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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Abbreviations: ACCα, acetyl-CoA carboxylase alpha; bHLH-Zip, basic-helix-loop-helix-leucine zipper; E-box, enhancer box; ER, endoplasmic reticulum; FAS, fatty acid synthase; 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; YY1, yin yang 1

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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 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 *accα*, *fas* and *scd*1) in a freshwater teleost, grass carp *Ctenopharyngodon idella*. We cloned and characterized the 1988 bp, 2043 bp, 1632 bp and 1889 bp sequences of *srebp*-1, *accα*, *scd*1, and *fas* promoters, respectively. A cluster of putative binding sites of transcription factors, such as SP, YY1, NF-Y, SREs and E-box element, were predicted on their promoter regions. nSREBP-1 overexpression reduced *srebp*-1 promoter activity, increased *scd*1 and *fas* promoter activity, but did not influence *accα* promoter activity. The site mutation and EMSA analysis indicated that *srebp*-1, *fas* and *scd*1 promoters, but not *accα* promoter, possessed SREs. In 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 these results above, 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; (2) there was a self-activating loop of SREBP-1 and miR-29.

Keywords: *Ctenopharyngodon idella*; sterol regulatory element binding proteins; promoter analysis; microRNA; lipid metabolism

1. Introduction

Deregulated lipid metabolism is an established hallmark of many diseases, such as fatty liver and obesity. Lipid metabolism are tightly regulated by 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 characterized, such as SREBP-1a and -1c. However, only a single form of the SREBP-1 gene has been characterized in fish [2, 3]. The SREBPs are synthesized as precursor proteins bound to the endoplasmic reticulum (ER) membrane [4]. After stimulation, the SREBP precursor undergoes proteolytic cleavage in the Golgi to release the transcriptionally active N-terminal domain

(nSREBP). Once mature, active SREBP1 translocates to the nucleus where they can bind to SREs (sterol response elements) 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), fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD1) [6, 7]. ACCα catalyzes the ATP-dependent carboxylation response of acetyl-CoA to form malonyl-CoA; FAS catalyzes the condensation of acetyl-CoA and malonyl-CoA to generate long-chain fatty acids [8]. SCD1 catalyzes the synthesis of long-chain unsaturated fatty acids. At present, the promoter regions of these genes have been partially isolated and characterized in mammals [9-11]. Meantime, Griffin *et al*. pointed out that 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 in 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. miRNAs are a class of small non-coding RNAs that regulate gene expression post-transcriptionally by binding to complementary regions in the 3'UTR of target mRNAs, resulting in mRNA degradation or attenuated translation [14]. Studies have shown that miRNAs play a role in TG 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 unraveled a negative feedback loop mediated by miR-29 in SREBP-1 signaling [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 *accα*, *fas*, *scd*1 and *srebp*-1, and investigate the functions of SREs on their promoter regions. To gain insight into the distinct roles of SREBP-1, over-expressing truncated, active nuclear forms of grass carp SREBP-1 were produced and characterized. 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.

2. Materials and Methods

2.1. Animals, cells and media

Juvenile grass carp were purchased from a commercial farm (Wuhan, China) and used for DNA and RNA extraction. HepG2 and grass carp 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) media containing 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (0.1 mg/mL) in an incubator at 37^{o}C with 5% CO_2. Grass carp CIK cells were cultured in Medium 199 (M199) media containing 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (0.1 mg/mL) in an incubator at 28^{o}C with 5% CO_2. All these culture media, 0.25% trypsin-EDTA and FBS were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Penicillin and streptomycin were purchased from Sigma-Aldrich (St. Louis, MA, USA). The present study was performed in accordance with the relevant institutional and national guidelines, and the manuscript must conform to the ARRIVE Guidelines for Reporting Animal Research. The Ethics Committee of Huazhong Agricultural University (HZAU) checked and approved our present experimental protocols on animals and cells.

2.2. Cloning and plasmids construction

We identified the 5' cDNA sequences and the transcription start sites (TSS) of *srebp*-1, *accα*, *scd*1 and *fas* of grass carp by 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 (Omega, USA). The promoter sequences of *srebp*-1, *accα*, *scd*1 and *fas* were amplified from extracted DNA using PCR and subcloned into pGl3-basic vector (Promega, Madison, WI, USA) using ClonExpress™ II One Step Cloning Kit (Vazyme, Piscataway, NJ, USA), and the PCR reactions were performed using TaKaRa PrimeSTAR® HS DNA Polymerase kit (TaKaRa, Otsu, Japan). Based on the distance from their TSS, we named the srebp1-1998 plasmid for -1998/+59 *srebp*-1 promoter, accα-2043 plasmid for -2043/+49 *accα* promoter, scd1-1632 plasmid for -1632/+57 *scd*1 promoter and fas-1889 plasmid for -1889/+111 *fas* promoter, respectively. Plasmids of srebp1-1493, srebp1-1098, srebp1-604, accα-1538, accα-1069, accα-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, accα-2043, scd1-1632 and fas-1889 plasmid, respectively.

Total RNA was extracted from hepatic samples of grass carp using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) 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 subcloned into pcDNA3.1 (+) vector with FLAG-tag sequence inserted at the C-terminus of n*srebp*-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 subcloned 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, Wuhan, China) and listed in Table S1.

2.3. Sequence analysis

Nucleotide sequences of *srebp*-1, *accα*, *scd*1 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 *accα*, *fas*, *scd*1 and *srebp*-1 from grass carp, putative transcription factor binding sites were predicted by online software MatInspector (http://www.genomatix.de/). Besides, SREs with relative score over 0.9 based on JASPAR database (http://jaspar.genereg.net/) were also considered as potential binding sites.

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

For expression of nSREBP-1 plasmid group, HepG2 cells were counted and seeded at a density of 1 x 10^6 in a 60-mm culture dish and then were cultured until 80% to 90% confluence before transfecting with 8 μg of nSREBP-1 plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacture's instruction. For the control, the same amount of pcDNA3.1(+) was transfected into HepG2 cells. Then, cells were harvested at 12-h, 24-h 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 to 1:10

with ddH_2O before use. Q-PCR was performed using the SYBR Premix Ex TaqTM II kit (Takara) in a quantitative thermal cycler (BIO-RAD, Hercules, CA, USA). A set of six common housekeeping genes (*ß-actin*, *18s-rRNA*, *gapdh*, *ef1a*, *hprt* and *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 normalizing 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 Table S2.

Proteins for Western Blot were extracted from transfected HepG2 cells using RIPA lysis and extraction buffer (Thermo Fisher Scientific, Waltham, MA, USA), 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). 30μg total protein per lane was loaded on SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF). After blocked with 8% skim milk for 1 h, the membranes were incubated with primary antibody at 4°C overnight. The primary antibodies used in this study were rabbit polyclonal of anti-GAPDH (1:2000, Abcam, Cambridge, UK), anti-FLAG (1:1000, Proteintech, Wuhan, china). After washed 5 times with Tris-buffered saline-Tween, the membrane was probed with HRP-conjugated Anti-Rabbit IgG (1:10000, Cell Signaling Technology, Cambridge, USA) for 1 h at room temperature. The protein bands were visualized by Vilber Fusion FX6 Spectra imaging system (Vilber Lourmat, Collegien, France), and quantified by Image-Pro Plus (Media Cybernetics, Sliver Spring, MD, USA).

2.5. 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 x 10^5 in 24-well plates, then cultured and transfected as our recent studies [19, 23]. Briefly, to study the nSREBP-1-induced changes of 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, scd*1 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 (pRL-TK)

per well was included in all transfections to normalize transfection efficiency. 6-h after the transfection, the cells were incubated with the fresh DMEM medium containing 10% FBS for 24-h. Then, the relative luciferase activity of *srebp*-1*, accα, scd*1 and *fas* promoters was measured using the Dual-luciferase Reporter Assay System (Promega) according to the manufacturer's instruction, respectively. 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.

2.6. Site-mutation assay of SRE sites on the srebp-1, accα, scd1 and fas promoters

To identify the corresponding SREs on the grass carp *srebp*-1*, accα, scd*1 and *fas* promoters, we performed site-directed mutagenesis of SREs on the plasmid of *srebp*-1*, accα, scd*1 and *fas* promoters according to the manufacture's instruction of QuickChange II Site-Directed Mutagenesis Kit (Vazyme), respectively. SRE-mutated plasmids of srebp-sre1, accα-sre1, scd1-sre1, scd1-sre2, fas-sre1, fas-sre2, fas-sre3, fas-sre4 and fas-sre3&4 were generated from the wild-type plasmids of srebp1-1998, accα-2043, scd1-1632 and fas-1889, respectively. Similar to promoter luciferase assay performed above, 300 ng of nSREBP-1 plasmid or the same amount of pcDNA3.1(+) plasmid (300 ng, control) was co-transfected with 500 ng of wild type plasmid or the same amount of SRE-mutated plasmid into HepG2 cells using Lipofectamine 2000 (Invitrogen) at 80%-90% confluence, respectively. For an internal control, 25 ng of *Renilla* luciferase vector (pRL-TK) per well was included in all transfections to normalize transfection efficiency. 6-h after the transfection, the cells were incubated with the same fresh DMEM medium containing 10% FBS for 24-h. Then, 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.

2.7. Electrophoretic mobility-shift assay (EMSA)

EMSA was performed to confirm the functional SREs 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 analyzed. Each oligonucleotide duplex of SREs was incubated with 5 μg nuclear extracts at room temperature according to the instruction of LightShift™ Chemiluminescent EMSA Kit (Invitrogen, USA), and each unlabeled probe was pre-incubated 10 min prior to the addition of biotin-labeled probe. The reaction was allowed to proceed for 20 min after addition of biotin-labeled probe at room temperature, and then were detected by electrophoresis on 6% native polyacrylamide gels. Competition analyses were performed by using 200-fold excess of unlabeled oligonucleotide duplex with or without the SRE-mutation. All the oligonucleotide sequences of EMSA were listed in Table S3.

2.8. 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 wild type pmirGLO-srebp1 plasmid (WT) using QuickChange II Site-Directed Mutagenesis Kit (Vazyme), and the generated plasmid was named as pmirGLO-srebp1-miR29mut (Mut). As site-mutation assay performed above, 20 pmol of miR-29 mimics or the same amount of non-coding microRNA (20 pmol, negative control, N.C.) was co-transfected with 500 ng of the wild-type pmirGLO-srebp1 plasmid (WT) or 500 ng of pmirGLO-srebp1-miR29mut plasmid (Mut) into HepG2 cells using Lipofectamine 2000 (Invitrogen) at 90% confluence, respectively. The cells were incubated, 6-h after the transfection, with the same fresh culture DMEM medium containing 10% FBS for 24-h. Then, 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.

2.9. miRNA, mRNA and protein expression induced by nSREBP-1 or miR-29 in CIK cells

Grass carp CIK cells were counted and seeded at a density of 1 x 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 microRNAs (100 pmol), respectively. Cells were harvested and washed with PBS. Then, total RNAs were extracted from the cells and reverse-transcribed to cDNA as templates. Real-time quantification of microRNA was performed by stem-loop RT-PCR [26], miR-29 stem-loop RT primers (5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACCGA-3') replaced Oligo dT/Random primers in Quantitect Reverse Transcription Kit (Takara, Tokyo, Japan). The resulting first-strand cDNA was diluted to 1:10 with ddH_2O before use. Expression level of microRNA was determined by comparative delta-delta Ct method normalized with U6. These gene-specific primers are listed in 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). 30 μg total protein per lane was loaded on SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF). After blocked with 8% skim milk for 1 h, the membranes were incubated with primary antibody at 4°C overnight. The primary antibodies used in this study were rabbit polyclonal of anti-GAPDH (1:2000, Abcam), rabbit polyclonal of anti-SREBP1 (1:1000, Abcam). After washed 5 times with Tris-buffered saline-Tween, the membrane was probed with HRP-conjugated Anti-Rabbit IgG (1:10000, Cell Signaling Technology) for 1 h at room temperature. The protein bands were visualized by Vilber Fusion FX6 Spectra imaging system (Vilber Lourmat), and quantified by Image-Pro Plus (Media Cybernetics).

2.10. Statistical analysis

The data were analyzed using SPSS 19.0 (SPSS Inc., Chicago, IL). Results are presented as means ± SEM (standard error of mean) at 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 $p < 0.05$.

3. Results

3.1. Cloning and sequence analysis of the promoter regions of srebp-1, accα, scd1 and fas

In the present study, we identified the transcription start sites (TSS) of *srebp*-1 (GenBank accession no.: KJ162572), *accα* (GenBank accession no.: GU908475), *scd1* (GenBank accession no.: AJ243835) and *fas* (GenBank accession no.: MK111644), and the first nucleotide of *srebp*-1, *accα*, *fas* and *scd*1 was designated as +1. Then, the 1988 bp, 2043 bp, 1632 bp and 1889 bp sequences of *srebp*-1, *accα*, *scd*1, and *fas* promoters were cloned and analyzed, respectively. A cluster of putative binding sites of several transcription factors, such as Sp family, YY1, NF-Y, SREs and E-box element, were predicted on the promoters of *srebp*-1, *accα*, *scd*1 and *fas*. On the region of *srebp*-1 promoter (Figure 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), and one SRE (at -597 bp/ -611 bp) and one e-box element (at -508 bp/ -524 bp) were predicted, respectively. On the region of *accα* promoter (Figure 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 *scd*1 promoter (Figure S3), there were two NF-Y binding sites (at -950 bp/ -964 bp and -1040 bp/ -1054 bp), two SREs (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 (Figure 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 SREs (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).

3.2. 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 derived cells 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 analyzing the SREBP-1 function in lipogenic genes 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-h, 24-h and 48-h. Compared to the control, nSREBP-1 overexpression significantly up-regulated the mRNA and protein levels of nSREBP-1 at 24-h (Figure 1). Thus, to investigate the SREBP-1 function in gene expression, 24-h was used to determine the luciferase activities of *srebp*-1*, accα, fas* and *scd*1 promoters below.

3.3. 5'-deletion assay of the promoter regions of srebp-1, accα, 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 24h and performed the 5'- deletion assay of *srebp*-1*, accα, scd*1, *and fas* promoters, respectively (Figure 2). Compared to 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 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 (Figure 2A). Overexpressed nSREBP-1 showed no effect on the wild type *accα* promoter activity, and no significant differences were found in relative luciferase activity of *accα* promoter between different plasmid groups, indicating that the region of *accα* promoter was not influenced by nSREBP-1 (Figure 2B). Overexpression of SREBP-1 markedly increased the *scd*1 promoter activity by 2.6-fold compared to the control. Deleting the sequence from -1632 bp to -1160 bp and from -1160 bp to -602 bp decreased the SREBP-1-induced *scd*1 promoter activity by 38%, and further deleting the sequence between -602 bp to -273 bp recovered the SREBP-1-induced *scd*1 promoter activity, suggesting that -1632/-1160, -273/-602 and -273/+57 region of *scd*1 promoter were influenced by nSREBP-1 (Figure 2C). Overexpression of SREBP-1 significantly increased the *fas* promoter activity by 1.5-fold compared to 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 (Figure 2D).

3.4. Site-mutation analysis of SREs on the promoters of srebp-1, accα, fas and scd1

To further elucidate whether the regions of *srebp*-1*, accα, fas* and *scd*1 promoters possessed SREs, we performed the site mutation at these regions of *srebp*-1*, accα, fas* and *scd*1 promoters that potentially possessed SREs (Figure 3). Overexpressed nSREBP-1 resulted in a reduction of *srebp*-1 promoter activity by 43% compared to 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 (Figure 3A). Consistent with 5'-deletion assays of the promoter region from -2043 to +49bp of *accα*, overexpressed nSREBP-1 showed no stimulatory effect on the wild type *accα* promoter activity, and mutation of acc1-sre1 (-661/-675) site did not affect SREBP-1-induced *accα* promoter activity, indicating that the -2043/+49 region of *accα* did not possess any SRE (Figure 3B). The promoter activity of wild type *scd*1 construct was enhanced by nSREBP-1 overexpression for 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 *scd*1 promoter activity, suggesting scd1-sre2 site up-regulated SREBP-1-induced *scd*1 transcription (Figure 3C). Overexpressed nSREBP-1 markedly enhanced the wild type *fas* promoter activity by 2.9-fold, but only the mutation plasmid of fas-sre3 and fas-sre3 & sre4, which was related to the mutation of fas-sre3 (-133/-142) site, caused a marked reduction of *fas* promoter activity and completely abolished the stimulatory effect of SREBP-1, indicating that fas-sre3 enhanced the SREBP-1-induced *fas* transcription (Figure 3D).

3.5. EMSA analysis of Each SREBP-1 Binding Sequence

Based on the results of the site-mutation assay above, we further used EMSA assay to explore their ability to interact with SREBP-1. We made a probe by using biotin to label the srebp1-sre1 sequence. The results indicated that the 200-fold unlabeled srebp1-sre1 sequence competed the labeled probe for nSREBP-1 and reduced the brightness of the labeled probe (Lane 3, Figure 4A). In contrast, the 200-fold unlabeled mutated srebp1-sre1 sequence did not compete for the labeled probe for nSREBP-1 (Lane 4,

Figure 4A), indicating that srebp1-sre1 site could be bound by SREBP-1 (Figure 4A). For accα-sre1 site of accα promoter, the 200-fold unlabeled accα-sre1 sequence did not compete for the labeled probe, confirming that accα-sre1 site could not interact with SREBP-1 (Lane 3, Figure 4B). Similarly, the unlabeled scd1-sre1 and scd1-sre2 site of scd1 promoter did not compete for the labeled probe for nSREBP-1 (Lane 3, Figure 4C, D), indicating that scd1-sre2 site need a synergistic action of SREBP-1 assisted with other factors. In addition, EMSA analysis of four SREs on *fas* promoter indicated that the fas-sre1 (-1235/-1249) site presented as a faint binding site of SREBP-1 (Figure 4E); the unlabeled fas-sre2 (-1185/-1194) site and fas-sre4 site (-63/-72) did not compete for the labeled probe, respectively (Lane 3, Figure 4F, H). Only at the fas-sre3 (-133/-142) site (Figure 4G), the 200-fold unlabeled sequence competed for the labeled probe for SREBP-1 binding (Lane 3, Figure 4G) and the 200-fold mutated fas-sre3 did not compete for the labeled probe for nSREBP-1 binding (Lane 4, Figure 4G), 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 *accα*, were the target genes of SREBP-1.

3.6. Analysis of nSREBP-1 overexpression in CIK cells

The results above indicated that the promoter regions of genes involved in lipid metabolism (*srebp*-1, *fas* and *scd*1) possessed SREs. 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 to 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 *accα* and miR-29. mRNA expression of *fas* and *scd*1 tended to increase after nSREBP1 overexpression but the differences were not statistically significant between two groups (Figure 5A). Moreover, the protein level of nSREBP-1 tended to up-regulate during the transfection of nSREBP-1 plasmid into CIK cells although the differences did not reach statistical significance (Figure 5B).

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

The predicted miR-29 binding sites on the 3'UTR of *srebp*-1 were presented in Figure 6A. The luciferase reporter assay was used to verify whether miR-29 could influence the transcription of *srebp*-1. Compared to the N.C., 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 (Figure 6B).

3.8. Analysis of miR-29 transfection in CIK 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 to the N.C., 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 *accα*, *fas* and *scd*1 (Figure 7A). Moreover, the protein level of nSREBP-1 tended to up-regulate during the transfection of miR-29 mimics into CIK cells although the differences did not reach statistically significant (Figure 7B).

4. Discussion

In fish, several authors have overexpressed mature active SREBP-1, which activates the expression of target genes by translocating to the nucleus and binding to SREs within the promoters of target genes [6, 27]. Here, we prepared an NH_2-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 (*srebp*1, *accα*, *fas* and *scd*1).

In the present study, we cloned the 1988 bp, 2043 bp, 1632 bp and 1889 bp sequences of *srebp*-1, *accα*, *scd*1, and *fas* promoters, respectively. To our best knowledge, this is the first time to clone and characterize 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, *accα*, *scd*1, 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 coactivating factor with SREBP-1a [30]. YY1 is a multifunctional zinc-finger transcription factor which can act as a transcriptional repressor, activator or initiator element binding protein [10, 31]. NF-Y factor was presented to be an essential coactivator 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 SREs on the promoters of *srebp*-1, *accα*, *scd*1, and *fas* of grass carp. Multiple lines of evidence from 5'-deletion assay, site-mutation and EMSA analysis of each SREs on *accα*, *fas*, *scd*1 and *srebp*-1 promoters further confirmed that those SREs actually presented as functional sites for SREBP-1 regulation and that *srebp*-1, *fas* and *scd1*, but not *accα*, 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 bind SREBP-1 [33]. Accordingly, SREBP-1 directly controls the expression of FAS [11, 34]. Li et al. [29] reported that the 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 analysis of SRE on *accα* promoters, the present study clearly indicated that *accα* was not the direct target gene of SREBP-1 although 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 analysis of SRE on *scd*1 promoter.

In mammals, studies suggested that SREBP-1 contained SREs in their enhancer/promoter regions, and the nuclear forms of SREBPs can activate their own genes in an autoregulatory 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 zinc 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 SREBP binding to the SREs 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 *accα*, *fas* and *scd*1 promoters. Thus, we concluded that it may be YY1 that cause 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 *accα*. 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 down-stream target genes. Similarly, Kim *et al*. pointed out that SREBP1 translocates to the nucleus where it activates lipogenic genes by binding to the SREs of target genes [7]. The present study also indicated that mRNA expression of *fas* and *scd*1, 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 *scd*1. It is well reported that some microRNAs regulate the expression of target genes involving 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 *accα*, *fas* and *scd*1. The up-regulation of *srebp*-1 mRNA level by miR-29 was not expected since miRNAs are believed to bind through partial homologous sequence to a target gene at 3' UTR and cause translation repression. However, other studies suggest that microRNA 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.

5. Conclusion

In summary, we identified and characterized the promoter regions of sr*ebp*-1*, acca, fas* and *scd*1 genes from grass carp. The present study demonstrated that *fas* and *scd*1 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; (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.

Conflicts of interest: The authors declare no conflict of interest.

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Author Contributions: Zhi Luo and Yi-Huan Xu designed the experiment; Yi-Huan Xu conducted the experiment with the help of Xiao-Ying Tan, Yi-Chuang Xu, Tao Zhao and Li-Han Zhang; Yi-Huan Xu, Xiao-Ying Tan and Zhi Luo analyzed the data; Yi-Huan Xu drafted the manuscript and Zhi Luo revised the manuscript. All the authors read and approved the manuscript.

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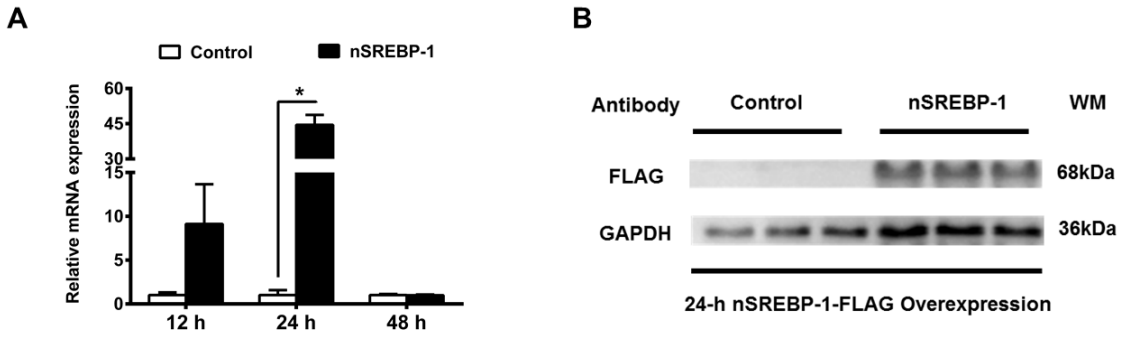

Figure 1. Overexpression of nSREBP-1 in HepG2. (A) The mRNA expression of nSREBP-1 plasmid at 12, 24, and 48 h in HepG2. Asterisk (*) indicates significant differences between nSREBP-1 overexpression group and the control ($p < 0.05$). *β-actin* and *gapdh* were chosen as the housekeeping genes (M=0.345) (B) Western blot of FLAG antibody for nSREBP-1 in HepG2 cells at 24-h.

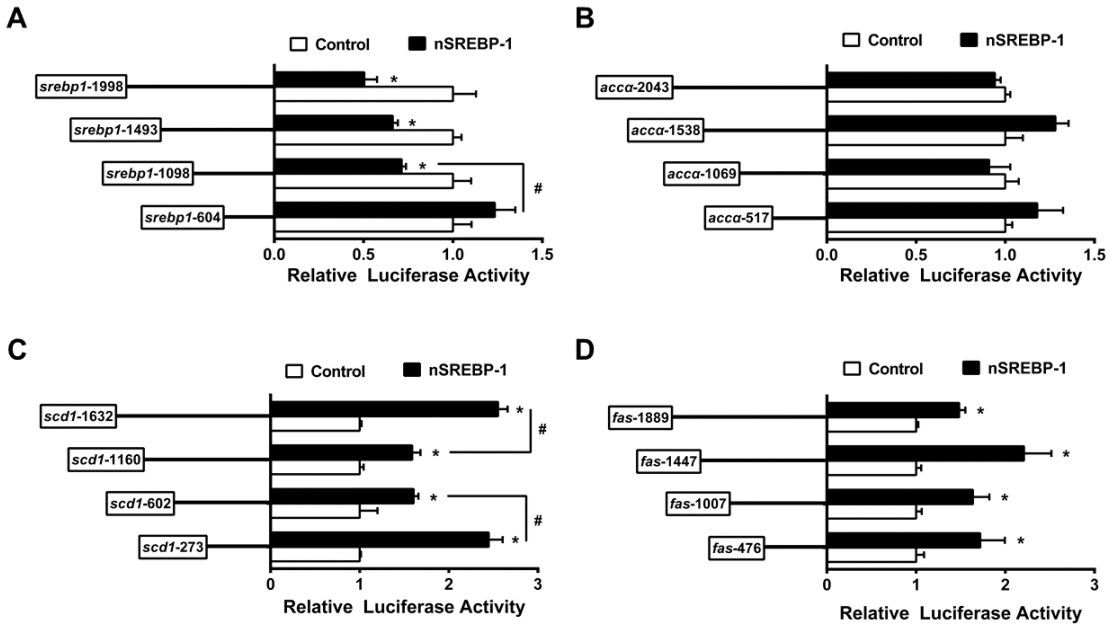

Figure 2. 5'-unindirectional deletion assays for promoter regions of *srebp*-1, *accα*, *scd*1 and *fas* at 24-h. (A) Assay for *srebp*-1 promoter region; (B) assay for *accα* promoter region; (C) assay for *scd*1 promoter region; (D) assay for *fas* promoter region. Values are presented as mean ± SEM (n=3). Asterisk (*) indicates significant differences in relative luciferase activities between the group nSREBP-1 overexpression and the control ($p < 0.05$). Hash symbol (#) indicates significant difference in nSREBP-1-induced changes of relative luciferase activity between two promoter regions ($p < 0.05$). The relative luciferase activity was presented as the fold activated by nSREBP-1 compared with the control.

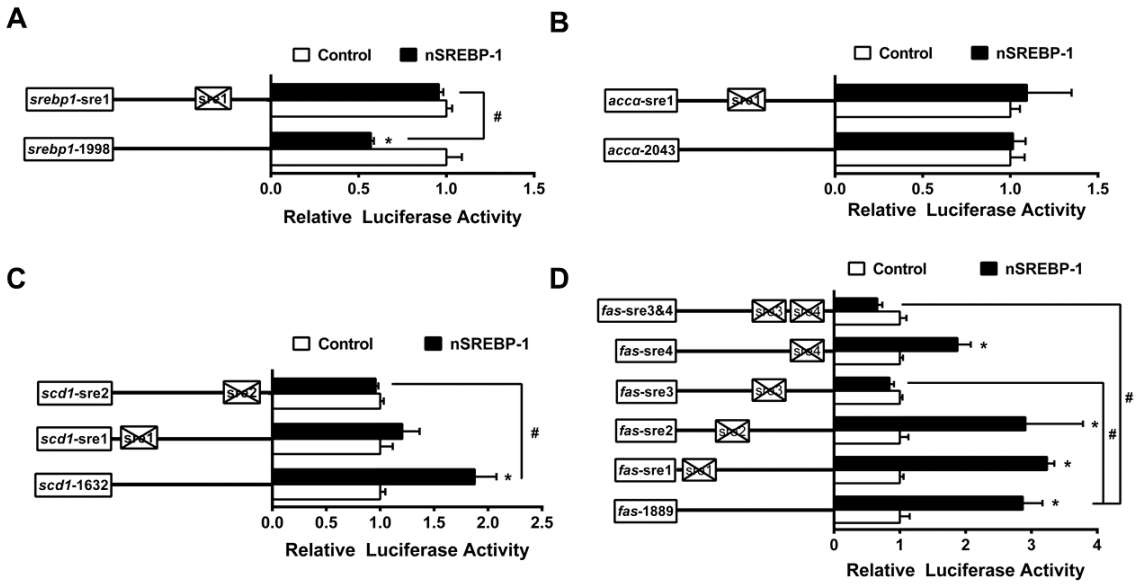

Figure 3. Promoter activities of site mutagenesis on predicted SREs at 24-h. (A) Site mutagenesis on -1998/+58 *srebp*-1 promoter; (B) Site mutagenesis on -2043/+49 *accα* promoter; (C) Site mutagenesis on -1632/+57 *scd*1 promoter; (D) Site mutagenesis on -1889/+111 *fas* promoter. Values are presented as mean ± SEM (n=3). Asterisk (*) indicates significant differences between the group of nSREBP-1 overexpression and the control ($p < 0.05$). Hash symbol (#) indicates significant difference in the nSREBP-1-induced changes of relative luciferase activity between two site mutagenesis ($p < 0.05$). The relative luciferase activity was presented as the fold activated by nSREBP-1 compared with the control.

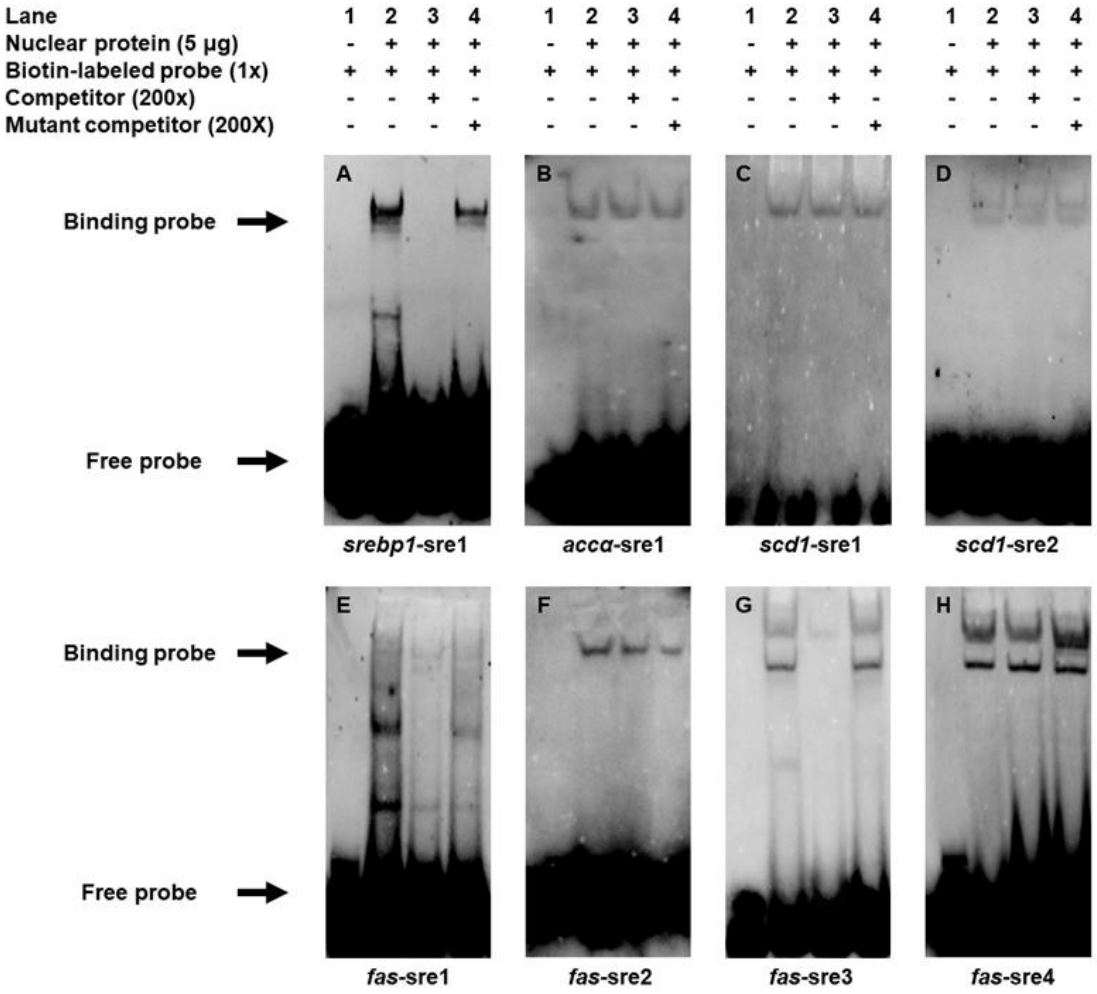

Figure 4. EMSA analysis of predicted SREs. (A) -597/-611 binding site of *srebp*-1 (srebp1-sre1); (B) -661/-675 binding site of *accα* (accα-sre1); (C) -1569/-1583 binding site of *scd*1 (scd1-sre1); (D) -42/-56 binding site of *scd*1 (scd1-sre2); (E) -1235/-1249 binding site of *fas* (fas-sre1); (F) -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).

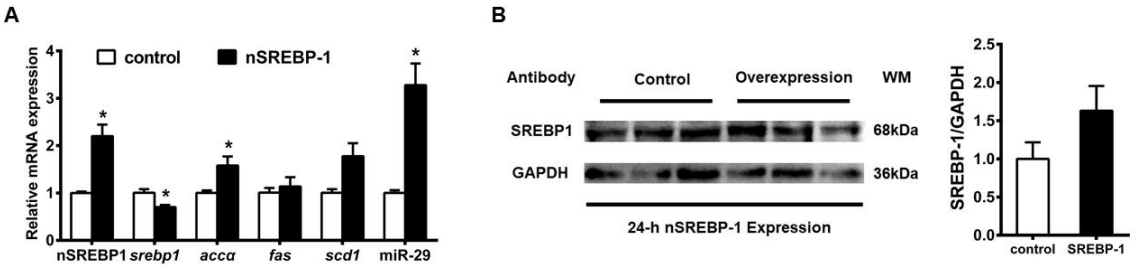

Figure 5. Expression of *srebp*-1, *acca*, *fas* and *scd*1 after nSREBP-1 overexpression in CIK cells for 24-h. (A) The mRNA expression of *srebp*-1, *acca*, *fas* and *scd*1 and miR-29 in CIK cells for 24-h overexpression. *β-actin* and *ef1α* were chosen as the housekeeping genes (M=0.253). (B) The protein expression of nSREBP-1 in CIK cells for 24-h overexpression. Asterisk (*) indicates significant differences between nSREBP-1 overexpression group and the control ($p < 0.05$).

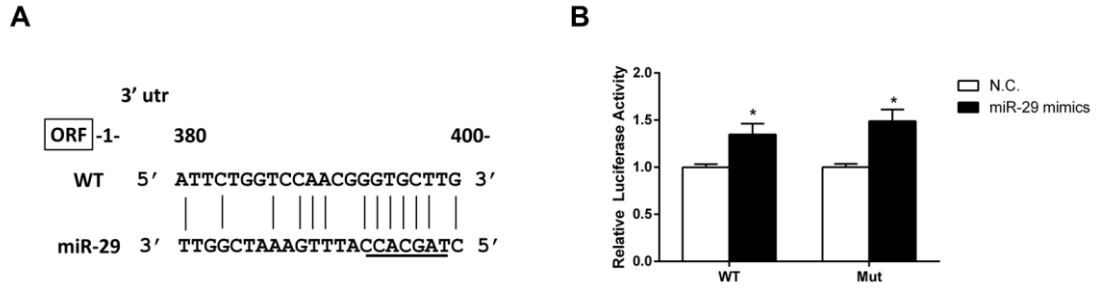

Figure 6. Prediction and luciferase assay of 3'UTR of SREBP-1 by miR-29. (A) Schematic representation of the miR-29 target sequence within the 3' UTR of *srebp*-1 gene. Vertical line (|) indicates nucleotides that are reversely complementary to miR-29. The core seed of miR-29 is underlined. The numbers indicate the positions of the nucleotides in the *srebp*-1 3' UTR region. (B) The luciferase activities of the 3'UTR of *srebp*-1 at 24-h. The relative luciferase activity was presented as the fold activated by miR-29 mimics compared with the negative control (N.C). Asterisk (*) indicates significant differences of luciferase activity between the miR-29 mimics group and the N.C. ($p < 0.05$).

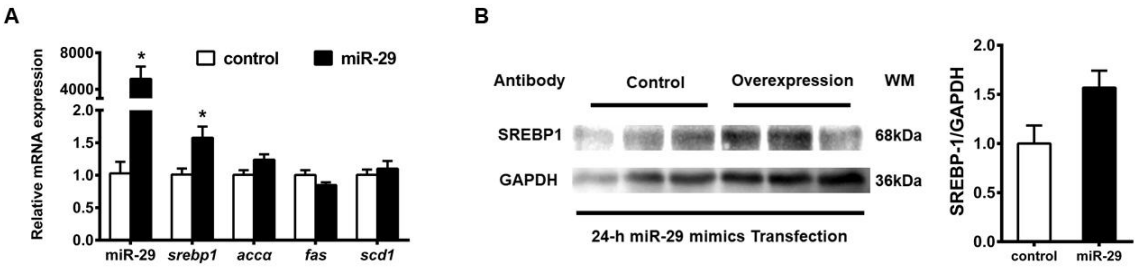

Figure 7. Expression of *srebp*-1, *accα*, *fas* and *scd*1 after miR-29 mimics transfection in CIK cells for 24-h. (A) The mRNA expression of *srebp*-1 (nuclear part sequence of *srebp*-1), *accα*, *fas* and *scd*1 and miR-29 in CIK cells after miR-29 transfection at 24-h. *β-actin* and *ef1α* were chosen as the housekeeping genes (M=0.242). (C) The protein expression of nSREBP-1 in CIK cells after miR-29 transfection at 24-h. Asterisk (*) indicates significant differences between the group of nSREBP-1 overexpression and the control ($p < 0.05$).