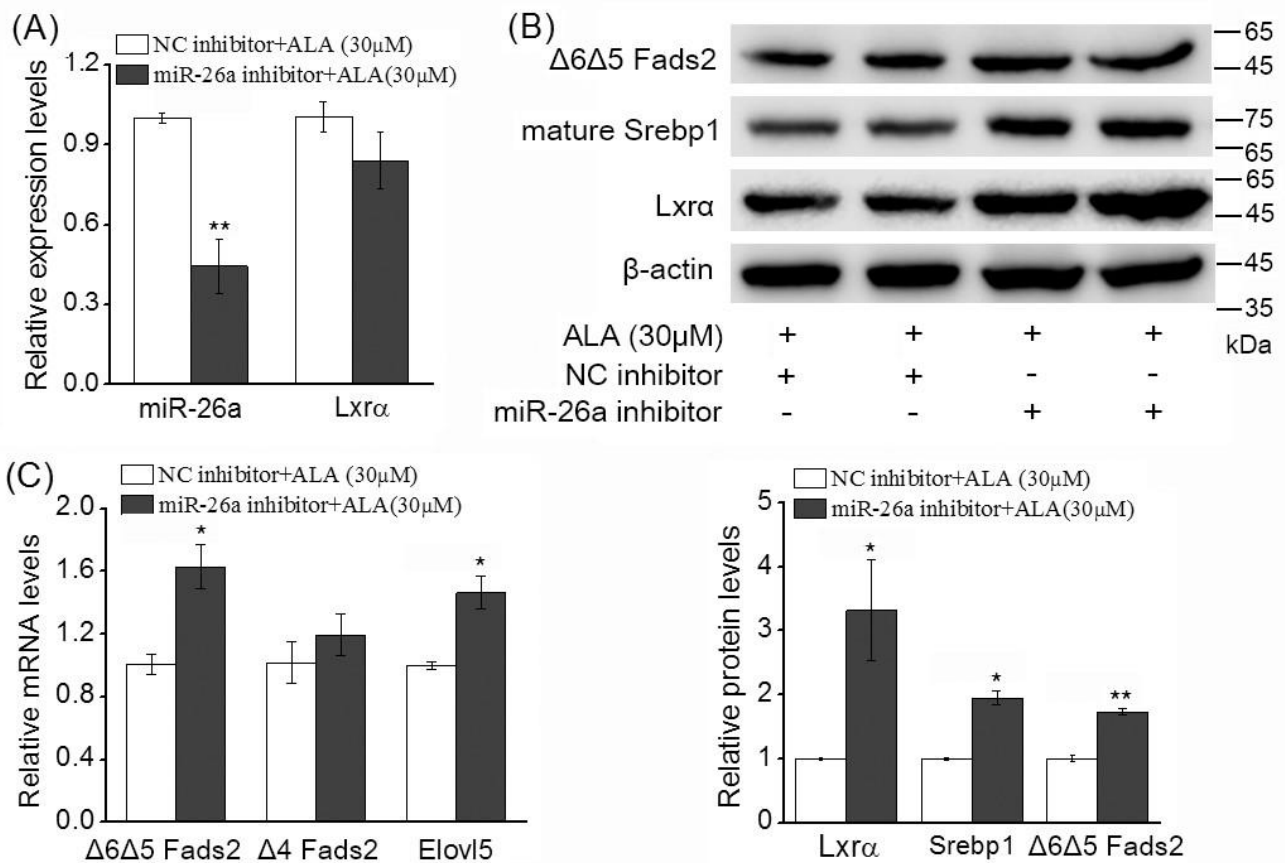
**Figure 3. Rabbitfish *lxra* is a target of miR-26a.** (A, B) Sequence alignment of miR-26a and pre-miR-26a, and the construction plasmids. (C) The HEK 293T cells were co-transfected with pmirGLO empty plasmid, wild-type *lxra* 3'UTR (WT) and the mutated-type of *lxra* 3'UTR (MT), together with miR-26a mimic or negative control mimic (miR-NC) and pre-miR-26a plasmid or control plasmid (pEGFP-C3) for 48 h. (D) HEK 293 T cells were co-transfected with *lxra* 3'UTR (WT), together with miR-26a or miR-NC and miR-26a inhibitor or NC inhibitor 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  $\pm$  SEM from three independent experiments. \*  $P < 0.05$ , \*\*  $P < 0.01$ .



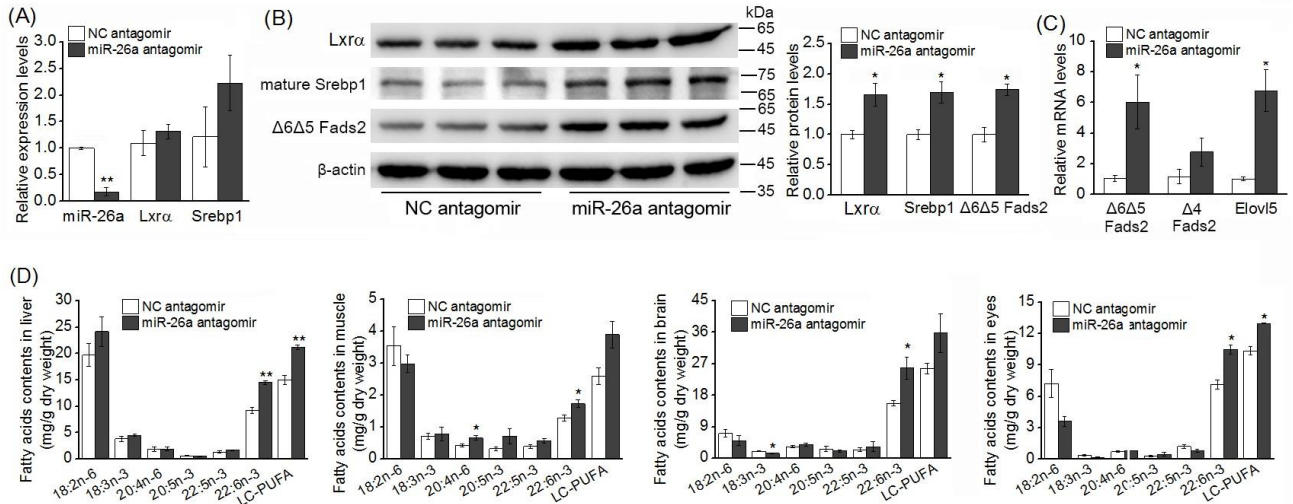
**Figure 4. MiR-26a decreases the abundance of *lxrα* at the post-transcriptional level.** (A) Rabbitfish SCHL cells were transfected with miR-26a mimic or NC mimic within the concentration gradient. After 24 h, the expression of *lxrα* mRNA was determined by qPCR and normalized to  $\beta$ -actin (left). After 48 h, aliquots of proteins from cells were subjected to 10% SDS-PAGE gels and immunoblot analysis of the protein levels of Lxr $\alpha$  (~50 kDa) and normalized to  $\beta$ -actin (~42 kDa) as described in Materials and Methods (middle and right). (B) Rabbitfish SCHL cells were transfected with miR-26a inhibitor or NC inhibitor within the concentration gradient. After 24 h, the expression of *lxrα* mRNA was determined by qPCR as described above (left). After 48 h, the Lxr $\alpha$  protein levels were determined by Western blotting as described above (middle and right). (C) Rabbitfish SCHL cells were transfected with 10nM miR-26a mimic or NC mimic. After 24 h, the cells were treated with DMSO or TO901317 (2  $\mu$ M) for another 24 h. The qPCR was conducted for *lxrα* mRNA level (left) and Western blotting was conducted for Lxr $\alpha$  protein level (middle and right). The Image Studio Software Ver 5.2 was used to quantify the intensity of the Western blotting bands. The intensity ratio between Lxr $\alpha$  and  $\beta$ -actin was calculated as an indication of endogenous Lxr $\alpha$  protein expression change. Data are means  $\pm$  SEM as fold change from the controls. \*  $P < 0.05$  versus the controls and \*\*  $P < 0.01$  (n=3 or 6 for each group).



**Figure 5. The inhibition of miR-26a on Srebp1 activation and expression of genes responsible for LC-PUFA biosynthesis is mediated by *Lxra*.** (A) Rabbitfish SCHL cells were treated with DMSO or TO901317 (2  $\mu$ M) for 24 h. The expression of *srebp1*,  $\Delta 4$  *fads2*,  $\Delta 6\Delta 5$  *fads2* and *elovl5* were analyzed by qPCR. The indicated gene expression was normalized to  $\beta$ -actin mRNA expression. The relative level of indicated gene expression was determined using the  $2^{-\Delta\Delta C_t}$  method. \*  $P < 0.05$  and \*\*  $P < 0.01$  versus the controls (n=3 for each group). (B) SCHL cells were transfected with 10 nM miR-26a mimic or NC mimic. After 24 h, the cells were treated with DMSO or TO901317 (2  $\mu$ M) for another 24 h. Then the expression of mature *Srebp1* (~68 kDa) and  $\Delta 6\Delta 5$  *Fads2* (~48 kDa) protein were determined by Western blotting. (C) Rabbitfish SCHL cells were transfected with 40 nM miR-26a inhibitor or NC inhibitor or co-transfected with 40 nM of miR-26a inhibitor and si-*Lxra*. After 48 h, the protein levels of *Lxra*, *Srebp1* and  $\Delta 6\Delta 5$  *Fads2* were determined by Western blotting. The Image Studio Software Ver 5.2 was used to quantify the intensity of the Western blotting bands. The intensity ratios between *Lxra*/*Srebp1*/ $\Delta 6\Delta 5$  *Fads2* and  $\beta$ -actin were calculated as the indication of endogenous *Lxra*/*Srebp1*/ $\Delta 6\Delta 5$  *Fads2* protein expression changes. \*  $P < 0.05$ , \*\*  $P < 0.01$  (n = 3 or 6 for each group).



**Figure 6. Knockdown of miR-26a promotes LC-PUFA biosynthesis through facilitating *Lxra*-dependent *Srebp1* activation in rabbitfish hepatocytes.** The SCHL cells were transfected with 40 nM of miR-26a inhibitor or NC inhibitor for 24 h, and then treated with 30 μM precursor ALA for another 48 h. (A) The expression of miR-26a and *lxra* mRNA was determined by qPCR. (B) The protein levels of *Lxra*, *Srebp1* and Δ6Δ5 *Fads2* were determined by Western blotting. (C) The expression of Δ4 *fads2*, Δ6Δ5 *fads2* and *elov15* was also analyzed by qPCR. The relative level of indicated gene expression was determined using the  $2^{-\Delta\Delta Ct}$  method. The Image J software v1.8.0 was used to quantify the intensity of the Western blotting bands. The Image Studio Software Ver 5.2 was used to quantify the intensity of the Western blotting bands. The intensity ratios between *Lxra*/*Srebp1*/Δ6Δ5 *Fads2* and β-actin were calculated as the indication of endogenous *Lxra*/*Srebp1*/Δ6Δ5 *Fads2* protein expression changes. \*  $P < 0.05$  and \*\*  $P < 0.01$  versus the controls (n=3 or 6 for each group).



**Figure 7. Antagonizing miR-26a increases LC-PUFA accumulation in tissues of rabbitfish by facilitating *Lxra*-dependent *Srebp1* activation.** Rabbitfish juveniles (~15g) were injected intraperitoneally with 100  $\mu$ l of total antagonists (miR-26a antagonist or the negative control antagonist) diluted in PBS to 50 nmol/ml twice weekly per fish for 3 weeks. (A) The expression of miR-26a and *Lxra* mRNA in liver were determined by qPCR. (B) The protein levels of *Lxra*, *Srebp1* and  $\Delta 6\Delta 5$  *Fads2* in liver were determined by Western blotting. (C) The expression of  $\Delta 4$  *fads2*,  $\Delta 6\Delta 5$  *fads2* and *elovl5* in liver was also analyzed by qPCR. (D) The main fatty acid contents (mg/g dry weight) in liver, muscle, brain and eyes tissues of fish were examined by gas chromatography (GC). Individual fatty acids were identified with retention indices by comparing of known commercial standards and the content of each fatty acid (mg) in the dry weight of tissues (g) was quantified relative to the internal standard (17:0). \*  $P < 0.05$  and \*\*  $P < 0.01$  versus the controls.