

Novel insights for SREBP-1 as a key transcription factor in regulating lipogenesis in a freshwater teleost, grass carp *Ctenopharyngodon idella*

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Abstract

Disturbances in lipid metabolism are at the core of several health issues facing modern society, including fatty liver and obesity. The sterol regulatory element-binding protein 1 (SREBP-1) is one important transcription factor regulating lipid metabolism, but the relevant mechanism still remains unknown. The present study determined the transcriptional regulation of SREBP-1 and its target genes (including acetyl-CoA carboxylase α (*acc α*), fatty acid synthase (*fas*) and stearoyl-CoA desaturase 1 (*scd1*)) in a freshwater teleost, grass carp *Ctenopharyngodon idella*. We cloned and characterised the 1988 bp, 2043 bp, 1632 bp and 1889 bp sequences of *srebp-1*, *acc α* , *scd1* and *fas* promoters, respectively. A cluster of putative binding sites of transcription factors, such as specific protein, yin yang 1, nuclear factor Y, sterol response elements (SRE) and enhancer box (E-box) element, were predicted on their promoter regions. Overexpression of nSREBP-1 reduced *srebp-1* promoter activity, increased *scd1* and *fas* promoter activity but did not influence *acc α* promoter activity. The site-mutation and electrophoretic mobility shift assay analysis indicated that *srebp-1*, *fas* and *scd1* promoters, but not *acc α* promoter, possessed SRE. In *Ctenopharyngodon idella* kidney (CIK) cells of grass carp, nSREBP-1 overexpression significantly reduced *srebp-1* mRNA expression and up-regulated miR-29 mRNA expression. The 3'UTR of *srebp-1* possessed the potential miR-29 binding site and miR-29 up-regulated the luciferase activity of *srebp-1* 3'UTR and *srebp-1* mRNA expression, implying a self-activating loop of SREBP-1 and miR-29 in grass carp. Based on the above-mentioned results, we found two novel transcriptional mechanisms for SREBP-1 in grass carp: (1) the auto-regulation sited on the SREBP-1 promoter regions was suppressive and (2) there was a self-activating loop of SREBP-1 and miR-29.

Key words: *Ctenopharyngodon idella*: Sterol regulatory element binding proteins: Promoter analysis: microRNA: Lipid metabolism

Deregulated lipid metabolism is an established hallmark of many diseases, such as fatty liver and obesity. Lipid metabolism is tightly regulated by sterol regulatory element-binding protein 1 (SREBP-1), an important transcriptional factor that regulates the transcription of most genes involved in lipogenesis⁽¹⁾. To date, two forms of mammalian SREBP-1 have been characterised, such as SREBP-1a and -1c. However, only a single form of the *SREBP-1* gene has been characterised in fish^(2,3). The SREBPs are synthesised as precursor proteins bound to the endoplasmic reticulum membrane⁽⁴⁾. After stimulation, the SREBP precursor undergoes proteolytic cleavage in the Golgi to release the transcriptionally active N-terminal domain (nSREBP). Once

mature, the active SREBP1 translocates to the nucleus where it binds to sterol response elements (SRE) in the promoter regions of target genes to modulate their transcription^(1,4,5).

The target genes of SREBPs included SREBP itself, acetyl-CoA carboxylase α (ACC α), fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD1)^(6,7). ACC α catalyses the ATP-dependent carboxylation response of acetyl-CoA to form malonyl-CoA, and FAS catalyses the condensation of acetyl-CoA and malonyl-CoA to generate long-chain fatty acids⁽⁸⁾. SCD1 catalyses the synthesis of long-chain unsaturated fatty acids. At present, the promoter regions of these genes have been partially isolated and characterised in mammals^(9–11). Meantime, Griffin *et al.* pointed out that

Abbreviations: ACC α , acetyl-CoA carboxylase α ; cDNA, complementary DNA; CIK, *Ctenopharyngodon idella* kidney; DMEM, Dulbecco's modified Eagle's medium; E-box, enhancer box; EMSA, electrophoretic mobility shift assay; FAS, fatty acid synthase; FBS, fetal bovine serum; miR, microRNA; NF-Y, nuclear factor Y; SCD1, stearoyl-CoA desaturase 1; SP, specific protein; SRE, sterol response elements; SREBP-1, sterol regulatory element-binding protein 1; UTR, untranslated region; WT, wild type; YY1, yin yang 1.

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their regulation occurs mainly at the transcriptional level⁽¹²⁾. However, in fish, no reports investigated the mechanism of SREBP-1 regulating its target genes related to lipid metabolism.

On the other hand, the mechanisms involved in the activation of the SREBPs have been investigated in mammals^(1,13), but not in fish. MicroRNA (miR) are a class of small non-coding RNAs that regulate gene expression post-transcriptionally by binding to the complementary regions in the 3'UTR of target mRNAs, resulting in mRNA degradation or attenuated translation⁽¹⁴⁾. Studies have shown that miR play a role in TAG homeostasis⁽¹⁵⁾. Recently, Ru *et al.* reported that SREBP-1 can activate miR-29 expression and in turn miR-29 inhibited *srebp-1* expression, which unravelled a negative feedback loop mediated by miR-29 in SREBP-1 signalling⁽¹⁶⁾. However, in fish, no reports explore the molecular mechanism of SREBP regulation by miR-29.

Fish are by far the largest group of vertebrates in the world. Grass carp (*Ctenopharyngodon idella*) was an important herbivorous freshwater fish widely farmed all over the world. In some countries of European and Northern America, grass carp were widely used to control aquatic plants because of their aggressive feeding on vegetation⁽¹⁷⁾. Recently, the draft genome of the grass carp has been released, which is considered a convenient tool for identifying genomic structure of genes involved in lipid metabolism⁽¹⁸⁾. The present study was conducted to identify the promoter regions of *acca*, *fas*, *scd1* and *srebp-1* and investigate the functions of SRE on their promoter regions. To gain insight into the distinct roles of SREBP-1, overexpressing truncated, active nuclear forms of grass carp SREBP-1 were produced and characterised. The post-transcriptional regulation of *srebp-1* by miR-29 was explored. Our study offers innovative insights into the regulatory mechanism of SREBP1 and provides direct evidence for SREBP-1 regulating itself and its downstream lipogenic genes in fish.

Materials and methods

Animals, cells and media

Juvenile grass carp were purchased from a commercial farm and used for DNA and RNA extraction. HepG2 and grass carp *Ctenopharyngodon idella* kidney (CIK) cell lines were obtained from our Cell Resource Center in Huazhong Agricultural University. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (0.1 mg/ml) in an incubator at 37°C with 5% CO₂. Grass carp CIK cells were cultured in Medium 199 (M199) containing 10% FBS, penicillin (100 U/ml) and streptomycin (0.1 mg/ml) in an incubator at 28°C with 5% CO₂. All these culture media, 0.25% trypsin-EDTA and FBS were purchased from Gibco (Thermo Fisher Scientific). Penicillin and streptomycin were purchased from Sigma-Aldrich. The present study was performed in accordance with the relevant institutional and national guidelines, and the manuscript must conform to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. The ethics committee of Huazhong Agricultural University checked and approved our present experimental protocols on animals and cells.

Cloning and plasmids construction

We identified the 5' complementary DNA (cDNA) sequences and the transcription start sites of *srebp-1*, *acca*, *scd1* and *fas* of grass carp using RNA ligase-mediated rapid amplification of 5' cDNA ends (RLM-5'RACE) method. The promoter cloning was performed based on the published draft genome of grass carp⁽¹⁸⁾, and the protocols followed these described in our recent studies⁽¹⁹⁾. Genomic DNA was extracted from grass carp tail fins using a commercial kit (Tissue DNA Kit; Omega). The promoter sequences of *srebp-1*, *acca*, *scd1* and *fas* were amplified from extracted DNA using PCR and sub-cloned into pGL3-basic vector (Promega) using ClonExpress™ II One Step Cloning Kit (Vazyme), and the PCR were performed using TaKaRa PrimeSTAR® HS DNA Polymerase kit (TaKaRa). Based on the distance from their transcription start sites, we named the *srebp1*-1998 plasmid for -1998/+59 *srebp-1* promoter, *acca*-2043 plasmid for -2043/+49 *acca* promoter, *scd1*-1632 plasmid for -1632/+57 *scd1* promoter and *fas*-1889 plasmid for -1889/+111 *fas* promoter, respectively. Plasmids of *srebp1*-1493, *srebp1*-1098, *srebp1*-604, *acca*-1538, *acca*-1069, *acca*-517, *scd1*-1160, *scd1*-602, *scd1*-273, *fas*-1447, *fas*-1007 and *fas*-476, which contained unidirectional deletions of the promoter regions, were generated with the Erase-a-Base system (Promega) using templates of *srebp1*-1998, *acca*-2043, *scd1*-1632 and *fas*-1889 plasmid, respectively.

Total RNA was extracted from hepatic samples of grass carp using TRIzol reagent (Invitrogen) and reverse transcribed to cDNA as a template for constructing the nSREBP-1 expression plasmid and the 3'UTR of *srebp-1* plasmid. The open reading frame sequence encoding nSREBP-1 was amplified from cDNA using PCR and sub-cloned into pcDNA3.1 (+) vector with the DYKDDDDK peptide (FLAG-tag) sequence inserted at the C-terminus of *nsrebp-1* sequence using ClonExpress™ II One Step Cloning kit (Vazyme) and named as nSREBP-1 plasmid. For constructing 3'UTR of *srebp-1* plasmid, the 3'UTR sequence of *srebp-1* was amplified and sub-cloned into pmirGLO vector using ClonExpress™ II One Step Cloning Kit (Vazyme) and named as pmirGLO-*srebp1*. All the primers were sequenced in a commercial company (Tsingke) and listed in online Supplementary Table S1.

Sequence analysis

Nucleotide sequences of *srebp-1*, *acca*, *scd1* and *fas* promoters were compared with DNA sequences presented in the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>) and the UCSC Genome Browser (<http://genome.ucsc.edu/>). For sequence analysis of the promoters of *acca*, *fas*, *scd1* and *srebp-1* from grass carp, putative transcription factor binding sites were predicted by online software MatInspector (<http://www.genomatix.de/>). Besides, SRE with relative score over 0.9 based on JASPAR database (<http://jaspar.genereg.net/>) were also considered as potential binding sites.

mRNA and protein expression of grass carp nSREBP-1 in HepG2 cells

For expression of nSREBP-1 plasmid group, HepG2 cells were counted and seeded at a density of 1×10^6 in a 60-mm culture

dish and then were cultured until 80–90 % confluence before transfecting with 8 µg of nSREBP-1 plasmid using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instruction. For the control, the same amount of pcDNA3.1(+) was transfected into HepG2 cells. Then cells were harvested at 12-, 24- and 48-h incubation, respectively. Total RNAs from transfected HepG2 were extracted and reverse transcribed to cDNA with equal quantities of each total RNA (1 µg) as templates for real-time quantitative PCR (Q-PCR). The resulting first-strand cDNA was diluted 1:10 with ddH₂O before use. Q-PCR was performed using the SYBR Premix Ex Taq™ II kit (TaKaRa) in a quantitative thermal cycler (BIO-RAD). A set of six common housekeeping genes (*β-actin*, *18s-rRNA*, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), elongation factor 1-α (*ef1a*), hypoxanthine-guanine phosphoribosyl transferase (*hprt*) and β-2-microglobulin (*b2m*)) were selected in order to test their transcription stability. Two most stable housekeeping genes were selected by geNorm software⁽²⁰⁾. The relative expression levels were calculated with the delta–delta Ct method⁽²¹⁾, when normalising to the geometric mean of the best combination of two housekeeping genes as suggested by geNorm. To confirm amplification specificity, the PCR products from each sample were examined by melting curve analysis. All experiments were performed in triplicates. These gene-specific primers for each gene are listed in online Supplementary Table S2.

Proteins for Western blot were extracted from transfected HepG2 cells using RIPA lysis and extraction buffer (Thermo Fisher Scientific), and the protocols followed these described in our recent studies⁽²²⁾. Protein concentration was measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific). About 30 µg total protein per lane was loaded on SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocked with 8 % skimmed milk for 1 h, the membranes were incubated with primary antibody at 4°C overnight. The primary antibodies used in the present study were rabbit polyclonal of anti-GAPDH (1:2000; Abcam) and anti-FLAG (1:1000; Proteintech). After washing for five times with Tris-buffered saline-Tween, the membrane was probed with horseradish peroxidase-conjugated anti-rabbit IgG (1:10000; Cell Signaling Technology) for 1 h at room temperature. The protein bands were visualised by Vilber Fusion FX6 Spectra imaging system (Vilber Lourmat) and quantified by Image-Pro Plus (Media Cybernetics).

Luciferase assay of *srebp-1*, *acca*, *scd1* and *fas* promoters

For promoter luciferase assays, HepG2 cells were counted and seeded at a density of 1×10^5 in twenty-four well plates, then cultured and transfected as mentioned in our recent studies^(19,23). Briefly, to study the nSREBP-1-induced changes in promoter activities, we co-transfected 300 ng of nSREBP-1 plasmid or the same amount of pcDNA3.1(+) plasmid (300 ng, control) with 500 ng of each of these luciferase reporter plasmids of *srebp-1*, *acca*, *scd1* and *fas* promoters into HepG2 cells using Lipofectamine 2000 (Invitrogen) at 80–90 % confluence, respectively. For an internal control, 25 ng of *Renilla* luciferase vector the thymidine kinase promoter-*Renilla* luciferase reporter plasmid (pRL-TK) per well was included in all transfections to

normalise transfection efficiency. At 6 h after the transfection, the cells were incubated with the fresh DMEM containing 10 % FBS for 24 h. Then the relative luciferase activity of *srebp-1*, *acca*, *scd1* and *fas* promoters was measured using the Dual-luciferase Reporter Assay System (Promega), according to the manufacturer's instruction. The relative luciferase activity of these promoters was calculated using the ratio of *Firefly* luciferase activity:*Renilla* luciferase activity. All experiments were performed in triplicates.

Site-mutation assay of sterol response elements sites on the *srebp-1*, *acca*, *scd1* and *fas* promoters

To identify the corresponding SRE on the grass carp *srebp-1*, *acca*, *scd1* and *fas* promoters, we performed site-directed mutagenesis of SRE on the plasmid of *srebp-1*, *acca*, *scd1* and *fas* promoters, respectively, according to the manufacturer's instruction of QuickChange II Site-Directed Mutagenesis kit (Vazyme). SRE-mutated plasmids of *srebp-sre1*, *acca-sre1*, *scd1-sre1*, *scd1-sre2*, *fas-sre1*, *fas-sre2*, *fas-sre3*, *fas-sre4* and *fas-sre3&4* were generated from the wild-type (WT) plasmids of *srebp1-1998*, *acca-2043*, *scd1-1632* and *fas-1889*, respectively. Similar to the promoter luciferase assay performed previously, 300 ng of nSREBP-1 plasmid or the same amount of pcDNA3.1(+) plasmid (300 ng, control) was co-transfected with 500 ng of WT plasmid or the same amount of SRE-mutated plasmid into HepG2 cells using Lipofectamine 2000 (Invitrogen) at 80–90 % confluence. For an internal control, 25 ng of *Renilla* luciferase vector (pRL-TK) per well was included in all transfections to normalise the transfection efficiency. At 6 h after the transfection, the cells were incubated with the same fresh DMEM containing 10 % FBS for 24 h. Then the cells were harvested and the relative luciferase activity was measured using the Dual-luciferase Reporter Assay System (Promega), according to the manufacturer's instruction. The relative luciferase activity of these promoters was calculated using the ratio of *Firefly* luciferase activity:*Renilla* luciferase activity. All experiments were performed in triplicates.

Electrophoretic mobility shift assay

An electrophoretic mobility shift assay (EMSA) was performed to confirm the functional SRE of the promoters. HepG2 cells were transfected with nSREBP-1 plasmid as described above. Nuclear proteins for EMSA were extracted from HepG2 cells, and concentrations were determined using Pierce BCA protein assay kit (Thermo Fisher Scientific). These extracts were stored at –80°C until analysed. Each oligonucleotide duplex of SRE was incubated with 5 µg nuclear extracts at room temperature, according to the instruction of LightShift™ Chemiluminescent EMSA kit (Invitrogen), and each unlabelled probe was pre-incubated 10 min prior to the addition of biotin-labelled probe. The reaction was allowed to proceed for 20 min after the addition of biotin-labelled probe at room temperature and then were detected by electrophoresis on 6 % native polyacrylamide gels. Competition analyses were performed using 200-fold excess of unlabelled oligonucleotide duplex with or without the SRE mutation. All the oligonucleotide sequences of EMSA were listed in online Supplementary Table S3.

The prediction and luciferase assay of *srebp-1* for miR-29

To validate the mRNA level of *srebp-1* regulated by miR-29, we obtained the sequence of miR-29 of grass carp based on the recent publication⁽²⁴⁾. The target sites of miR-29 on the 3'UTR of *srebp-1* were predicted based on the principle of Targetscan⁽²⁵⁾. The miR-29 targeted seed sequence was mutated on the WT pmirGLO-*srebp1* plasmid using QuickChange II Site-Directed Mutagenesis kit (Vazyme), and the generated plasmid was named as pmirGLO-*srebp1*-miR29mut (Mut). Based on the site-mutation assay performed previously, 20 pmol of miR-29 mimics or the same amount of non-coding miR (20 pmol, negative control) was co-transfected with 500 ng of the WT pmirGLO-*srebp1* plasmid (WT) or 500 ng of pmirGLO-*srebp1*-miR29mut plasmid (Mut) into HepG2 cells using Lipofectamine 2000 (Invitrogen) at 90% confluence. At 6 h after the transfection, the cells were incubated, with the same fresh culture DMEM containing 10% FBS for 24 h. Then the cells were harvested and the relative luciferase activity was measured using the Dual-luciferase Reporter Assay System (Promega), according to the manufacturer's instruction. The relative luciferase activity of these plasmids was calculated using the ratio of *Firefly* luciferase activity: *Renilla* luciferase activity. All experiments were performed in triplicates.

miR, mRNA and protein expression induced by nSREBP-1 or miR-29 in *Ctenopharyngodon idella* kidney cells

Grass carp CIK cells were counted and seeded at a density of 1×10^6 in a 60-mm culture dish. They were then cultured until 90% confluence was achieved before transfection using Lipofectamine 2000 (Invitrogen) with plasmids (8 µg) or miR (100 pmol). Cells were harvested and washed with PBS. Then total RNA were extracted from the cells and reverse transcribed to cDNA as templates. Real-time quantification of miR was performed by stem-loop RT-PCR⁽²⁶⁾, miR-29 stem-loop RT primers (5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGA-TACGACAACCGA-3') replaced oligo dT/random primers in Quantitect Reverse Transcription kit (TaKaRa). The resulting first-strand cDNA was diluted to 1:10 with ddH₂O before use. The expression level of miR was determined by comparative delta-delta Ct method normalised with U6. These gene-specific primers are listed in online Supplementary Table S2. All experiments were performed in triplicates.

Proteins for Western blot were extracted from transfected cells using RIPA lysis and extraction buffer (Thermo Fisher Scientific). Protein concentration was measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific). About 30 µg total protein per lane was loaded on SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocking with 8% skimmed milk for 1 h, the membranes were incubated with primary antibody at 4°C overnight. The primary antibodies used in the present study were rabbit polyclonal of anti-GAPDH (1:2000; Abcam) and rabbit polyclonal of anti-SREBP1 (1:1000; Abcam). After washing for five times with Tris-buffered saline-Tween, the membrane was probed with horseradish peroxidase-conjugated anti-rabbit IgG (1:10000; Cell Signaling Technology) for 1 h at room temperature. The protein bands

were visualised by Vilber Fusion FX6 Spectra imaging system (Vilber Lourmat) and quantified by Image-Pro Plus (Media Cybernetics).

Statistical analysis

The data were analysed using SPSS 19.0 (SPSS Inc.). Results are presented as mean values with their standard errors for three independent experiments. Differences between two groups were assessed using the unpaired two-tailed Student's *t* test unless otherwise noted. The differences were considered to be significant at $P < 0.05$.

Results

Cloning and sequence analysis of the promoter regions of *srebp-1*, *acca*, *scd1* and *fas*

In the present study, we identified the transcription start sites of *srebp-1* (GenBank accession no.: KJ162572), *acca* (GenBank accession no.: GU908475), *scd1* (GenBank accession no.: AJ243835) and *fas* (GenBank accession no.: MK111644), and the first nucleotide of *srebp-1*, *acca*, *fas* and *scd1* was designated as +1. Then the 1988 bp, 2043 bp, 1632 bp and 1889 bp sequences of *srebp-1*, *acca*, *scd1* and *fas* promoters were cloned and analysed, respectively. A cluster of putative binding sites of several transcription factors, such as specific protein (SP) family, yin yang 1 (YY1), nuclear factor Y (NF-Y), SRE and enhancer box (E-box) element, were predicted on the promoters of *srebp-1*, *acca*, *scd1* and *fas*. On the region of *srebp-1* promoter (online Supplementary Fig. S1), two SP binding sites (at -60 bp/-72 bp and -451 bp/-467 bp), three YY1 binding sites (at -515 bp/-537 bp, -1902 bp/-1924 bp and -1925 bp/-1947 bp), six NF-Y binding sites (at -73 bp/-87 bp, -134 bp/-148 bp, -485 bp/-499 bp, -574 bp/-588 bp, -845 bp/-859 bp and -970/-984 bp), one SRE (at -597 bp/-611 bp) and one E-box element (at -508 bp/-524 bp) were predicted, respectively. On the region of *acca* promoter (online Supplementary Fig. S2), two SP binding sites (at -86 bp/-102 bp and -116 bp/-132 bp), one YY1 binding site (at -436 bp/-458 bp), nine NF-Y binding sites (at -62 bp/-76 bp, -125 bp/-143 bp, -389 bp/-403 bp, -974 bp/-988 bp, -1186 bp/-1200 bp, -1251 bp/-1265 bp, -1360 bp/-1374 bp, -1765 bp/-1779 bp and -1960 bp/-1974 bp), one SRE (at -661 bp/-675 bp) and two E-box elements (at -149 bp/-166 bp and -1232 bp/-1249 bp) were predicted, respectively. On the region of *scd1* promoter (online Supplementary Fig. S3), there were two NF-Y binding sites (at -950 bp/-964 bp and -1040 bp/-1054 bp), two SRE (at -42 bp/-56 bp and -1569 bp/-1583 bp) and three E-box elements (at -87 bp/-103 bp, -714 bp/-731 bp and -1477 bp/-1494 bp). On the region of *fas* promoter (online Supplementary Fig. S4), we discovered one SP binding site (at -1254 bp/-1270 bp), two YY1 binding sites (at -37 bp/-59 bp and -1517 bp/-1539 bp), seven NF-Y binding sites (at -90 bp/-104 bp, -127 bp/-141 bp, -785 bp/-799 bp, -1116 bp/-1130 bp, -1147 bp/-1161 bp, -1484 bp/-1498 bp and -1685 bp/-1699 bp), four SRE (at -63 bp/-72 bp, -133 bp/-142 bp, -1185 bp/-1194 bp and -1235 bp/-1249 bp) and four E-box elements (at -55 bp/-72 bp, -260 bp/-276 bp, -270 bp/-286 bp and -1059 bp/-1076 bp).

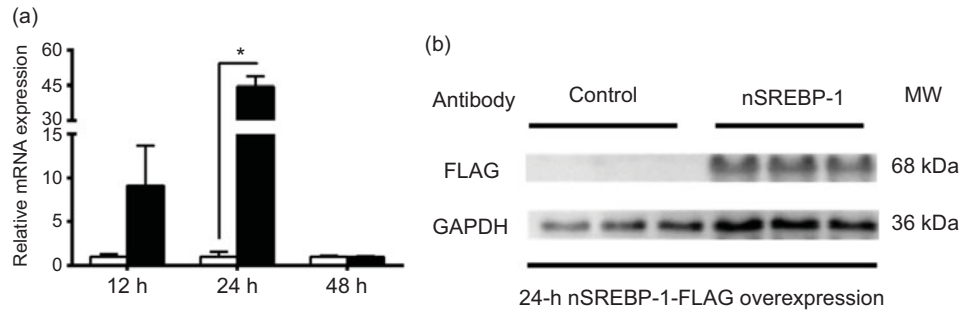


Fig. 1. Overexpression of the N-terminal domain of sterol regulatory element-binding protein 1 (nSREBP-1) in HepG2 cells. (a) mRNA expression of nSREBP-1 plasmid at 12, 24 and 48 h in HepG2 cells. * Significant difference between nSREBP-1 overexpression (■) and control (□) groups ($P < 0.05$). β -Actin and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) were chosen as the housekeeping genes ($M = 0.345$). (b) Western blot of FLAG antibody for nSREBP-1 in HepG2 cells at 24 h. MW, molecular weight.

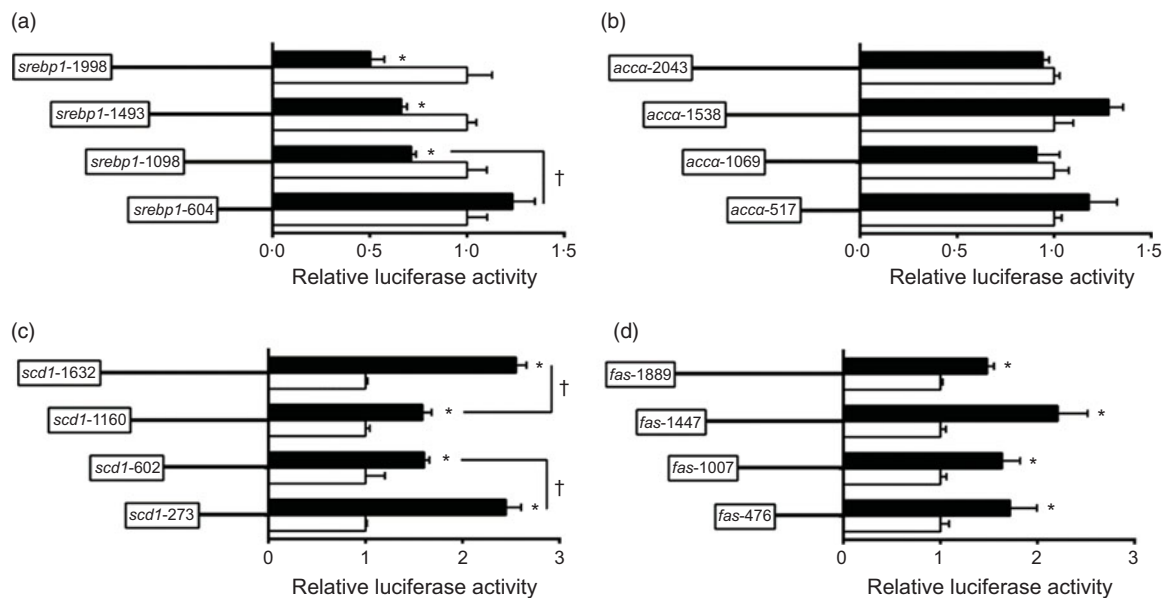


Fig. 2. 5'-Unidirectional deletion assays for promoter regions of sterol regulatory element-binding protein 1 (*srebp-1*), acetyl-CoA carboxylase α (*acca*), stearoyl-CoA desaturase 1 (*scd1*) and fatty acid synthase (*fas*) at 24 h. (a) Assay for *srebp-1* promoter region; (b) assay for *acca* promoter region; (c) assay for *scd1* promoter region; (d) assay for *fas* promoter region. Values are means ($n = 3$), with their standard errors represented by horizontal bars. * Significant difference in relative luciferase activities between the N-terminal domain of SREBP-1 (nSREBP-1) overexpression (■) and control (□) groups ($P < 0.05$). † Significant difference in nSREBP-1-induced changes in relative luciferase activity between the two promoter regions ($P < 0.05$). Relative luciferase activity was presented as the fold activated by nSREBP-1 compared with the control.

Overexpression analysis of grass carp nSREBP-1 in HepG2 cells

To reveal the mechanism of SREBP-1 regulating downstream target genes in grass carp, we tested the Dual-Luciferase Reporter system in cells derived from grass carp. However, compared with the canonical cells derived from mammals, the fish cells were not suitable for the Dual-Luciferase Reporter system, because the *Renilla* luciferase activities could not be detected. Thus, we chose HepG2 cells for analysing the SREBP-1 function in lipogenic gene expression. Therefore, to investigate the nSREBP-1 plasmid for grass carp *nSREBP-1* gene overexpressed in HepG2 cells, we determined the mRNA level of grass carp nSREBP-1 after transfection into HepG2 cells at 12-, 24- and 48 h. Compared with the control, nSREBP-1 overexpression significantly up-regulated the mRNA and protein levels of

nSREBP-1 at 24 h (Fig. 1). Thus, to investigate the SREBP-1 function in gene expression, 24 h was used to determine the luciferase activities of *srebp-1*, *acca*, *fas* and *scd1* promoters.

The 5'-deletion assay of the promoter regions of *srebp-1*, *acca*, *scd1* and *fas*

To investigate the activities of these promoters induced by nSREBP-1, we co-transfected nSREBP-1 plasmid along with the promoter constructs into HepG2 for 24 h and performed the 5'-deletion assay of *srebp-1*, *acca*, *scd1* and *fas* promoters (Fig. 2). Compared with the control, overexpression of nSREBP-1 resulted in a reduction of *srebp-1* promoter activity by 50 %, and the sequence deletion from -1998 bp to -1098 bp presented no significant influences on SREBP-1-induced promoter activity. However, further deleting the sequence

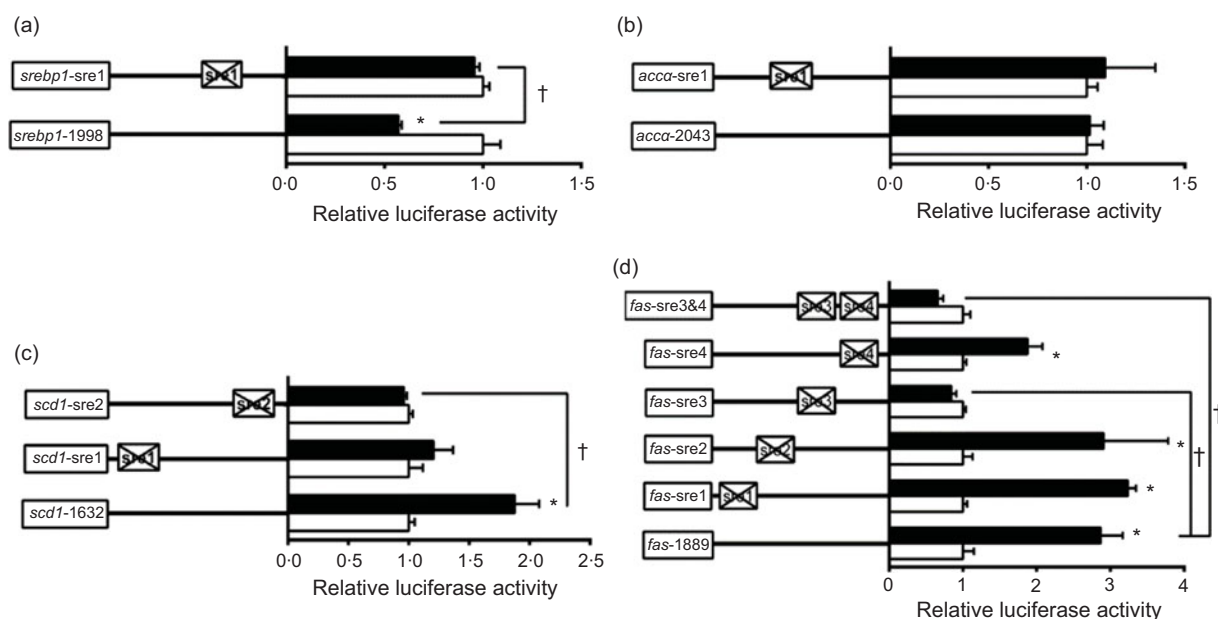


Fig. 3. Promoter activities of site mutagenesis on predicted sterol response elements at 24 h. (a) Site mutagenesis on -1998/+58 sterol regulatory element-binding protein 1 (*srebp-1*) promoter; (b) site mutagenesis on -2043/+49 acetyl-CoA carboxylase α (*acca*) promoter; (c) site mutagenesis on -1632/+57 stearoyl-CoA desaturase 1 (*scd1*) promoter; (d) site mutagenesis on -1889/+111 fatty acid synthase (*fas*) promoter. Values are means (n 3), with their standard errors represented by horizontal bars. * Significant difference between the N-terminal domain of SREBP-1 (nSREBP-1) overexpression (■) and control (□) groups ($P < 0.05$). † Significant difference in the nSREBP-1-induced changes of relative luciferase activity between two sites of mutagenesis ($P < 0.05$). The relative luciferase activity was presented as the fold activated by nSREBP-1 compared with the control.

between -1098 and -604 completely abolished the inhibitory effect by nSREBP-1, indicating that negative response element to nSREBP-1 existed on -1098/-604 region of *srebp-1* promoter (Fig. 2(a)). Overexpressed nSREBP-1 showed no effect on the WT *acca* promoter activity, and no significant differences were found in relative luciferase activity of *acca* promoter between different plasmid groups, indicating that the region of *acca* promoter was not influenced by nSREBP-1 (Fig. 2(b)). Overexpression of SREBP-1 markedly increased the *scd1* promoter activity by 2.6-fold compared with the control. Deleting the sequence from -1632 bp to -1160 bp and from -1160 bp to -602 bp decreased the SREBP-1-induced *scd1* promoter activity by 38 %, and further deleting the sequence between -602 bp to -273 bp recovered the SREBP-1-induced *scd1* promoter activity, suggesting that -1632/-1160, -273/-602 and -273/+57 region of *scd1* promoter were influenced by nSREBP-1 (Fig. 2(c)). Overexpression of SREBP-1 significantly increased the *fas* promoter activity by 1.5-fold compared with the control, and further deleting the sequence from -1889 bp to -476 bp presented no significant effects on SREBP-1-induced *fas* promoter activity. These results indicated that there are positive responsive elements at -476/+111 region of *fas* promoter to nSREBP-1 (Fig. 2(d)).

Site-mutation analysis of sterol response elements on the promoters of *srebp-1*, *acca*, *fas* and *scd1*

To further elucidate whether the regions of *srebp-1*, *acca*, *fas* and *scd1* promoters possessed SRE, we performed the site mutation at these regions of *srebp-1*, *acca*, *fas* and *scd1* promoters that potentially possessed SRE (Fig. 3). Overexpressed nSREBP-1

resulted in a reduction in *srebp-1* promoter activity by 43 % compared with the control, and its inhibitory effect was completely abolished when *srebp1-sre1* (-597/-611) was mutated, suggesting that *srebp1-sre1* site inhibited SREBP-1-induced *srebp-1* transcription (Fig. 3(a)). Consistent with 5'-deletion assays of the promoter region from -2043 to +49bp of *acca*, overexpressed nSREBP-1 showed no stimulatory effect on the WT *acca* promoter activity, and mutation of *acca-sre1* (-661/-675) site did not affect SREBP-1-induced *acca* promoter activity, indicating that the -2043/+49 region of *acca* did not possess any SRE (Fig. 3(b)). The promoter activity of WT *scd1* construct was enhanced by nSREBP-1 overexpression by 1.9-fold; mutation of *scd1-sre2* (-42/-56) site completely abolished the stimulatory effect of SREBP-1, whereas mutation of *scd1-sre1* (-1569/-1583) site showed no significant effect on SREBP-1-induced *scd1* promoter activity, suggesting *scd1-sre2* site up-regulated SREBP-1-induced *scd1* transcription (Fig. 3(c)). Overexpressed nSREBP-1 markedly enhanced the WT *fas* promoter activity by 2.9-fold, but only the mutation plasmid of *fas-sre3* and *fas-sre3* and *sre4*, which were related to the mutation of *fas-sre3* (-133/-142) site, caused a marked reduction in *fas* promoter activity and completely abolished the stimulatory effect of SREBP-1, indicating that *fas-sre3* enhanced the SREBP-1-induced *fas* transcription (Fig. 3(d)).

Electrophoretic mobility shift assay analysis of each SREBP-1 binding sequence

Based on the results of the site-mutation assay discussed above, we further used EMSA to explore their ability to interact with SREBP-1. We made a probe using biotin to label the *srebp1*-

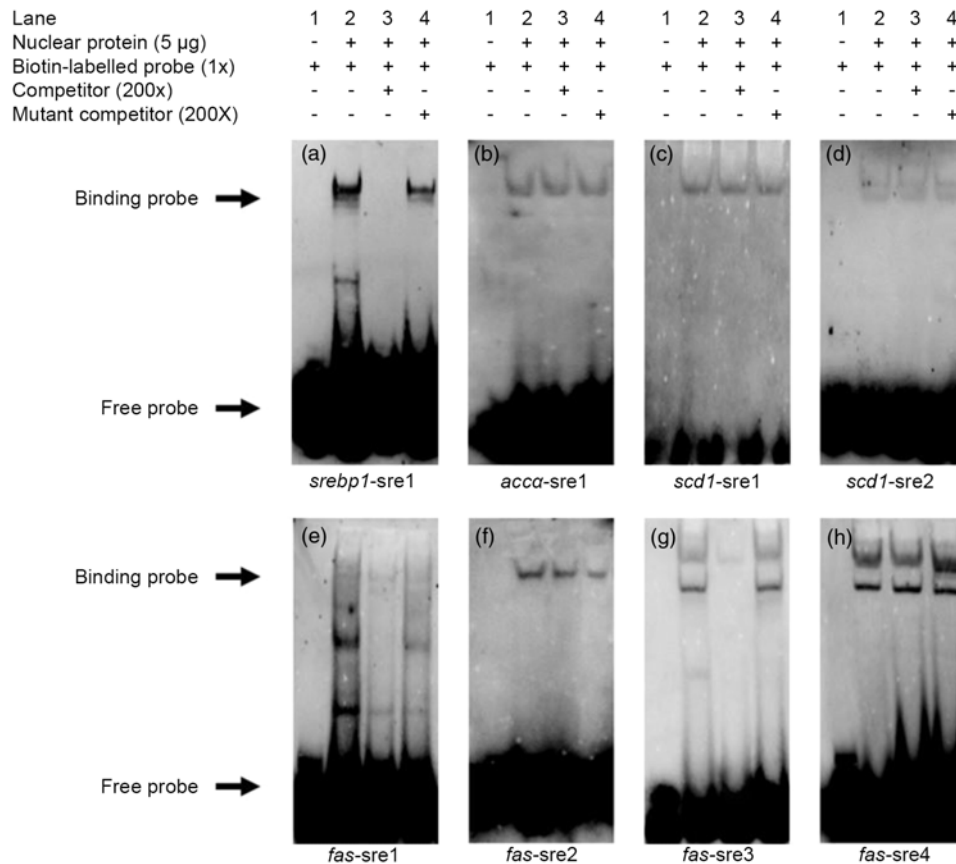


Fig. 4. Electrophoretic mobility shift assay (EMSA) analysis of predicted sterol response elements (SRE). (a) -597/-611 binding site of sterol regulatory element-binding protein 1 (*srebp-1*) (*srebp1-sre1*); (b) -661/-675 binding site of acetyl-CoA carboxylase α (*acc α*) (*acc α -sre1*); (c) -1569/-1583 binding site of stearoyl-CoA desaturase 1 (*scd1*) (*scd1-sre1*); (d) -42/-56 binding site of *scd1* (*scd1-sre2*); (e) -1235/-1249 binding site of fatty acid synthase (*fas*) (*fas-sre1*); (f) -1185/-1194 binding site of *fas* (*fas-sre2*); (g) -133/-142 binding site of *fas* (*fas-sre3*); (h) -63/-73 binding site of *fas* (*fas-sre4*).

sre1 sequence. The results indicated that the 200-fold unlabelled *srebp1-sre1* sequence competed the labelled probe for nSREBP-1 and reduced the brightness of the labelled probe (lane 3, Fig. 4(a)). In contrast, the 200-fold unlabelled mutated *srebp1-sre1* sequence did not compete for the labelled probe for nSREBP-1 (lane 4, Fig. 4(a)), indicating that *srebp1-sre1* site could be bound by SREBP-1 (Fig. 4(a)). For *acc α -sre1* site of *acc α* promoter, the 200-fold unlabelled *acc α -sre1* sequence did not compete for the labelled probe, confirming that *acc α -sre1* site could not interact with SREBP-1 (lane 3, Fig. 4(b)). Similarly, the unlabelled *scd1-sre1* and *scd1-sre2* sites of *scd1* promoter did not compete for the labelled probe for nSREBP-1 (lane 3, Fig. 4(c) and (d)), indicating that *scd1-sre2* site need a synergistic action of SREBP-1 assisted with other factors. In addition, EMSA analysis of four SRE on *fas* promoter indicated that the *fas-sre1* (-1235/-1249) site presented as a faint binding site of SREBP-1 (Fig. 4(e)); the unlabelled *fas-sre2* (-1185/-1194) site and *fas-sre4* site (-63/-72) did not compete for the labelled probe (lane 3, Fig. 4(f) and (h)). Only at the *fas-sre3* (-133/-142) site (Fig. 4(g)), the 200-fold unlabelled sequence competed for the labelled probe for SREBP-1 binding (lane 3, Fig. 4(g)) and the 200-fold mutated *fas-sre3* did not compete for the labelled probe for nSREBP-1 binding (lane 4, Fig. 4(g)), indicating that *fas-sre3* was a strong binding site for SREBP-1. Taken together, these results from

luciferase activity, 5'-deletion and mutation analysis demonstrated that *srebp-1*, *fas* and *scd1*, but not *acc α* , were the target genes of SREBP-1.

Analysis of nSREBP-1 overexpression in *Ctenopharyngodon idella* kidney cells

The results above indicated that the promoter regions of genes involved in lipid metabolism (*srebp-1*, *fas* and *scd1*) possessed SRE. These observations prompted us to investigate whether and how SREBP-1 regulated lipid metabolism in grass carp. We transfected the nSREBP-1 plasmid into CIK cells of grass carp and then determined the expression of its potential target genes and nSREBP-1. Compared with the control, the overexpression of nSREBP 1 significantly increased the mRNA expression of nSREBP-1, and significantly reduced *srebp-1* mRNA expression by 30 %, and up-regulated mRNA expression of *acc α* and miR-29. mRNA expression of *fas* and *scd1* tended to increase after nSREBP1 overexpression but the differences were not statistically significant between the two groups (Fig. 5(a)). Moreover, the protein level of nSREBP-1 tended to up-regulate during the transfection of nSREBP-1 plasmid into CIK cells, though the differences did not reach statistical significance (Fig. 5(b)).

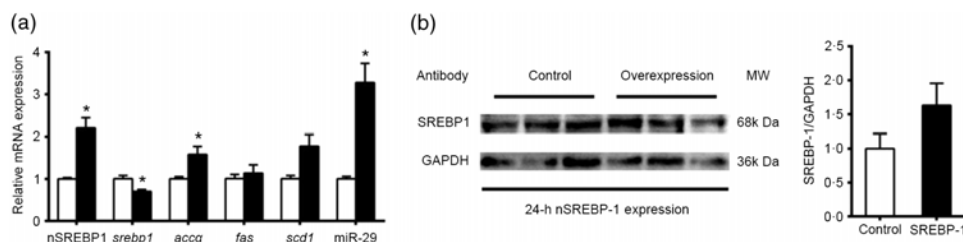


Fig. 5. Expression of sterol regulatory element-binding protein 1 (*srebp-1*), acetyl-CoA carboxylase α (*acca*), fatty acid synthase (*fas*) and stearyl-CoA desaturase 1 (*scd1*) after N-terminal domain of SREBP-1 (nSREBP-1) overexpression in *Ctenopharyngodon idella* kidney (CIK) cells for 24 h. (a) mRNA expression of *srebp-1*, *acca*, *fas* and *scd1* and microRNA-29 (miR-29) in CIK cells for 24-h overexpression. β -Actin and elongation factor 1- α (*ef1a*) were chosen as the housekeeping genes ($M = 0.253$). (b) Protein expression of nSREBP-1 in CIK cells for 24-h overexpression. Values are means ($n = 3$), with their standard errors represented by vertical bars. * Significant differences between nSREBP-1 overexpression (■) and control (□) groups ($P < 0.05$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MW, molecular weight.

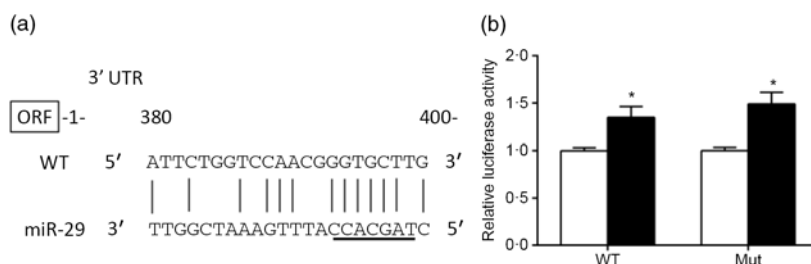


Fig. 6. Prediction and luciferase assay of 3'UTR (untranslated region) of sterol regulatory element-binding protein 1 (SREBP-1) by microRNA-29 (miR-29). (a) Schematic representation of the miR-29 target sequence within the 3'UTR of the *srebp-1* gene. Vertical lines (|) indicate nucleotides that are reversely complementary to miR-29. The core seed of miR-29 is underlined. The numbers indicate the positions of the nucleotides in the *srebp-1* 3'UTR region. (b) Luciferase activities of the 3'UTR of *srebp-1* at 24 h. Relative luciferase activity was presented as the fold activated by miR-29 mimics (■) compared with the negative control (□). Values are means ($n = 3$), with their standard errors represented by vertical bars. * Significant difference of luciferase activity between the miR-29 mimics and negative control groups ($P < 0.05$). WT, wild type.

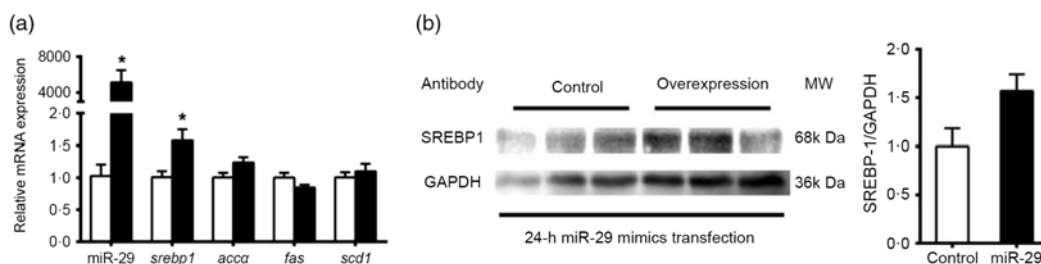


Fig. 7. Expression of sterol regulatory element-binding protein 1 (*srebp-1*), acetyl-CoA carboxylase α (*acca*), fatty acid synthase (*fas*) and stearyl-CoA desaturase 1 (*scd1*) after microRNA-29 (miR-29) mimics transfection in *Ctenopharyngodon idella* kidney (CIK) cells for 24 h. (a) mRNA expression of *srebp-1* (nuclear part sequence of *srebp-1*), *acca*, *fas* and *scd1* and miR-29 in CIK cells after miR-29 transfection at 24 h. β -Actin and elongation factor 1- α (*ef1a*) were chosen as the housekeeping genes ($M = 0.242$). (b) Protein expression of N-terminal domain of SREBP-1 (nSREBP-1) in CIK cells after miR-29 transfection at 24 h. Values are means ($n = 3$), with their standard errors represented by vertical bars. * Significant difference between the nSREBP-1 overexpression (■) and control (□) groups ($P < 0.05$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MW, molecular weight.

Luciferase assay of 3'UTR of SREBP-1 by miR-29

The predicted miR-29 binding sites on the 3'UTR of *srebp-1* are presented in Fig. 6(a). The luciferase reporter assay was used to verify whether miR-29 could influence the transcription of *srebp-1*. Compared with the negative control, co-transfection of miR-29 mimics with the pmirGLO-*srebp1* significantly increased the luciferase activity of *srebp-1* 3'UTR by 1.3-fold, and its stimulatory effect was not abolished when the miR-29 targeted seed sequence of the *srebp-1* 3'UTR was mutated, indicating that miR-29 up-regulated the transcription of *srebp-1* (Fig. 6(b)).

Analysis of miR-29 transfection in *Ctenopharyngodon idella* kidney cells

To support further the function of miR-29 in regulating the expression of SREBP-1 and its potential target genes, we transfected miR-29 mimics into the grass carp CIK cell lines. Compared with the negative control, miR-29 expression was significantly increased during the transfection of miR-29 mimics into CIK cells, and miR-29 mimics significantly increased the mRNA level of *srebp-1* by 1.6-fold but showed no effect on the mRNA expression of *acca*, *fas* and *scd1* (Fig. 7(a)). Moreover, the protein level of nSREBP-1 tended to up-regulate during the

transfection of miR-29 mimics into CIK cells, though the differences did not reach statistical significance (Fig. 7(b)).

Discussion

Several authors have overexpressed mature active SREBP-1 in fish, which activates the expression of target genes by translocating to the nucleus and binding to SRE within the promoters of target genes^(6,27). Here we prepared an NH₂-terminal fragment of grass carp SREBP-1 (amino acid residues 1–494 of the protein, defined as nuclear SREBP-1, or nSREBP-1) and transfected the nSREBP-1 overexpression plasmid along with promoter reporter system to study the activation of potential target genes (*srebp1*, *acca*, *fas* and *scd1*).

In the present study, we cloned the 1988 bp, 2043 bp, 1632 bp and 1889 bp sequences of *srebp-1*, *acca*, *scd1* and *fas* promoters, respectively. To our best knowledge, this is the first time to clone and characterise their promoter regions of these genes in fish. We predicted a cluster of putative binding sites of several transcription factors on the promoters of *srebp-1*, *acca*, *scd1* and *fas* of grass carp, such as SP, YY1, NF-Y, SREBP-1 itself and E-box element. Similar structures have been reported in mammals^(13,28,29). Sp1 has been shown to be a co-activating factor with SREBP-1a⁽³⁰⁾. YY1 is a multifunctional Zn-finger transcription factor that can act as a transcriptional repressor, activator or initiator element binding protein^(10,31). NF-Y was presented to be an essential co-activator of the sterol response^(11,32). The E-box is important for sterol regulation⁽³³⁾. Accordingly, these transcription factor binding sites were important for its basal activation and also activation through other pathways.

The functional importance of SREBPs in controlling transcription of lipid metabolism-related genes is well established, but the mechanism remains unknown. The present study found that there were SRE on the promoters of *srebp-1*, *acca*, *scd1* and *fas* of grass carp. Multiple lines of evidence from 5'-deletion assay, site-mutation and EMSA analyses of each SRE on *acca*, *fas*, *scd1* and *srebp-1* promoters further confirmed that those SRE actually presented as functional sites for SREBP-1 regulation and that *srebp-1*, *fas* and *scd1*, but not *acca*, were the target genes of SREBP-1. Similarly, several studies suggested that the SRE-1 and the E-box elements were existent in FAS promoter, which binds SREBP-1⁽³³⁾. Accordingly, SREBP-1 directly controls the expression of FAS^(11,34). Li *et al.*⁽²⁹⁾ reported that overexpression of SREBP-1 can increase FAS promoter activity and mRNA expression levels. SREBP-1 overexpression resulted in an increase in the mRNA levels of FAS and SCD^(29,35). Unexpectedly, based on the results from 5'-deletion assay, site-mutation and EMSA analyses of SRE on *acca* promoters, the present study clearly indicated that *acca* was not the direct target gene of SREBP-1 though mammal's study found that SREBP-1 overexpression resulted in an increase in the mRNA levels of ACC⁽³⁵⁾. Studies indicated that SREBPs required interaction with cofactors after binding to target DNAs to activate the downstream gene^(5,33). Similar results were also indicated by 5'-deletion assay, site-mutation and EMSA analyses of SRE on *scd1* promoter.

In mammals, studies suggested that SREBP-1 contained SRE in their enhancer/promoter regions, and the nuclear forms of

SREBPs can activate their own genes in an auto-regulatory loop^(5,28,36). However, in contrast with mammals, the present study clearly indicated that the activity of *srebp-1* promoter was strongly inhibited by SREBP-1 itself. The reasons remained unknown now. Studies showed that SREBP activation of gene expression, in some SREBP target genes, can be negatively regulated by YY-1 Zn-finger transcription factor in manner of repressing SREBP activation by displacing NF-Y^(37,38). Moreover, YY1 could bind to SREBP-1 with a high affinity and interferes with the SREBP binding to the SRE on target genes⁽³⁹⁾. Interestingly, we discovered that there are multiple potential YY1 binding sites on the promoter of *srebp-1* gene of grass carp, but not on *acca*, *fas* and *scd1* promoters. Thus, we concluded that it may be YY1 that causes the inhibition of *srebp-1* expression during nSREBP-1 overexpression, indicating that a potential pathway might participate in SREBP-1-mediated lipid homeostasis.

In the present study, in grass carp CIK cells, the overexpression of nSREBP-1 significantly reduced *srebp-1* mRNA expression and up-regulated mRNA expression of *acca*. Thus, again our study confirmed that the SREBP-1 suppressed its own expression; meantime, it is the nuclear form of SREBP-1, not SREBP-1, that regulates its downstream target genes. Similarly, Kim *et al.* pointed out that SREBP1 translocates to the nucleus where it activates lipogenic genes by binding to the SRE of target genes⁽⁷⁾. The present study also indicated that mRNA expression of *fas* and *scd1*, and the protein level of nSREBP-1 tended to up-regulate during the transfection of nSREBP-1 plasmid into CIK cells but the differences did not reach statistical significance. We speculated that it may be because increased SREBP-1 protein expression was not enough to up-regulate mRNA expression of its target genes *fas* and *scd1*. It is well reported that some miR regulate the expression of target genes involved in lipid metabolism⁽¹⁵⁾. Recently, in mammals, Ru *et al.* reported that SREBP-1 transcriptionally activated specific SRE motifs on the promoter of miR-29, and then miR-29 inversely suppressed SREBP-1 expression by binding to their 3'UTR region⁽¹⁶⁾. In contrast, in the present study, we found that transfection of miR-29 mimics significantly increased the mRNA level of *srebp-1* and miR-29 in CIK cells but showed no significant effects on the mRNA expression of *acca*, *fas* and *scd1*. The up-regulation of *srebp-1* mRNA level by miR-29 was not expected since miR are believed to bind through partial homologous sequence to a target gene at 3'UTR and cause translation repression. However, other studies suggest that miR can up-regulate translation of target genes⁽⁴⁰⁾. On the other hand, we also noticed that miR-29 mimic transfection tended to up-regulate the protein expression of nSREBP1 and that nSREBP-1 overexpression up-regulated mRNA expression of miR-29, implying that a self-activating loop for SREBP-1 and miR-29 existed in grass carp. Further study is still needed to elucidate the details of the self-activating mechanism of SREBP-1 and miR-29 in grass carp.

Conclusion

In summary, we identified and characterised the promoter regions of *srebp-1*, *acca*, *fas* and *scd1* genes from grass carp. The present study demonstrated that *fas* and *scd1* were the direct target genes of SREBP-1. Furthermore, we found two novel



transcriptional mechanism for regulating SREBP-1 expression: (1) the auto-regulation sited on the SREBP-1 promoter regions was suppressive and (2) SREBP-1 overexpression up-regulated miR-29 expression, and SREBP-1 expression was up-regulated by miR-29 as well, implying a self-activating loop of SREBP-1 and miR-29 in grass carp. Our study shed us new sight into the regulation of lipid metabolism.

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Z. L. and Y.-H. X. designed the experiment; Y.-H. X. conducted the experiment with the help of X.-Y. T., Y.-C. X., T. Z. and L.-H. Z.; Y.-H. X., X.-Y. T. and Z. L. analysed the data; Y.-H. X. drafted the manuscript and Z. L. revised the manuscript. All the authors read and approved the manuscript.

The authors declare no conflicts of interest.

Supplementary material

For supplementary material/s referred to in this article, please visit <https://doi.org/10.1017/S0007114519001934>

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