Title

MicroRNAs involved in the regulation of LC-PUFA biosynthesis in teleosts: miR-33 enhances LC-PUFA biosynthesis in *Siganus canaliculatus* by targeting *insig1* which in turn up-regulates *srebp1*

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Abstract

Post-transcriptional regulatory mechanisms play important roles in the regulation of LC-PUFA biosynthesis. Our previous study revealed that miR-33 could increase the expression of fatty acyl desaturases (*fads2*) in the rabbitfish *Siganus canaliculatus*, but the specific mechanism is unknown. Here, we confirmed that miR-33 could target the 3'UTR of insulin-induced gene 1 (*insig1*), resulting in down-regulation of its protein level in the rabbitfish hepatocyte line (SCHL). *In vitro* overexpression of miR-33 inhibited the mRNA level of *insig1* and increased the mRNA levels of *Δ6Δ5 fads2* and *elovl5*, as well as *srebp1*. In SCHL cells, proteolytic activation of sterol-regulatory-element-binding protein-1 (Srebp1) was blocked by Insig1, with overexpression of *insig1* decreasing mature Srebp1 level, while inhibition of *insig1* led to the opposite effect. Srebp1 could enhance the promoter activity of *Δ6Δ5 fads2* and *elovl5*, whose expression levels decreased with knockdown of *srebp1* in SCHL. Overexpression of miR-33 also resulted in a higher conversion of 18:3n-3 to 18:4n-3 and 20:5n-3 to 22:5n-3, linked to desaturation and elongation via Δ6Δ5 Fads2 and Elovl5, respectively. The results suggested that the mechanism by which miR-33 regulates LC-PUFA biosynthesis in rabbitfish is through enhancing the expression of *srebp1* by targeting *insig1*. The findings here provide more insight to the mechanism of miRNAs involvement in the regulation of LC-PUFA biosynthesis in teleosts.

Key words: miR-33; *insig1*; *srebp1*; *Δ6Δ5fads2*; *elovl5*; LC-PUFA biosynthesis

1. Introduction

Long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA) are highly bioactive forms of PUFA. The LC-PUFA with crucial physiological functions in humans and other animals (Janssen and Kiliaan 2014; Calder 2015) include arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). As important components of cell membranes, LC-PUFA reduce membrane phase-change temperatures and enhance membrane fluidity, as well as playing crucial roles in maintaining the normal physiological function of biofilms (Xiao et al. 2001). LC-PUFA are also important in growth, survival, pigmentation, stress and disease resistance of fish, as well as in the development of brain, vision and the nervous system (Sargent et al. 2002; Tocher 2010).

The biosynthesis of LC-PUFA involves desaturation and chain elongation reactions that convert the C_{18} PUFA precursors, linoleic acid (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3), into the physiologically important ARA, EPA and DHA. Fish are important sources of n-3 LC-PUFA in the human diet and, consequently, LC-PUFA biosynthesis in fish has been investigated extensively in recent years (Castro et al. 2016; Monroig et al. 2018). Generally, freshwater fish have the ability to convert LA and ALA to $C_{20\text{-}22}$ LC-PUFA (ARA, EPA and DHA), with this process requiring enzymes such as fatty acyl desaturases (Fads) and elongation of very-long-chain fatty acids (Elovl) proteins. Fads and Elovl enzymes involved in LC-PUFA biosynthesis can also be found in marine fish, but lower activity and/or absence of key enzymatic capacities is associated with low LC-PUFA biosynthesizing capacity and thus dietary provision of

EPA and DHA is required to sustain growth and development (Tocher et al. 2003). The rabbitfish *Siganus canaliculatus* is an exception to this pattern since this marine herbivore has the ability to biosynthesize LC-PUFA from C_{18} PUFA (Li et al. 2010; Monroig et al. 2012). *S. canaliculatus*, in addition to its complement of elongases (Monroig et al. 2012), possesses two distinct *fads2* genes encoding Δ6Δ5 and Δ4 desaturases enabling all desaturation reactions involved in the LC-PUFA biosynthesis pathway (Castro et al. 2016; Monroig et al. 2018). Consequently, *S. canaliculatus* and has become a valuable model for studying regulatory mechanisms of LC-PUFA biosynthesis in teleosts (Li et al. 2010; Monroig et al. 2012).

MicroRNAs (miRNAs) are a class of highly conserved, small non-coding, ~22 nucleotides (nt) RNA molecules that are widespread in organisms. In land animals, miRNAs generally exhibit a negative regulatory effect on gene expression and are involved in a number of biological processes (Alvarezgarcia and Miska 2005; Carrington et al. 2003; Xu et al. 2003). Post-transcriptional regulatory mechanisms have been shown to play important roles in LC-PUFA biosynthesis and glycolipid metabolism, as well as growth, development, reproduction and immune function in teleosts (Gong et al. 2015; Her et al. 2011; Siddique et al. 2016; Škugor et al. 2014; Tao et al. 2018; Zhang et al. 2014; Zhu et al. 2015). In rabbitfish, miR-17 was found to be involved in the regulation of LC-PUFA biosynthesis by targeting *Δ4 fads2* (Zhang et al. 2014). Moreover, our previous study revealed that miR-33 was involved in the regulation of LC-PUFA biosynthesis by increasing the expression of *fads2* in rabbitfish (Zhang et al. 2016b), although the underlying mechanism was not clearly established.

In mammals, miR-33 exists as two distinct isoforms, namely miR-33a and b, which differ from each other in two bases outside the seed region of the mature versions (Najafi-Shoushtari et al. 2010). Both miR-33a and miR-33b are located in the sterol regulatory element-binding protein (*SREBP*) intron region (Najafi-Shoushtari et al. 2010; Rayner et al. 2010; Goldstein et al. 2002). In rabbitfish, the miR-33 gene was identified within intron 16 of the gene encoding *srebp1*, and miR-33 overexpression suppressed the expression of *insig1*, which is predicted to be the target gene of miR-33 (Zhang et al. 2016b). However, whether this is a direct effect or not is unclear. Moreover, miR-33 overexpression led to an increase in the mRNA levels of *fads2* and *srebp1* and, thus, it is believed to be involved in the regulation of LC-PUFA biosynthesis, but the specific mechanism is unknown (Zhang et al. 2016b).

The Insulin-induced gene protein (Insig) is an important factor in the regulation of lipid metabolism (Jo et al. 2011). The two subtypes of Insig, namely Insig1 (Radhakrishnan et al. 2007) and Insig2 (Lee et al. 2005), combine with Srebp and Srebp cleavage activating protein (SCAP) in the endoplasmic reticulum (ER) as an Insig-SCAP-Srebp complex. Insig1 has a stronger affinity for SCAP compared to Insig2, and is reported to block the proteolytic cleavage of Srebp proteins by retaining Srebp precursors in the ER membrane, consequently decreasing lipogenesis (Gong et al. 2006; Engelking et al. 2004). In particular, the mechanisms detailed above apply to mammals, and it remains unclear whether Insig1 can block Srebp proteolytic activation in teleost fish.

In mammals, miR-33 has been found within the intron of *srebp* genes and reported

115 to function in cooperation with its host (Horie et al. 2013). *Srebp1* is an important
116 transcription factor involved in regulating the expression of key enzymes in LC-PUFA
117 synthesis in liver (Nara et al. 2002; CarmonaAntoñanzas et al. 2014). Srebp1 affects
118 the synthesis of LC-PUFA via activation of acetyl CoA carboxylase (ACC), fatty acid
119 synthetase (FAS), stearoyl-CoA desaturase (SCD) and other enzymes related to fatty
120 acid metabolism in mouse liver (Shimomura et al. 1998). *Srebp1c* is also reported to
121 promote the expression of *Δ5* and *Δ6 fads-like* genes in the liver (Qin et al. 2009). In
122 rabbitfish, our previous studies showed there might be potential interaction between
123 Srebp1 and the key enzymes of LC-PUFA synthesis, especially Δ6Δ5 Fads2 and the
124 PUFA elongase, Elovl5, and the sterol regulatory element (SRE) of Srebp protein
125 predicted in the promoter region of *Δ6Δ5 fads2* and *elovl5* (Zhang et al. 2016; Dong et
126 al. 2018).

127 The aim of the present study was to investigate the mechanism underpinning the
128 regulation of LC-PUFA biosynthesis by miR-33. Firstly, dual luciferase assay and
129 western blotting were performed to determine whether *insig1* was a direct target gene
130 of miR-33. Secondly, to explore the functional relationship between Insig1 and Srebp1
131 in rabbitfish, overexpression and inhibition of *insig1* followed by western blotting was
132 used to detect the protein abundances of Insig1 and mature Srebp1. Subsequently, in
133 order to further elucidate the roles of Srebp1 in the regulation of LC-PUFA biosynthesis
134 in rabbitfish, changes in the expression, as well as the promoter activity, of *Δ6Δ5 fads2*
135 and *elovl5*, in response to changes in the *in vitro* expression level of *srebp1* were
136 explored. The data provide the basis for elucidating the mechanism of miR-33

involvement in the regulation of LC-PUFA biosynthesis in rabbitfish, as well as providing the theoretical basis for the participation of miRNA in the regulation of LC-PUFA biosynthesis in teleosts.

2. Materials & Methods

2.1 Rabbitfish hepatocyte culture

The rabbitfish *S. canaliculatus* hepatocyte line (SCHL) was successfully established in our laboratory (Liu et al. 2017) SCHL cell line was cultured at 28 ºC in Dulbecco's modified Eagle's medium/nutrient F12 (DMEM/F12, Gibco, Life Technologies, USA) containing 20 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulphonic acid (HEPES, Sigma-Aldrich, USA), 10 % fetal bovine serum (FBS, Gibco, Life Technologies, USA), 0.5 % rainbow trout *Oncorhynchus mykiss* serum (Caisson Labs), penicillin (100 U ml^{-1}, Sigma-Aldrich, USA) and streptomycin (100 U ml^{-1}, Sigma-Aldrich, USA).

2.2 Plasmid construction

In order to achieve overexpression of *insig1*, we constructed the pcDNA-Insig1 eukaryotic expression vector at the *EcoR*I and *Xho*I (New England Biolabs, Ipswich, USA) restriction sites, with full-length sequence of *insig1* amplified using pcDNA-Insig1-F/R primers (Table 1). Similarly, we constructed the pcDNA3.1-SREBP overexpression vector at the *Xba*I and *Hin*dⅢ restriction sites, with full-length sequence of *srebp1* amplified using LG-SREBP-F/R primers (Table 1). The *Δ6Δ5 fads2*

and *elovl5* promoter deletion dual luciferase reporter vectors were constructed previously in our laboratory (Dong et al., 2018). For heterologous expression of rabbitfish miR-33, a DNA fragment encompassing rabbitfish pre-miR-33 was digested with *Eco*RI and *Bam*HI and inserted into the pEGFP-C3 plasmid. To construct the dual luciferase reporter vectors, DNA fragments were inserted into pmirGLO dual-luciferase miRNA target expression vector (Promega, Madison, WI, USA) at the *Sac*I and *Xba*I restriction sites. The recombinant vectors were: (1) pmirGLO-Insig-3'UTR, pmirGLO including an insert consisting of a partial DNA fragment of the rabbitfish *insig1* 3'UTR, which includes the binding site of miR-33 in rabbitfish, amplified with Insig-3'UTR-F/R primers (Table 1); (2) pmirGLO -Insig-3'UTR-MU, pmirGLO including an insert consisting of the predicted binding site of miR-33 in *insig1* 3'UTR (5'-AATGCA-3') mutated into 5'-TAAGGA-3' to prevent complementarity of miR-33, and amplified with mutation primers Insig-3'UTR-Mu-F/R designed following the instructions of the Muta-direct TM site-Directed Mutagenesis Kit (SBS Genetech Co., Ltd., Beijing, China); (3) pmirGLO-R33 (positive control), pmirGLO including an insert consisting of a synthesized oligonucleotide containing a 100 % match to miR-33 (Sangon Biotech, Shanghai, China). Sequences of primers and oligonucleotides used for cloning are provided in Table 1.

2.3 RNA isolation and quantitative real-time PCR (qPCR)

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) followed by determination of the concentration and quality of the total RNA on

NanoDrop 2000 (Thermo Scientific, USA). cDNA was synthesized with 1 μg total RNA using the miScript II RT Kit (Qiagen, Hilden, Germany), and the expression of miR-33 determined using the miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) with miR-33 specific primer (qPCR-miR-33) (Table 1) and universal primers. For the qPCR determination of the mRNA expression levels of *insig1* (KU598855), *srebp1* (JF502069.1), *Δ6Δ5 fads2* (EF424276.2) and *elovl5* (GU597350.1), LightCycler® 480 SYBR Green I Master (Roche, Germany) was used with rabbitfish gene-specific primers (Table 1). The relative RNA level of each gene was normalized to that of 18s rRNA (AB276993), and calculated using the comparative threshold cycle method (Livak and Schmittgen 2012). All reactions were run on LightCycler® 480 thermocycler (Roche, Germany) using qPCR programs according to manufacturer's specifications.

2.4 Dual-luciferase assay

To determine whether *insig1* was a direct target gene of miR-33, a dual luciferase assay was performed using human embryonic kidney (HEK 293T) cells (Chinese Type Culture Collection, Shanghai, China). The HEK 293T cells were seeded into 96-well cell culture plates in 100 μl High Glucose Dulbecco's Modified Eagle Medium (DMEM) (Gluta MAX) (Gibco, Life Technologies, USA) with 10 % fetal bovine serum per well (FBS, Sijiqing Biological Engineering Material Company, China). The HEK 293T cells were grown for 24 h to 80 % confluence, and then co-transfected with either pEGFP-miR-33 (50 ng) or pEGFP-empty (50 ng) with different recombinant dual luciferase

reporter vectors (50 ng) using Lipofectamine® 2000 Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Firefly and Renilla luciferase activities were quantified after 48 h transfection using a microplate reader (Infinite M200 Pro, Tecan, Switzerland) and firefly luciferase activity was normalized to Renilla luciferase activity. Eight replicate wells were used for each treatment.

2.5 Western blotting

In order to further study the potential relationship between miR-33 and the target gene Insig1 at the protein level, Western blotting was carried out. miR-33 was up-regulated by transfection with miR-33 mimics (dsRNA oligonucleotides) and negative control (NC) oligonucleotides were obtained from Genepharma (Shanghai, China). The sequences were as follows: miR-33 mimic, sense, 5'-CGUGCAUUGUAGUUGCAUUG-3'; antisense, 5'-AUGCAACUACAAUGCACGUU -3'. SCHL cells were seeded onto 100 mm plates (2×10^6 cells per plate), grown for 24 h to 80 % confluence, and then transfected with 300 pmol miR-33 mimics or NC using Lipofectamine® 2000 Reagent, in triplicate. Total protein was extracted at 48 h post-transfection using cell total protein extraction kit (Sangon Biotech, Shanghai, China) and concentrations quantified with non-interference protein assay kit (Sangon Biotech, Shanghai, China). Next, 30 μL of each sample was loaded and separated on a 12 % SDS/PAGE, transferred onto PVDF membranes, and then incubated with anti-Insig1 rabbit pAb (Wanleibio, Shenyang, China) at 1:500 dilution. Actin level, determined using anti-actin antibody (Beyotime,

225 Haimen, China) at 1:2000 dilution, was used for normalization. After three washes with
226 Tris-Buffered Saline Tween (TBST), membranes were incubated with goat-anti-rabbit
227 and goat-anti-mouse (Millipore, Bedford, MA, USA) secondary antibodies at a ratio of
228 1:15000. Membranes were washed three times with TBST, and the immunoreactive
229 bands visualized using the Odyssey infrared imaging system 2.1 (LI-COR, USA), and
230 analyzed by Image studio (ver 5.2) software with the quantity of Insig1 and Actin
231 protein converted into intensity values.
232
233 **2.6 Overexpression of miR-33 to investigate the functional relationship between**
234 **miR-33 and LC-PUFA biosynthesis-related genes**
235 To further investigate the potential role of miR-33 in the regulation of gene
236 expression in LC-PUFA biosynthesis, miR-33 was up-regulated by transfection with
237 miR-33 mimics. SCHL cells were seeded into six-well plate (5×10^5 cells per well),
238 grown for 24 h to 80 % confluence, and then transfected with 100 pmol miRNA mimics
239 or NC with Lipofectamine® 2000 Reagent, each process consisting of six replicates. At
240 24 h post transfection, SCHL cells were harvested, RNA extracted and subjected to
241 qPCR analysis of expression levels of *insig1*, *srebp1*, *Δ6Δ5 fads2* and *elovl5* mRNA.
242
243 **2.7 Overexpression and inhibition of *insig1* to explore the functional relationship**
244 **between Insig1 and Srebp1**
245 SCHL cells were seeded onto 100 mm plates (2×10^6 cells per plate), grown for 24
246 h to 80 % confluence, and then transfected with 4 μg pcDNA-Insig1 overexpression

plasmid using X-tremeGENE HP DNA Transfection Reagent (Roche, Germany), in triplicate. Silencing of *insig1* expression was performed using small interfering RNA (siRNA) duplexes obtained from Ribobio (Guangzhou, China) with the following sequences: si-insig1 sense, 5'-CAAAGCUGAAGAAAUGAUdTdT-3'; si-insig1 antisense, 5'- AUCAUUUCUUCAGCUUUGGdTdT-3', and SCHL transfected with 50 nM of each siRNA. The *insig1*-specific siRNA (si-insig1) or negative control (si-NC) was performed with Lipofectamine® 2000 Reagent. The cells were harvested at 48 h post transfection and subsequently subjected to Western blotting analysis as described above. In addition, the anti-Srebp1 rabbit pAb was purchased from Wanleibio (Shenyang, China) and used as a dilution of 1:500.

2.8 Promoter analysis to explore the functional relationship between *srebp1*, *Δ6Δ5 fads2* and *elovl5*

To investigate the functional relationship between srebp1 and the promoters of *Δ6Δ5 fads2* and *elovl5*, a dual luciferase assay was performed. The relationship between these constructs was investigated using HEK 293T cells, which were co-transfected with 100 ng pcDNA3.1-SREBP eukaryotic expression vector and 50 ng *Δ6Δ5 fads2* or *elovl5* promoter deletion dual luciferase reporter vectors with Lipofectamine® 2000 Reagent (Invitrogen, Carlsbad, CA, USA). The Renilla reporter vector pGL4.75 plasmid was used as the internal reference. Firefly and Renilla luciferase activities were quantified after 48 h transfection by a microplate reader (Infinite M200 Pro, Tecan, Switzerland) and firefly luciferase activity was normalized to Renilla luciferase activity.

2.9 Inhibition of *srebp1* in rabbitfish hepatocytes

For *srebp1* RNAi in SCHL cells, three pairs of siRNAs, namely siRNA-638, siRNA-1211 and siRNA-1303, were designed (numbers represented the location of the target site of the siRNA on the gene) and synthesized by a commercial company (Genepharma, Shanghai, China). SCHL cells were seeded onto six-well plates with 5×10^5 cells per well, grown for 24 h to 80 % confluence, and then transfected with the siRNAs or NC using the RNAiMAX Reagent (Invitrogen, Carlsbad, CA, USA) in triplicate. At 48 h post transfection, SCHL cells were harvested, RNA extracted and subjected to qPCR determination of the mRNA expression levels of *srebp1*.

2.10 Fatty acid analysis

SCHL cells were seeded onto 100 mm plates at a density of 2×10^6 cells per plate, grown for 24 h to 80 % confluence, and then transfected with 300 pmol miRNA mimics or NC with Lipofectamine® 2000 Reagent triplicates. After 48h incubation, cells were harvested for fatty acid composition analysis by gas chromatography (GC) after chloroform/methanol extraction, saponification and methylation with boron trifluoride (Sigma-Aldrich, USA) as described previously (Li et al., 2010; Chen et al., 2016). For identification, the retention times of the fatty acids were compared to those of standard methyl esters (Sigma-Aldrich, USA), with quantification of each fatty acid in a certain number of cells being estimated using the signal of the internal standard 17:0 (heptadecanoic acid) (Sigma-Aldrich, USA). Fatty acid contents were expressed as

291 percentage of total fatty acids (Table 2).

292

293 **2.11 Statistical analysis**

294 All the data are presented as means ± SEM. The dual-luciferase assay and qPCR
295 expression data were analyzed by one-way analysis of variance (ANOVA) followed by
296 Tukey's multiple comparison test or Student's t-test using Origin 7.0. A significance of
297 $P < 0.05$ was applied to all statistical tests performed.

298

299 # 3. Results

300 **3.1 miR-33 targets to the 3'UTR of *insig1***

301 Bioinformatic analyses showed that potential binding sites of *S. canaliculatus* miR-
302 33 (sca-miR-33) were found in the 3'UTR of *insig1* in rabbitfish (Fig. 1a) (Zhang et al.,
303 2016). Based on this, a dual luciferase assay was used to further verify the interaction
304 between miR-33 and *insig1*. Results from the qPCR analysis revealed that HEK 293T
305 cells transfected with pEGFP-miR-33 had a 140-fold higher level of rabbitfish miR-33
306 expression than the endogenous background of miR-33 ($P < 0.01$) (Fig. 1b). The results
307 of the dual luciferase reporter assay showed that: i) in negative control groups, there
308 was no difference between the pEGFP-empty /pmirGLO-empty co-transfected group
309 (Fig. 1c: lane 1) and the pEGFP-miR-33/pmirGLO-empty co-transfected group (Fig.
310 1c: lane 2); ii) in positive control groups, the pEGFP-miR-33/pmirGLO-R33 co-
311 transfected group (Fig. 1c: lane 4) showed significantly lower normalized Luc activity
312 than the pEGFP-empty/pmirGLO-R33 group (Fig. 1c: lane 3) ($P < 0.01$). If the

heterologous expression of miRNA interacts with the inserted target fragment, the Luc activity would decrease and, therefore, this result proved that the dual luciferase assay report system worked well; iii) in the experimental groups (Fig. 1c: lanes 5-8), the pEGFP-miR-33/pmirGLO-Insig-3'UTR co-transfected group (Fig. 1c: lane 6) showed significantly lower normalized Luc activity than the pEGFP-empty/pmirGLO-Insig-3'UTR group ($P < 0.05$) (Fig. 1c: lanes 5). However, when a mutation is introduced into the predicted binding sites of miR-33 at 3'UTR of *insig1* mRNA, the inhibition was eliminated (Fig. 1c: lanes 7-8).

3.2 Overexpression of miR-33 inhibits the activity of Insig1 protein in SCHL

Western blotting was used to further study the potential relationship between miR-33 and its target protein Insig1. After overexpression of miR-33, the protein level of Insig1 decreased by 40 % compared with the NC group (Fig. 2). It was observed that miR-33 had an inverse expression pattern with Insig1 protein level in rabbitfish.

3.3 Overexpression of miR-33 impacts on *insig1* expression inducing *srebp1* downstream genes including *Δ6Δ5 fads2* and *elovl5*

To further investigate the potential role of miR-33 in regulating the expression of genes involved in LC-PUFA biosynthesis, miR-33 was overexpressed by transfection with miR-33 mimics. At 24 h post-treatment, the expression of *insig1* was inhibited whereas the expression of *srebp1* and *Δ6Δ5 fads2* and *elovl5* were up-regulated, with the expression of *elovl5* significantly increased ($P < 0.05$) (Fig. 3).

3.4 Insig1 can block the formation of mature Srebp1 protein in rabbitfish hepatocytes

In order to provide direct proof for Insig1 being able to block Srebp proteolytic activation, Western blotting was used to further study the potential relationship between Insig1 and Srebp1. The protein levels of mature Srebp1 and Insig1 were detected after overexpression and inhibition of *insig1*. With overexpression of *insig1*, the protein level of Insig1 increased by 1.5-fold, while the protein level of mature Srebp1 decreased by 50 % compared with the pcDNA3.1 group (Fig. 4a). With inhibition of *insig1*, the protein level of Insig1 decreased by 30 %, while the protein level of mature Srebp1 increased by 1.5-fold compared with the si-NC group (Fig. 4b).

3.5 Functional relationship between *srebp1* and *Δ6Δ5 fads2* and *elovl5*.

Our previous studies predicted that the SRE binding element of Srebp protein was present in the promoter regions of *Δ6Δ5 fads2* and *elovl5* in rabbitfish (Fig. 5) (Dong et al., 2018). Thus, the pcDNA3.1-SREBP eukaryotic expression plasmid, *Δ6Δ5 fads2* and *elovl5* promoter deletion dual luciferase reporter vectors were co-transfected into HEK 293T cells. The dual luciferase reporter assay results showed that overexpression of *srebp1* resulted in significantly increased fluorescence activity of *Δ6Δ5 fads2* promoter deletion D2 and D4 ($P < 0.05$ and $P < 0.01$), while the fluorescence activity of D1 and D3 was not significantly different from the control group ($P > 0.05$) (Fig. 6). At the same time, when *srebp1* was overexpressed, the fluorescence activity of *elovl5*

promoter deletion D1, D2 and D3 significantly increased ($P < 0.05$) (Fig. 7).

3.6 *Srebp1* regulates the expression of *Δ6Δ5 fads2* and *elovl5*

In order to knockdown *srebp1*, the silencing efficiency of three pairs of siRNA, namely siRNA-638, siRNA-1211 and siRNA-1303, were determined. The results showed that siRNA-638 and siRNA-1211 had significant knockdown effects on *srebp1* mRNA expression ($P < 0.05$) (Fig. 8), with about 16 % and 18 % knockdown efficiency, respectively, while there was no significant difference between siRNA-1303 and the control group ($P > 0.05$). Thus, siRNA-1211 was used for the RNAi experiments. Following successful *srebp1* knockdown with siRNA-1211 in SCHL cells, the expression levels of *Δ6Δ5 fads2* and *elovl5* were also significantly decreased ($P < 0.05$) (Fig. 9).

3.7 Up-regulation of miR-33 and biosynthesis of LC-PUFA in rabbitfish hepatocytes

The effects of miR-33 on LC-PUFA biosynthesis in SCHL cells was assessed by determining the effects of overexpressing miR-33 on fatty acid composition. It was observed that miR-33 overexpression resulted in a higher conversion of 18:3n-3 to 18:4n-3, and 20:5n-3 to 22:5n-3, as well as higher levels of ARA, EPA and DHA in rabbitfish hepatocytes. Compared with the NC group, the conversion of 18:3n-3 to 18:4n-3 increasing significantly ($P < 0.05$) (Table 2).

4. Discussion

miR-33 is highly conserved in animals and plays a crucial role in the regulation of lipid metabolism, such as reverse cholesterol transport and fatty acid oxidation (Gerin et al. 2010; Horie et al. 2010; Najafi-Shoushtari et al. 2010; Rayner et al. 2010). We reported previously that miR-33 has a potential role in the regulation of LC-PUFA biosynthesis in fish (Zhang et al. 2016b). Hence, the present study aimed to further explore the mechanisms underpinning this finding. In our previous research, we found that overexpression of miR-33 up-regulated the transcription of *Δ4 fads2* and *Δ6Δ5 fads2*, but suppressed the expression of *insig1*. In mammals, INSIG1 could bind to the sterol-sensing domain of SCAP (SREBP cleavage activating protein), which makes the SCAP/SREBP complex reside longer in the ER, ultimately blocking SREBP entry into the nucleus and preventing its action as a transcription factor (Gong et al. 2006; Engelking et al. 2004). Accordingly, it was necessary to demonstrate the direct effect of miR-33 on *insig1*, including whether Insig1 can block Srebp proteolytic activation, and whether Srebp1 directly up-regulates the expression of genes involved in LC-PUFA biosynthesis. We herein report compelling evidence corresponding to each of these points.

Our data confirmed that miR-33 down-regulated the protein abundance of Insig1 in rabbitfish through direct targeting of the 3'UTR of *insig1*. Generally, miRNAs depend on the "seed sequence" to identify and partially combine with the 3'UTR of target genes, thereby inducing target mRNA degradation or inhibiting protein translation, manifested by the reduction of function or activity of target genes. The dual

401 luciferase assay revealed that miR-33 could repress the 3'UTR luciferase activity of
402 *insig1*, which provided evidence that *insig1* was a direct target gene of miR-33. In
403 rabbitfish hepatocytes, the overexpression of miR-33 decreased the protein abundance
404 of Insig1, therefore indicating that a negative regulation of miR-33 occurred at the
405 translational level, in agreement with the general mechanism of animal miRNA (Bartel
406 2009; Pillai et al. 2005).

407 Lowering the protein abundance of Insig1 by miR-33 indirectly increased the level
408 of Srebp1 mature protein because the inhibitory effect of Insig on Srebp activation was
409 reduced. Our previous studies showed that, in SCHL cells, miR-24 enhanced the
410 expression of *srebp1* mRNA and the production of mature Srebp1 protein by targeting
411 *insig1*, while opposite results were observed with knockdown of miR-24 in rabbitfish
412 hepatocytes suggesting that *insig1* may inhibit the formation of mature Srebp1 (Chen
413 et al. 2019). In the present study, we confirmed Insig1 can block Srebp proteolytic
414 activation, with overexpression of *insig1* decreasing abundance of mature Srebp1
415 protein, while inhibition of *insig1* leads to an increase in abundance of mature Srebp1
416 protein. This suggested that Insig proteins dissociated from the Insig-SCAP-SREBP
417 complex, so that the SCAP-SREBP complex could transfer to the Golgi (Gong et al.
418 2006; Engelking et al. 2004). It is reported that, under the shear processing of S1P and
419 S2P, the mature protein of Srebp1 is formed, which then enters the nucleus and exerts
420 its regulatory functions (Yang et al. 2002; Yabe et al. 2003). In rabbitfish hepatocytes,
421 overexpression of miR-33 decreased the protein abundance of Insig1, coupled with
422 decreased mRNA expression of *insig1* and increased expression of *srebp1* mRNA. With

423 an increase in the mRNA expression level of *srebp1*, there would be a corresponding
424 increase in the protein abundance of Srebp1. It is known that miR-33 is found within
425 an intron of the *srebp* gene in both mammals and teleosts (Horie et al. 2013; Zhang et
426 al. 2016). In rabbitfish, increased transcription of *srebp1* elevated the abundance of
427 miR-33 in primary hepatocytes (Zhang et al. 2016b). This was interesting as it suggests
428 that transcription of *srebp1* helps to bring about self-proteolysis. In addition, it was
429 observed in the present study that, when miR-33 was overexpressed, the level of *srebp1*
430 also increased. The question here is, how did miR-33 enhance the expression of *srebp1*
431 to participate in LC-PUFA biosynthesis? Research in mice has shown that *srebp1c* can
432 promote the expression of the *Δ5* and *Δ6 fads-like* genes (Qin et al. 2009). Similarly, it
433 has been reported that Srebp1 can be activated by TO901317, a liver X receptor (*lxr*)
434 agonist, that activates the Lxr-Srebp1 pathway, and further promotes the expression of
435 Δ5 and Δ6 *fads* in Atlantic salmon (*Salmo salar*) (Minghetti et al. 2011). Studies have
436 also found that SRE elements are present in the human *FADS2* (Δ6) promoter (Nara et
437 al. 2002). In Atlantic salmon, Atlantic cod (*Gadus morhua* L.) and zebrafish (*Danio*
438 *rerio*), SRE elements have been predicted in the *Δ6 fads2* promoter (Zheng et al. 2009).
439 In rabbitfish, our previous studies characterized the upstream promoter sequences of
440 *Δ6Δ5 fads2* and *elovl5* genes (Dong et al. 2018) and predicted SRE elements in the
441 upstream promoter sequences. These data indicated that *srebp1* might participate in the
442 regulation of LC-PUFA biosynthesis by regulating *Δ6Δ5 fads2* and *elovl5*. When further
443 exploring the functional relationship between *srebp1* and the promoters of *Δ6Δ5 fads2*
444 and *elovl5*, dual luciferase reporter assays showed that overexpression of *srebp1*

445 significantly enhanced the fluorescence activity of *Δ6Δ5 fads2* and *elovl5* promoter
446 containing SRE element. These results demonstrated that *srebp1* can activate the
447 expression of the rabbitfish *Δ6Δ5 fads2* and *elovl5* depending on the SRE element. We
448 previously revealed that *lxr* agonist (TO901317) could significantly increase the mRNA
449 expression level of *srebp1*, together with an increase in the expression of *fads2* in
450 rabbitfish (Zhang et al. 2016a). Here, when the expression of *srebp1* was depleted by
451 RNAi, the expression levels of *Δ6Δ5 fads2* and *elovl5* also decreased. Therefore, miR-
452 33 enhances LC-PUFA biosynthesis by up-regulating srebp1, which acting on *Δ6Δ5*
453 *fads2* and *elovl5* in rabbitfish.

454 Analysis of the differences in the fatty acid composition reflects the ability of C_{18}
455 PUFA to be converted into LC-PUFA through the desaturation and elongation pathway
456 (Brown 2005). We observed that overexpression of miR-33 resulted in higher
457 production of $C_{20\text{-}22}$ LC-PUFA compared to the NC group, as the level of ARA, EPA
458 and DHA increased in rabbitfish hepatocytes. These findings indicated that miR-33
459 promoted LC-PUFA biosynthesis. Moreover, overexpression of miR-33 increased the
460 conversion of 18:3n-3 to 18:4n-3 and 20:5n-3 to 22:5n-3. Specifically for rabbitfish,
461 the conversion of 18:3n-3 to 18:4n-3 requires the action of the Δ6Δ5 Fads2, while the
462 conversion of 20:5n-3 to 22:5n-3 requires that of Elovl5, hence, these results suggested
463 an increase in the enzymatic activities of Δ6/Δ5 Fads2 and Elovl5. These results have
464 therefore shown that miR-33 promotes LC-PUFA biosynthesis at the physiological
465 level, which might be achieved by regulating the Δ6Δ5 Fads2 and Elovl5, key enzymes
466 in LC-PUFA biosynthesis.

In summary, we propose a putative mechanism (Fig. 10), suggesting that miR-33 inhibits the translation of Insig1 protein, thereby indirectly increasing the level of mature Srebp1 protein. Since Srebp1 upregulates the expression of key enzymes in LC-PUFA biosynthesis (i.e. Δ6Δ5 Fads2 and Elovl5), so miR-33 can increase the activity of LC-PUFA biosynthesis in rabbitfish. As miR-33 is expressed with transcription of *srebp1*, the interaction between Srebp1 and miR-33 might be a self-promoting mechanism, which helps to enhance the function of Srebp1.

Acknowledgements

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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642 muscle of Chinese perch (*Siniperca chuatsi*). Mar Biotechnol (NY) 17(2): 180-189.
643

644 **Table 1.** Primers or oligonucleotides used for vector reconstruction or qPCR.

Aim	Gene/Vector name	Primers/Oligonucleotides	Nucleotide sequence
Construction of reporter vectors	pEGFP-miR-33	pEG-pmiR-33-F	CGGAATTCTAGATAACTGAAGGTATTATTCAGCTGAGTGGA
		pEG-pmiR-33-R	CGGGATCCTATATAATCAACTTCCACACTAAAG
	PGLO-Insig -3'UTR	Insig -3'UTR-F	CCCGGGTCTAGAAACTGCTAAGACTATGGCTAATGCTA
		Insig -3'UTR-R	CCCGGGGAGCTCCTTTTACAACACCTGAAACATATCTC
	PGLO-Insig -3'UTR-MU	Insig -3'UTR-MU-F	CAAGGTTGATGGTAAGGAGAGGGACAGGAA
		Insig-3'UTR-MU-R	TTCCTGTCCCTCTCCTTACCATCAACCTTG
	pmirGLO-R33	pmirGLO-R33-F	CACATGCAATGCAACTACAATGCACCACAGT
		pmirGLO-R33-R	CTAGACTGTGGTGCATTGTAGTTGCATTGCATGTGAGCT
	pcDNA3.1-SREBP	LG-SREBP-F	GCTCTAGAGAAAGATGAATAGCCTGG
		LG-SREBP-R	GGGAAGCTTGCCTAGCTGTTGGTGACC
	pcDNA-Insig1	pcDNA-Insig1-F	CAGGAATTCATGTCCAGACTAGAGGATCA
		pcDNA-Insig1-R	GAACTCGAGTCAGTCCATGTGGGGCTTCTCGA
QPCR	miR-33	qPCR-miR-33	CGTGCATTGTAGTTGCATTG
	Δ6Δ5 fads2	*Δ6△5fads2* -F	TCACTGGAACCTGCCCACAT
		Δ6△5fads2 -R	TTCATTCTCAGACAGTGCAAACAG
	elovl5	*elovl5*-F	GCACTCACCGTTGTGTATCT
		elovl5-R	GCAGAGCCAAGCTCATAGAA
	insig1	Insig -1-F	AGTTACGCCGCCTGAGAAGA
		Insig -1-R	TGGGCATCACCACTGCTTTC
	srebp1	Srebp1 -F	CGGAGCCAAGACAGAGGAGTG
		Srebp1 -R	GTCTCCCAGCTTCTCCAAGGTAC
	18S	18S-F	CGCCGAGAAGACGATCAAAC
		18S-R	TGATCCTTCCGCAGGTTCAC

645 Notes:The underscore in the table indicates the restriction site in primers.

646 **Table 2.** Fatty acid composition (% total fatty acid) in rabbitfish hepatocytes transfected with
647 miR-33 mimics and negative control (NC) mimics

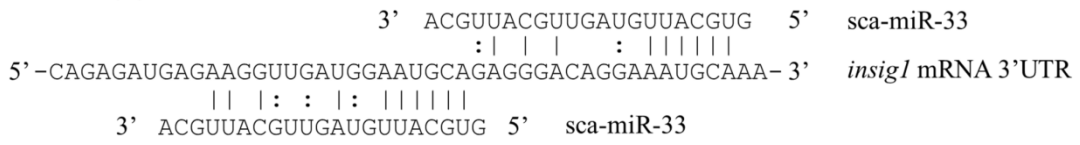
Fatty acids composition	Treatments	
	NC	miR-33 Mimic
C14:0	0.45±0.13	0.55±0.06
C16:0	8.89±0.77	10.49±0.79
C18:0	7.11±1.07	9.05±0.81
C18:1n-9	12.52±1.08	13.79±1.81
C18:2n-6	2.22±0.16	2.49±0.34
C18:3n-6	0.73±0.05	0.73±0.21
C18:3n-3	0.54±0.07	0.39±0.02
C18:4n-3	0.49±0.02	0.56±0.05
C20:2n-6	0.78±0.13	0.78±0.13
C20:4n-6(ARA)	2.24±0.43	2.70±0.48
C20:3n-3	0.31±0.07	0.33±0.02
C20:5n-3(EPA)	1.69±0.11	1.72±0.25
C22:4n-6	0.26±0.02	0.29±0.06
C22:5n-3	1.75±0.26	1.95±0.36
C22:6n-3(DHA)	7.11±0.94	7.76±1.23
∑SFA	16.45±1.83	20.08±1.60
∑MUFA	12.52±1.08	13.79±1.81
∑LC-PUFA	13.36±1.79	14.75±2.18
C18:4n-3/C18:3n-3 (Δ6Δ5 Fads2)	0.94±0.09[a]	1.42±0.01[b]
C22:5n-3/C20:5n-3 (Elovl5)	1.00±0.06	1.12±0.09

648 Notes: Data are means ± SEM (n = 3), different superscript letters at the same line represent higher
649 significance to each other ($P < 0.05$; t-test). SFA: saturated fatty acids; MUFA: monounsaturated
650 fatty acid; LC-PUFA: long-chain polyunsaturated fatty acid.
651

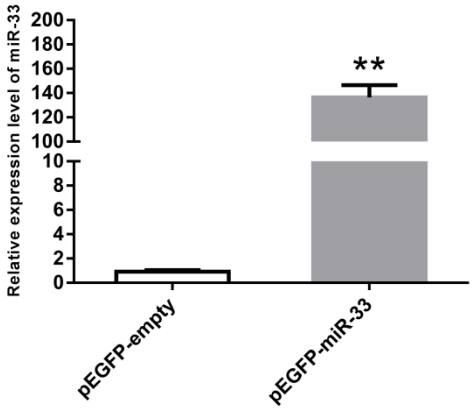
Figures

Fig. 1

(a)



(b)



(c)

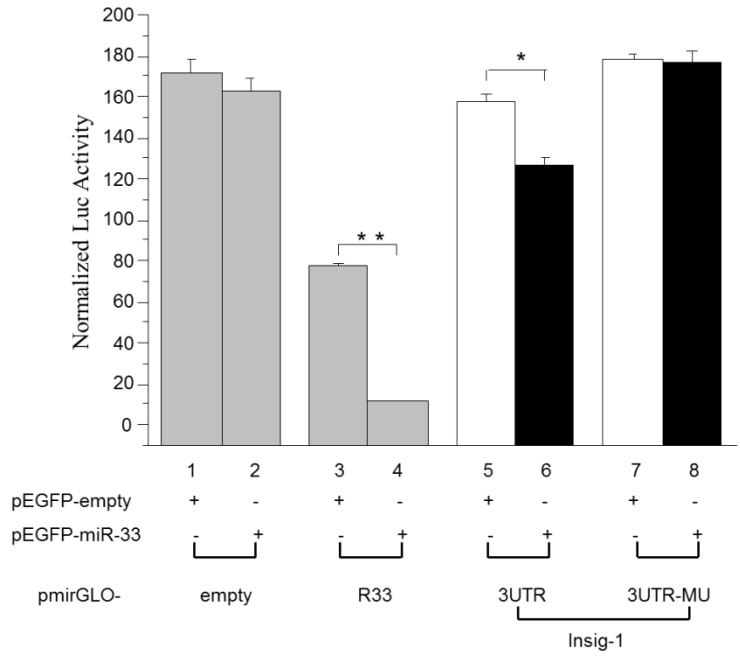


Fig. 1 (**a**) Scheme of miR-33 base pairing the 3’UTR of the rabbitfish *insig1*. (**b**) Rabbitfish miR-33 is over-expressed in HEK 293T cells by transfecting with pEGFP-miR-33. (**c**) Luciferase activity in HEK 293T cells co-transfected with pEGFP-miR-33 or pEGFP-empty with different recombinant dual luciferase reporter vectors: pmirGLO-empty as negative control (lanes 1-2); pmirGLO-R33 as

positive control (lanes 3-4); pmirGLO-Insig-3'UTR containing 3'UTR of *insig1* (lanes 5-6); pmirGLO-Insig-3'UTR-MU with 4 nt site-directed mutation in 3'UTR of *insig1* (lanes 7-8). The Renilla luciferase activity was used to normalize that of firefly luciferase. Data are shown as means ± SEM (n = 8) and asterisks represent significant differences (**P* < 0.05; ***P* < 0.01; t-test)

Fig. 2

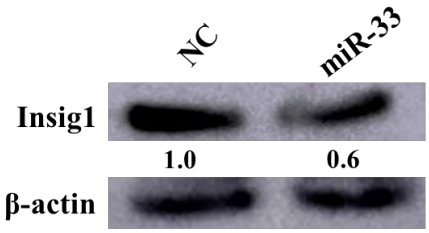


Fig. 2 miR-33 is a negative regulator of rabbitfish Insig1 at the protein level. The SCHL cells were transfected with miR-33 or NC mimics, western blotting detected protein expression levels of Insig1 and normalized by Actin. The numbers on the graph refer to the grey value ratio of the target gene to the reference gene, and the value indicated the relative expression level of the protein.

Fig. 3

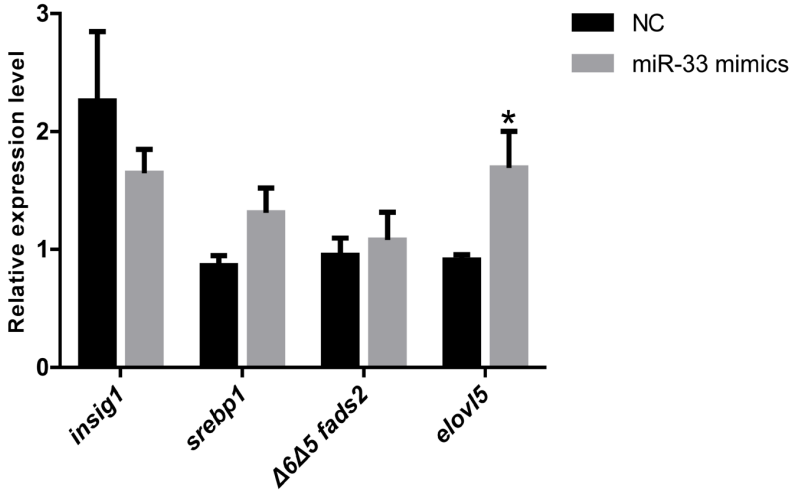
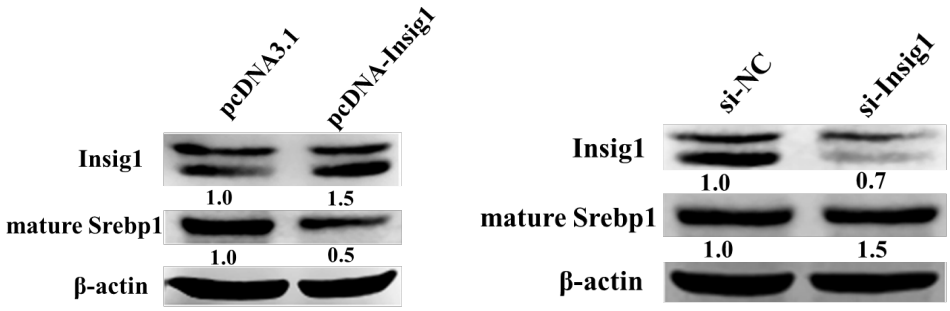


Fig. 3 Effects of miR-33 overexpression on the mRNA level of *insig1*, *srebp1*, *Δ6Δ5 fads2* and *elovl5* in SCHL cells. The gene expression was determined by qPCR in SCHL cells transfected with miR-33 mimics or NC mimics for 24 h. Data are means ± SEM (n = 6). Asterisks represent significant differences (*P* < 0.05; ANOVA, Tukey's test)

Fig. 4

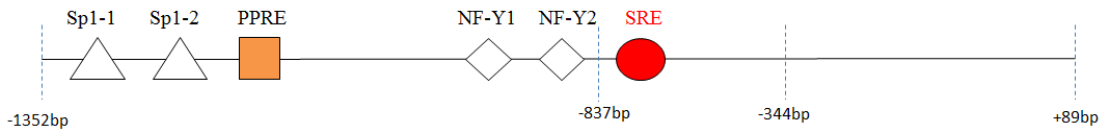


(a) **(b)**

Fig. 4 (**a**) Effects of *insig1* overexpression on the protein level of Insig1 and Srebp1. (**b**) Effects of *insig1* inhibition on the protein level of Insig1 and Srebp1. The numbers on the graph refer to the grey value ratio of the target gene to the reference gene, and the value indicated the relative expression level of the protein.

Fig. 5

(a)



(b)

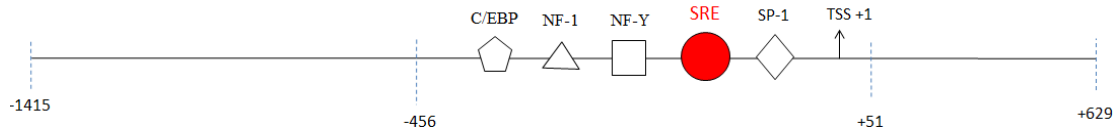
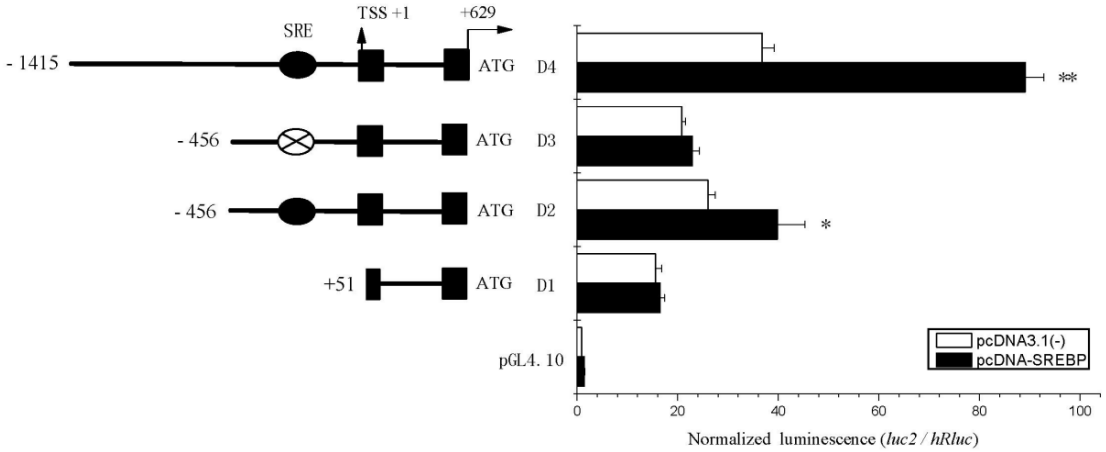


Fig. 5 Bioinformatics predicted the binding elements of the upstream promoter sequence of *elovl5* (**a**) and *Δ6Δ5 fads2* (**b**), where SRE was the acting element of *srebp1*

Fig. 6

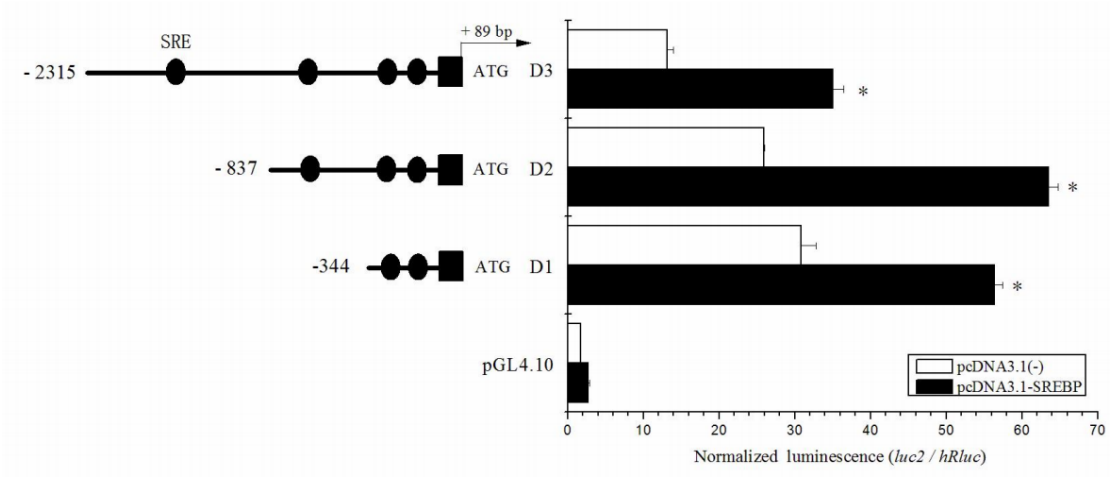


706

707 **Fig. 6** Effects of *srebp1* over-expression on activity of *Δ6Δ5 fads2* promoter deletion. Here is a dual-
708 luciferase report experiment in HEK 293T cells, with 0.04 ng pGL4.75 plasmid as the internal
709 reference, and pGL4.10 as the negative control. The black ellipse in the figure represents the position
710 of the SRE element predicted on the promoter sequence. Data are shown as means ± SEM and
711 asterisks represent significant differences (*$P < 0.05$; **$P < 0.01$; t-test)

712

713 **Fig. 7**



714

715 **Fig. 7** Effects of *srebp1* over-expression on activity of *elovl5* promoter deletion. Here is a dual-
716 luciferase report experiment in HEK 293T cells, with 0.04 ng pGL4.75 plasmid as the internal
717 reference, and pGL4.10 as the negative control. The black ellipse in the figure represents the position
718 of the SRE element predicted on the promoter sequence. Data are shown as means ± SEM and
719 asterisks represent significant differences (*$P < 0.05$; **$P < 0.01$; t-test)

720

721 **Fig. 8**

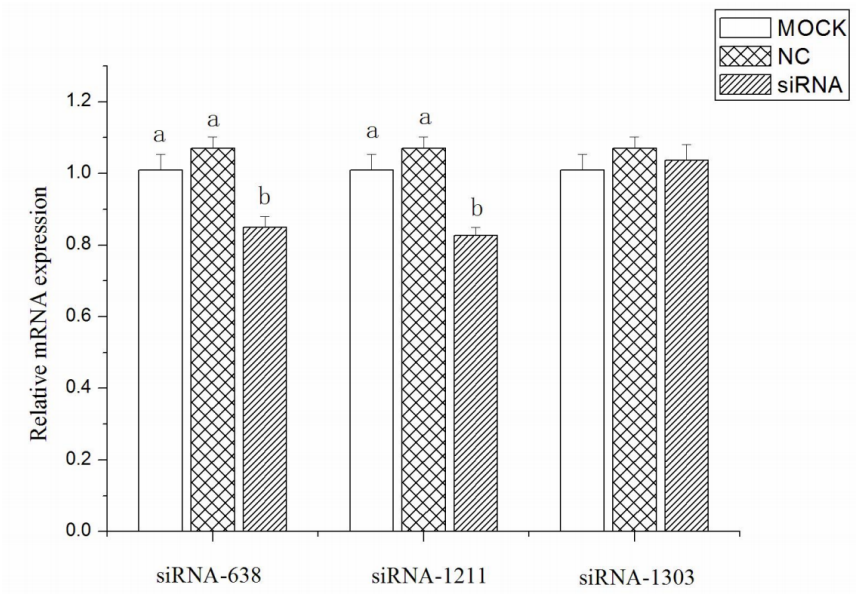


Fig. 8 Relative expression of *srebp1* after silenced by different siRNAs. Mock means adding only transfection reagent groups; NC means adding siRNA negative control group; SiRNA was the experimental group. Data are means ± SEM (n = 3) and different superscripts indicate significant differences ($P < 0.05$)

Fig. 9

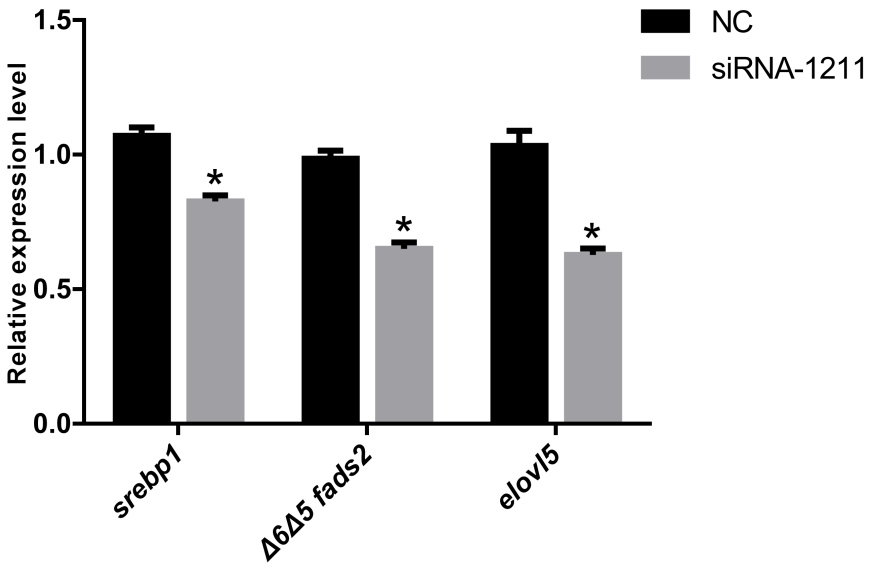
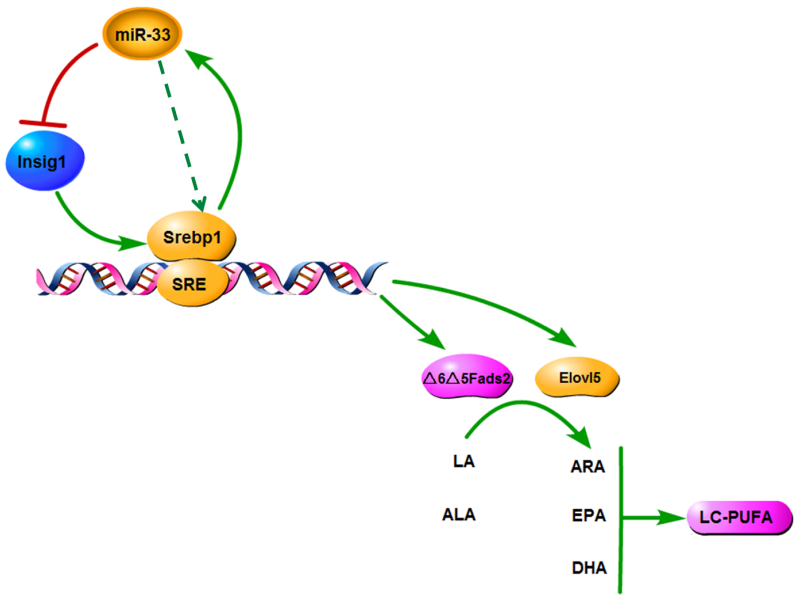


Fig. 9 Relative expression of *srebp1*, *Δ6Δ5 fads2*, *elovl5* gene after silenced by siRNA-1211 in SCHL cells. NC means adding siRNA negative control group; SiRNA-1211 was the experimental group. Data are means ± SEM (n = 3) and asterisks represent significant differences ($P < 0.05$; ANOVA, Tukey's test)

736 **Fig.10**

737



738

739 **Fig. 10** Diagrammatic representation of the roles of miR-33 in regulation of LC-PUFA biosynthesis.
740 The putative mechanism diagram is proposed for effects of miR-33 overexpression on target genes,
741 host genes and LC-PUFA biosynthesis related genes. Red arrows denote suppression; green arrows
742 indicate promotion

743