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

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MicroRNAs have been recently shown to be important regulators of lipid metabolism. However, the mechanisms of microRNA-mediated regulation of long-chain polyunsaturated fatty acid (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 brackish water and in S. canaliculatus hepatocyte line (SCHL) incubated with the LC-PUFA precursor α-linolenic acid, suggesting that miR-26a may be involved in LC-PUFA biosynthesis because of its abundance being regulated by factors affecting LC-PUFA biosynthesis. Opposite patterns were observed in the expression of liver X receptor α (lxrα) and sterol regulatory element-binding protein-1 (srebp1), as well as the LC-PUFA biosynthesis–related genes (Δ4 fads2, Δ6Δ5 fads2, and elovls) in SCHL cells incubated with α-linolenic acid. Luciferase reporter assays revealed rabbitfish lxrα as a target of miR-26a, and over-expression of miR-26a in SCHL cells markedly reduced protein levels of Lxrα, Srebp1, and Δ6Δ5 Fads2 induced by the agonist T0901317. Moreover, increasing endogenous Lxrα 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 Lxrα–Srebp1 pathway and provide new insights into the regulatory network controlling LC-PUFA biosynthesis and accumulation in vertebrates.

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). Because the capacity for fatty acyl desaturation and elongation of the C18 polyunsaturated fatty acids (PUFA) precursors such as α-linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6) to C20/22 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 in 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 (VOs) are the most sustainable alternatives to replace FO in aquafeed. However, unlike FO, VOs are devoid of C20/22 LC-PUFA but often rich in monounsaturated fatty acids and C18 PUFA (10, 11). The extent to which fish can convert C18 PUFA to C20/22 LC-PUFA varies with species and is associated with many other factors, including age, sex, and gene polymorphisms, among others (8, 12). Therefore, it is essential to understand the regulatory mechanisms of LC-PUFA biosynthesis to enable fish to make effective use of dietary VO.

It is well-known that C18 PUFA can be converted to C20/22 LC-PUFA through a series of carbon chain elongation and desaturation processes in the endoplasmic reticulum, but little is known about how these processes occur and are regulated in vivo (1, 13). In recent years, a variety of fatty acyl desaturases (Δ6, Δ5, and/or Δ4 Fads2) and elongases (Elovls1, Elovls5, Elovls6, and Elovls8), critical enzymes in the LC-PUFA biosynthesis pathway, have been cloned and functionally characterized from a range of vertebrates, including freshwater and marine teleosts (14–16). 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, 17, 18) and environmental factors (e.g. salinity) (18–20) have been demonstrated clearly as...
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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 diets rich in C_{18} PUFA (such as ALA and/or LA; i.e. VO-based) compared with fish reared in sea water and/or fed with LC-PUFA–rich diets (i.e. FO-based) (17, 18, 21). Moreover, several transcriptional factors, including Srebp1 (sterol regulatory element-binding protein 1) (22, 23), NF-Y (nuclear factor Y) (24), Hnf4α (hepatic nuclear factor 4α) (25, 26), Pparγ (peroxisome proliferator-activated receptor γ) (27), and Sp1 (stimulatory protein 1) (28), 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 regulation of LC-PUFA biosynthesis (29). There are two Lxr isoforms, Lxrα and Lxrβ, that 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 in the promoters of Lxr target genes (30). 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 (31–33). Recently, we found that microRNAs (abbreviated as miRNAs or miR) also regulate the expression of fads and elovl genes in fish (22, 23, 34, 35), 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 noncoding RNAs with ~22 nucleotides that regulate gene expression at the post-transcriptional level by binding to specific miRNAs 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 Ref. 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 Δ4 fads2 and elovl5, respectively (34, 35), whereas miR-24 and miR-33 can enhance LC-PUFA biosynthesis through activating the Srebp1 pathway by targeting Insig1 (insulin-induced gene protein 1) (22, 23). 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 (19, 36, 37). Thus, the rabbitfish provides a good model to investigate the regulatory mechanisms of LC-PUFA biosynthesis in marine teleosts. Here, in addition to the above miRNAs reported in rabbitfish, we found that 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 (38–40). 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′-UTR of rabbitfish Lxrα mRNA. Because the activation of Lxrα can increase expression of srebp1 and its downstream fads and elovl genes involved in LC-PUFA biosynthesis (29–31, 41, 42), the present study aimed to validate and characterize the potential roles of miR-26a in the regulation of LC-PUFA biosynthesis by targeting Lxrα in rabbitfish S. canaliculatus.

Results
Expression profiles of miR-26a, lxrα, 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 a salinity of 32 ppt compared with those 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 with 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 µM ALA–BSA complex compared with 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.
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To investigate whether miR-26a was involved in the regulation of Lxra expression, miR-26a was overexpressed and knocked down by transfecting with gradient concentrations of miR-26a mimics and inhibitors into rabbitfish SCHL cells, respectively. As shown in Fig. 4 (A 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 down-regulate 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 and then treated with T0901317. As expected, both mRNA and protein levels of Lxra were successfully up-regulated 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.

Down-regulation 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 (31). As expected, Lxra expression stimulated by T0901317 in rabbitfish hepatocyte line, SCHL, resulted in significant up-regulation of srebpl, Δ4 fads2, Δ6Δ5 fads2, and elolv5 (p < 0.05) (Fig. 5A). Moreover, the mature Srebpl1 and Δ6Δ5 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 Srebpl-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 Srebpl and Δ6Δ5 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 Srebpl activation and expression of

Rabbitfish lxta is a target of miR-26a

To explore the relationships between miR-26a and Lxra, srebpl, and 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 (Fig. 3S) and its sequence were 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 WT Lxra 3′-UTR reporter plasmid into HEK 293T cells, but this effect was largely restored for the co-transfected plasmid containing mutant 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

Our previous studies reported that both gene expression of srebpl, fads, and elolv and enzymatic activity of LC-PUFA biosynthesis were higher in liver of rabbitfish reared at 10-ppt salinity or fed VO diets when compared with fish reared at 32-ppt salinity or fed FO diets, respectively (19, 20, 31). Rabbitfish fed a FO diet displayed higher expression of Lxra in liver than fish fed VO diets, whereas ambient salinity produced no significant change in the expression of Lxra (31). However, in vitro, the Lxra mRNA level was significantly increased when the ALA concentration increased (p < 0.05) (Fig. 1C), which was similar to the expression patterns of srebpl, Δ4 fads2, Δ6Δ5 fads2, and elolv5 previously reported in rabbitfish SCHL cells incubated with ALA (22). In addition, tissue-specific distribution of rabbitfish miR-26a was determined in selected tissues by real-time quantitative PCR (qPCR). As shown in Fig. 2, miR-26a was highly (ΔCt < 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.
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its downstream LC-PUFA biosynthesis–related genes. These observations led us to conclude that the cross-talk between miR-26a and the Lxr–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 Lxrα 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 with NC inhibitor, along with a 3-fold increase of Lxrα protein level (Fig. 6, A and B). However, the Lxrα mRNA level was marginally decreased. Compared with 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, whereas the proportion of saturated fatty acids, 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 antagonir specifically targeting miR-26a or negative control antagonir for 21 days. We observed an 83% reduction of hepatic miR-26a expression in rabbitfish that received miR-26a antagonist compared with the negative control and a 1.7-fold increase of Lxrα protein level, but no statistically significant difference was observed in lxrα and srebp1 mRNA levels (Fig. 7, A and B). Treatment with miR-26a antagonist 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).

Knockdown of miR-26a facilitates lxrα-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 Lxrα–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 Lxrα and subsequent mature Srebp1 and ΔΔ5 Fads2 protein levels in ALA-treated rabbitfish cells (Fig. 6B).

Simultaneously, the expression levels of three Srebp1-targeted enzyme genes, Δ4 fads2, ΔΔ5 fads2, and elovl5, were also up-regulated 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 ΔΔ5 Fads2 protein and the transcripts of ΔΔ5 fads2 and elovl5 in liver (p < 0.05) (Fig. 7, B and C). Together, these results indicate that miR-26a is involved in the regulation of LC-PUFA biosynthesis by targeting the Lxrα–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 C18 PUFA precursors is possible, the conversions and efficiencies are specific to cell types and species (14). 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 C18 PUFA precursors into C20/22 LC-PUFA because of the absence of certain enzymes 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 (1, 13). With increasing use of VO sources in feeds used in fish farming, it is critical to understand the regulatory mechanisms of LC-PUFA biosynthesis to enable fish to make effective use of dietary C18 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 (43), and recently, we demonstrated that miRNAs are also involved in the regulation of LC-PUFA biosynthesis in the marine teleost rabbitfish S. canaliculatus (22, 23, 34, 35). 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 that it may be involved in the regulation of LC-PUFA biosynthesis.
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In mammals, the miR-26 family (miR-26a/b) has been reported to be involved in adipogenesis (38, 40), and they can control Lxr-dependent cholesterol efflux by targeting Lxr target genes that play critical roles in cholesterol metabolism (39). Based on the expression profiles of LC-PUFA biosynthesis–related genes in vivo and in vitro (31), we found that miR-26a showed an inverse expression pattern with srebp1 in liver of rabbitfish fed two different lipid diets and with both of lxrα and srebp1 in rabbitfish SCHL cells treated with ALA (22). Moreover, because tissue expression of miRNA might, to some extent, reflect the function of miRNA (44), 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 lxrα was relatively high in liver (31). These data suggest that there might be an interaction between miR-26a and lxrα 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 lxrα 3'UTR, and in vitro luciferase reporter assays confirmed that rabbitfish lxrα was a novel target gene of miR-26a. In addition, knockdown of miR-26a up-regulated the expression of Lxrα, 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 Lxrα in rabbitfish.

Lxrα is a member of the nuclear hormone receptor superfamily that plays a critical role in the transcriptional regulation of lipid metabolism (45). It was found that Lxrα activation promoted LC-PUFA biosynthesis through direct regulation of Elov5 and Srebp1-dependent regulation of key enzymes (Elov5 and Fads) in human macrophages (33). Elov5 is the direct Lxrα target gene in human macrophages (33), whereas indirect regulation of elov5 by Lxrα through a Srebp1-dependent pathway has been reported in mouse liver and salmon head kidney cell line (SHK-1) (32, 46). Similarly, in rabbitfish primary hepatocytes and the hepatocyte cell line SCHL, we found that...
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Figure 6. Knockdown of miR-26a promotes LC-PUFA biosynthesis through facilitating Lxrα-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 lxrα mRNA was determined by qPCR. B, the protein levels of Lxrα, Srebp1, and ΔΔΔΔ Fads2 were determined by Western blotting. C, the expression of Δ Fads2, ΔΔΔΔ Fads2, and elovl5 was also analyzed by qPCR. The relative level of indicated gene expression was determined using the 2−ΔΔCt method. ImageJ software, version 1.8.0 was used to quantify the intensity of the Western blotting bands. Image studio software, version 5.2 was used to quantify the intensity of the Western blotting bands. The intensity ratios between Lxrα/Lxrα and β-actin were calculated as the indication of endogenous Lxrα/Srebp1/ΔΔΔΔ Fads2 protein expression changes. *, P < 0.05; **, P < 0.01.

Table 1
Fatty acid composition (% total fatty acid) of rabbitfish S. canaliculatus hepatocyte line (SCHL) (a)

| Fatty acid | Mock cells (b) | NC inhibitor | miR-26a inhibitor | P value |
|------------|----------------|--------------|------------------|---------|
| 16:0       | 12.71          | 12.73 ± 0.12 | 11.42 ± 0.40     | 0.005   |
| 18:0       | 14.57          | 14.29 ± 0.52 | 12.42 ± 0.20     | 0.028   |
| 16:1n-7    | 1.19           | 1.42 ± 0.18  | 1.46 ± 0.06      | 0.868   |
| 16:1n-9    | 1.34           | 1.53 ± 0.10  | 1.49 ± 0.06      | 0.739   |
| 18:1n-9    | 21.04          | 19.63 ± 1.28 | 18.83 ± 0.24     | 0.572   |
| 20:1n-9    | 0.47           | 0.48 ± 0.03  | 0.54 ± 0.03      | 0.190   |
| 18:2n-6 (LA)| 2.52          | 3.46 ± 0.64  | 3.57 ± 0.32      | 0.883   |
| 18:3n-6    | nd             | nd           | 0.12 ± 0.06      | 0.116   |
| 20:2n-6    | 0.66           | 1.14 ± 0.20  | 1.56 ± 0.01      | 0.101   |
| 20:3n-6    | 1.33           | 1.33 ± 0.03  | 1.35 ± 0.03      | 0.794   |
| 20:4n-6 (ARA)| 6.10             | 6.69 ± 0.32  | 7.07 ± 0.17      | 0.358   |
| 22:0n-6    | 0.58           | 0.58 ± 0.02  | 0.81 ± 0.05      | 0.015   |
| 18:3n-3 (ALA)| 1.57          | 1.86 ± 0.34  | 1.76 ± 0.26      | 0.827   |
| 20:3n-1    | 0.52           | 0.93 ± 0.24  | 0.85 ± 0.48      | 0.886   |
| 20:4n-3    | 0.23           | 0.19 ± 0.10  | 0.39 ± 0.07      | 0.166   |
| 20:5n-3 (EPA)| 2.47          | 2.29 ± 0.09  | 2.76 ± 0.09      | 0.021   |
| 22:5n-3    | 2.17           | 2.21 ± 0.23  | 2.77 ± 0.01      | 0.071   |
| 22:6n-3 (DHA)| 7.34          | 7.08 ± 0.04  | 8.83 ± 0.04      | 0.001   |
| SFA        | 27.28          | 28.02 ± 0.63 | 23.85 ± 0.60     | 0.009   |
| MUFA       | 24.94          | 23.06 ± 1.30 | 22.31 ± 0.25     | 0.604   |
| PUFA       | 25.46          | 27.74 ± 0.91 | 31.84 ± 0.49     | 0.016   |
| LC-PUFA (c)| 20.71          | 21.29 ± 0.45 | 24.83 ± 0.53     | 0.007   |
| n-6 LC-PUFA| 7.98           | 8.61 ± 0.33  | 9.23 ± 0.16      | 0.166   |
| n-3 LC-PUFA| 12.73          | 12.69 ± 0.26 | 15.61 ± 0.39     | 0.003   |

(a) SCHL cells were treated with 30 μM ALA for another 48 h after transfection with 20 nM NC inhibitor or miR-26a inhibitor for 24 h. Data presented as mean ± SEM (n = 3). nd: not detected, < 0.01. SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUTA: Polyunsaturated fatty acids.

(b) Mock cells are SCHL cells treated with 30 μM ALA for another 48 h after not transfection with any oligonucleotides for 24 h.

(c) 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.

Activation of Lxrα by agonist T0901317 can stimulate the expression of srebp1 and Srebp1 target genes, such as Δ4 fads2, ΔΔΔΔ fads2, and elovl5 (31), and these effects were markedly attenuated by miR-26a mimics. However, in the core promoters (~200 bp) of the key enzyme genes, we did not find any Lxr response elements using the bioinformatics software TRANSFAC® and TF binding® (25, 26, 47). To further examine whether miR-26a suppressed the expression of the key enzyme genes through a Srebp1-dependent pathway by targeting Lxrα, we used siRNA to knock down the endogenous expression of Lxrα induced by transfecting miR-26a inhibitors into rabbitfish SCHL cells. The results showed that knockdown of miR-26a markedly increased Lxrα, mature Srebp1, and ΔΔΔΔ Fads2 protein levels, and this was attenuated by subsequent Lxrα knockdown, which established that miR-26a may suppress the expression of LC-PUFA biosynthesis–related genes through a Srebp1-dependent pathway by targeting Lxrα.

Fatty acid profile analysis performed on rabbitfish SCHL cells in vitro and rabbit tissues in vivo after knockdown of miR-26a supported the above hypothesis, because the amounts of LC-PUFA, especially DHA, were markedly increased in both cells and fish knocked down of miR-26a compared with controls, with increased expression levels of mature Srebp1 protein and enzyme genes. It was important to note that, consistent with our previous study (20), 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 (37). 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 β-oxidation of DHA than that of EPA and ARA (48, 49). Although the mechanism by which miR-26a controls LC-PUFA biosynthesis and accumulation requires further investigation, our study
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Figure 7. Antagonizing miR-26a increases LC-PUFA accumulation in tissues of rabbitfish by facilitating Lxrα-dependent Srebp1 activation. Rabbitfish juveniles (15 g) were injected intraperitoneally with 100 μl of total antagonim (miR-26a antagomir or the negative control antagonist) diluted to 50 nmol/ml twice weekly for 3 weeks. A, the expression of miR-26a and lxrα mRNA in liver was determined by qPCR. B, the protein levels of Lxrα, Srebp1, and ΔΔΔΔ Fads2 in liver were determined by Western blotting. C, the expression of Δ4 Fads2, ΔΔΔΔ Fads2, and elov15 in liver was also analyzed by qPCR. D, the main fatty acids content (mg/dry weight) in liver, muscle, brain, and eyes tissues of fish were examined by GC. Individual fatty acids were identified with retention indices by comparison 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; ** P < 0.01 versus the controls.

revealed an important role for the interaction between miR-26a and Lxrα–Srebp1 pathway in rabbitfish in vivo.

miRNAs are small noncoding 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 (43). In the present study, we found that overexpression of miR-26a significantly reduced the protein level of target Lxrα, but no corresponding decrease of lxrα mRNA level was observed. This suggested that miR-26a might target the 3′-UTR of rabbitfish lxrα mRNA and down-regulate 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 (50, 51), thereby demonstrating that the miRNA will be assigned to 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 (52, 53). There was a mismatch in position 8 between miR-26a seed region and lxrα 3′-UTR, and this may further support the above inference about translation inhibition of lxrα by miR-26a. Although some interactions between LC-PUFA metabolism and Lxr-mediated pathways have been suggested (54, 55), 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 (55, 56), and LC-PUFA can selectively suppress Srebp1 transcription through proteolytic processing and autoloop regulatory circuit (55). The present study also suggested that there may be an autoregulatory loop in the activation of Lxrα–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 lxrα 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 lxrα and srebp1 mRNA levels occurred in fish receiving miR-26a antagomir than that of the NC antagonist group, no statistical differences of lxrα 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, lxrα showed a higher expression level in the livers 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, Lxrα is not the only physiological regulator for Srebp1 expression in rabbitfish physiologically, and the complexity of the molecular mechanisms of Lxrα 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 Lxrα–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 (10–20 g) for the feeding trial and miRNA antagonist injection study were captured from the coast near Nan Ao Marine Biology Station of Shantou University. All procedures performed on fish were in accordance with
the National Institutes of Health guidelines for the care and use of laboratory animals (National Institutes of Health Publication 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 (FO and VO) and reared at two salinities (10 and 32 ppt) were obtained from the feeding trial, which is described in detail by Chen et al. (22). At the end of the feeding trial, the fish were fasted for 24 h and anesthetized with 0.01% MS222 (tricaine methanesulfonate) and subsequently stored at −80°C until further analysis.

Reagents, cells, and antibodies

Cells from the S. canaliculatus hepatocyte line (SCHL), initially established in 2017 (57), were cultured in Dulbecco’s modified Eagle’s medium/nutrient F12 (DMEM/F12; Gibco) with 20 mM HEPES (Sigma–Aldrich), 10% fetal bovine serum (FBS; Gibco), 0.2% rainbow trout Oncorhynchus mykiss serum (Caisson Laboratory), streptomycin (100 units/ml; Sigma–Aldrich) and penicillin (100 units/ml; Sigma–Aldrich). The cells were maintained in a normal atmosphere incubator at 28°C. The HEK 293T cells were cultured in DMEM (Gibco) containing 10% FBS and maintained at 37°C with 5% CO2. The Lxr agonist T0901317 was obtained from Sigma. The mouse mAb against rabbitfish ΔΔ5 Fads2 (~48 kDa) and rabbit polyclonal antibody against rabbitfish Lxrα (~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 mass, ~68 kDa; WL02093) and mouse mAb against β-actin (1:3000; ~42 kDa; WL01372) were purchased from Wanleibio (Shenyang, China).

Incubation of rabbitfish SCHL cells with ALA

The ALA (Cayman)–BSA (fatty acid-free; Cayman) complex at 10 mM concentration (10% BSA) was prepared according to the method described by Ou et al. (55) 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, the cells were incubated for 2 h in serum-free DMEM/F12 prior to treatment with 0 (BSA alone), 50, and 100 μM 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%).

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miR-26a mimics, inhibitors, siRNA, transient transfection, and Lxr 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′-UUUGUACUCACAAAGAUCUG-3′; antisense, 5′-GAUACUUUUUGAGUAGACAA-3′; miR-26a mimic, sense, 5′-UCAAGAUAUCGAGGUACCU-3′; antisense, 5′-AGCCUAUCCUGAUACUUGA-3′; negative control miRNA inhibitor, 5′-CAGUACUUUUUGAGUAGACAA-3′; and miR-26a inhibitor, 5′-AGCCUAUCCUGAUACUUGA-3′. Silencing of rabbitfish lxrα expression was performed using siRNA (siRNA) duplexes (Hippobio, Huzhou, China) with the following sequences: si-lxrα sense, 5′-GCAGCGUGACUGCAUGAUUTT-3′; si-lxrα antisense, 5′-UAUAGAUGCGAGUGCCAGCGAC-3′. After rabbitfish SCHL cells were cultured to 90% confluence in six-well plates or 90-mm vessels overnight, the 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™ (Invitrogen). After transfection with 10 nM miR-26a or negative control mimics for 24 h, the cells were treated with Lxrα agonist T0901317 (2 μM) for a further 24 h. Cells treated with DMSO (DMSO; Sigma) were the negative control, whereas T0901317 was the positive control. After incubation, the cells were harvested for qPCR and Western blotting analysis.

Plasmid construction and Dual-Luciferase reporter assays

The pre–miR-26a sequence (NCBI accession no. 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) to construct the pre-miRNA expression plasmid. To generate the WT 3′-UTR-luciferase plasmid of lxrα, the entire 3′-UTR of rabbitfish lxrα (JF502074.1) gene was amplified by PCR and inserted into the pmirGLO luciferase reporter vector (Promega) between the SacI and XbaI sites. The MT of lxrα-3′-UTR reporter vector was generated using Muta-direct™ site-directed mutagenesis kit (SBS Genetech, Beijing, China). The sequences of primers and oligonucleotides used for cloning are provided in Table S1.

For miR-26a target identification, HEK 293T cells were co-transfected with lxrα-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. The cells were subsequently transfected with 100 ng of plasmids or 100 nM oligonucleotides using Lipofectamine 2000™ (Invitrogen), 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) 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.
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In vivo miR-26a antagonor injection experiment

After acclimation in an indoor seawater (32 ppt) tank for 2 weeks at the Nan Ao Marine Biology Station, rabbitfish juveniles (~15 g) were then acclimated from seawater to brackish water (10 ppt) for a further 2 weeks. The rabbitfish were subsequently divided into two groups, with eight fish per group. One group was treated with miR-26a antagonors, and the other was treated with the negative control antagonors. The miRNA antagonors were chemically modified antisense oligonucleotides complementary to the mature miRNAs, which can inhibit the function of target miRNAs and are stable in vivo for at least 2 weeks (58). The miR-26a antagonist and the negative control antagonist were commercially synthesized from Hippobio (Huzhou, China). The fish were injected intraperitoneally twice weekly for 3 weeks with 100 μl of total antagonors diluted in PBS to 50 nmol/ml or with the negative control antagonist. During the in vivo injection experiment, the fish were fed a commercial diet, with the fatty acid composition of the diet presented in Table S2. 21 days after the first antagonist injection, the fish were fasted for 24 h and anesthetized with 0.01% 2-phenox-yethanol (Sigma–Aldrich). Liver, muscle, brain, and eye samples were collected, 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) following the manufacturer’s protocol. After DNase I digestion (Takara) at 37 °C for 30 min, 1 μg of high-quality RNA was reverse-transcribed using miScript II RT kit (Qiagen). All qPCR assays were performed in a LightCycler® 480 thermocycler (Roche) as described previously (22). The relative expression levels of miRNAs were normalized by β-actin, whereas miRNAs were normalized by 18S rRNA. All amplification reactions were carried out in triplicate using the primers designed by Primer 3 software (RRID:SCR_003139) and are listed in Table S2.

Western blotting

Samples of tissues and cultured cells were lysed in radioimmunoprecipitation assay buffer (Thermo Fisher) and centrifuged at 12,000 × g for 10 min at 4°C. After determination of protein concentration, aliquots of protein (20–40 μg) were separated on 10% SDS-polyacrylamide gels and transferred to 0.45 μm polyvinylidene fluoride membranes (Roche). The membranes were blocked for 1 h at room temperature with 5% non-fat milk in 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 (horseradish peroxidase goat anti-rabbit/mouse IgG; Abcam). Immunoreactive bands were visualized using the Odyssey IR imaging system (LI-COR), and the intensity of each band was analyzed with Image Studio Software (version 5.2, LI-COR). The optical density of each sample run on each blot was normalized to the expression level of β-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 NC inhibitor using Lipofectamine 2000™ (Invitrogen) for 24 h before incubation with 30 μM ALA–BQA complexes. After 48 h of incubation, the cells were harvested for qPCR, Western blotting, and fatty acid composition analysis.

Fatty acid composition of cultured cells and tissue samples was analyzed by GC after extraction of total lipid by chloroform/methanol, saponification, and methylation with boron trifluoride (Sigma–Aldrich), all according to the methods described in detail previously (36, 59). Individual fatty acids were identified by retention indices compared with known commercial standards (Sigma–Aldrich) and quantified relative to the internal standard (17:0).

Statistical analysis

The data on relative gene expression were obtained using the 2−ΔΔCt method, and comparisons were performed by the independent samples t test between pairs of groups or one-way analysis of variance followed by Tukey’s test for multiple groups using SPSS version 19.0 (SPSS Inc., Chicago, IL). All data are presented as means ± S.E. 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. The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBank®/EBI Data Bank with accession number MN443954.

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Abbreviations—The abbreviations used are: LC-PUFA, long-chain polyunsaturated fatty acid; ALA, α-linolenic acid; LA, linoleic acid; VO, vegetable oil; FO, fish oil; miRNA, microRNA; Lxr, liver X receptor; Srebp1, sterol regulatory element-binding protein 1; LXRE, Lxr response elements; NC, negative control; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ARA, arachidonic acid; MT, mutant type; qPCR, real-time quantitative PCR; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum.

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