

## Article

# RNA-Seq Analysis of the Key Long Noncoding RNAs and mRNAs Related to the Regulation of Hepatic Lipid Metabolism in *Oreochromis niloticus*

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**Abstract:** Genetically improved farmed tilapia (*Oreochromis niloticus*, GIFT) is prone to hepatic metabolic imbalances and fatty liver disease during intensive farming. Long non-coding RNAs (lncRNAs) perform essential roles in various biological processes, including lipid metabolism. However, the lncRNAs involved in hepatic lipid metabolism in tilapia have not yet been identified. In this study, Illumina sequencing and bioinformatic analyses were performed on the liver of juvenile male GIFT fed a high-fat diet (HFD, 18.5% lipid) or a normal-fat diet (NFD, 8% lipid) for 56 days. RNA-seq analyses revealed 299 differentially expressed (DE)-mRNAs and 284 DE-lncRNAs between these two groups. The transcript profiles of 14 candidates (seven DE-mRNA and seven DE-lncRNAs) were verified by qRT-PCR, and the results were consistent with the RNA-seq results. Furthermore, 65 *cis* target genes and 3610 *trans* target genes of DE-lncRNAs were predicted. Functional analyses suggested that multiple metabolic pathways are affected by a high fat intake, including the PPAR signaling, fatty acid degradation, and fatty acid metabolism pathways. A co-expression network analysis indicated that many lncRNAs interact with numerous genes involved in lipid metabolism, and that some genes are regulated by multiple lncRNAs. The expression patterns of three lncRNAs (MSTRG.14598.1, MSTRG.6725.3, and MSTRG.13364.2) and their potential target genes (*faldh*, *slc25a48*, and *fabp7a*) in the PPAR signaling pathway were investigated. Our study provides new information about lncRNAs associated with lipid metabolism in tilapia.

**Keywords:** lncRNA; mRNA; GIFT; lipid metabolism; fatty liver



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## 1. Introduction

Genetically improved farmed tilapia (GIFT, *Oreochromis niloticus*) is a widely farmed fish in China, especially in southern China, because of its rapid growth and excellent stress resistance [1]. However, fatty liver disease, which results from an imbalance of hepatic lipid metabolism, often occurs in intensively farmed tilapia [2]. There are many causes of fatty liver disease in fish, including nutritional imbalances, environmental stress, and various physiological disorders [3]. Among them, imbalanced nutrients in feed is the main cause of fatty liver disease in farmed tilapia [4,5]. Lipids, as a non-protein nutrient, are essential for fish. High-fat feeds are widely used in fish farming because they provide energy while saving feed protein [6,7]. However, an inappropriately high lipid intake can slow the growth of tilapia and reduce its stress resistance [5,8], thereby causing economic losses to the tilapia industry.

Lipid metabolism is an intricate biological process. Genes identified as crucial regulators of lipid metabolism include those encoding fatty acid synthetase (FAS) [9], peroxisome proliferator-activated receptor (PPAR) [10], and stearoyl-coa desaturase [11]. In our previous studies, we found that some non-coding RNAs, microRNA (miR)-34a, miR-205-5p,

and miR-23a-3p, participate in the regulation of lipid metabolism in tilapia [4]. However, long non-coding RNAs (lncRNAs) have been rarely studied in tilapia, especially in terms of their involvement in lipid metabolism.

lncRNAs are RNAs more than 200 nucleotides (nt) in length and lack protein-coding ability. They are widely found in animals and plants [12]. Most of them are functional and regulate gene expression at epigenetic, transcriptional, and post-transcriptional levels [13–15]. Many studies have demonstrated that lncRNAs are key regulators of lipid metabolism. For example, lncNEAT1 was found to promote the expression of *ACC*, encoding acetyl-CoA carboxylase, and *FAS*, encoding fatty acid synthase, in hepatocytes by activating the mTOR/S6K1 signaling pathway. Interference with lncNEAT1 lentivirus reduced hepatic fat deposition in rats [16]. A study on human hepatic cells showed that lncHR1 affects fat synthesis by regulating the expression of the gene encoding sterol regulatory element binding protein-1c [17]. In mice, depletion of a liver-specific lncRNA (lncLSTR) was found to enhance the transcript levels of *apoC2*, leading to increased lipoprotein lipase activity and decreased serum triglyceride content [18]. Lipid metabolism-related lncRNAs have been identified in several fishes, including Chinese tongue sole [19] and zebrafish [20]. However, the lncRNAs associated with hepatic lipid metabolism in tilapia have not yet been explored. Therefore, to explore their functions in more detail, it is important to first identify and characterize them.

Previously, we reported a model system wherein fatty liver disease can be induced in juvenile male GIFT by a high-fat diet (HFD, 18.5% *w/v* lipid). Using this system, we compared metabolites between fish in a HFD group and those fed a normal-fat diet (NFD, 8% *w/v* lipid) [21]. Based on that study, hepatic profiles of mRNAs and lncRNAs were determined after 56 days of a HFD or NFD by RNA-seq. Then, bioinformatics techniques were conducted to predict the target mRNAs of the lncRNAs. Quantitative real-time PCR (qRT-PCR) was used to further analyze and investigate the expression patterns of several key lncRNAs involved in lipid metabolism and their target mRNAs on days 14, 35, and 56 of a HFD. Our findings provide valuable information for further analyses of the functional roles of lncRNAs in fat metabolism of tilapia.

## 2. Materials and Methods

### 2.1. Experimental Design and Sampling

Healthy male GIFT juveniles, which were purchased from the YangZhong Research Station (Yangzhong, China), were stocked in 600-L aerated water tanks (27–29 °C and pH 7.4–7.6) for 1 week. Fish were fed a commercial diet (Tongwei, China) two times per day during this 1-week acclimation period.

After acclimation, 180 healthy fish (average weight,  $5.02 \pm 0.01$  g) were randomly divided into NFD (8% *w/v* lipid) and HFD (18.5% *w/v* lipid) groups in triplicate. Each tank housed 30 fish ( $n = 90$  per group). The NFD and HFD were formulated as previously described [4]. The fish were fed to apparent satiation two times per day. The feed formulae are listed in Table S1.

The feeding trial lasted for 56 days. On days 14, 35, and 56, three fish from each tank were randomly caught and anaesthetized with 100 mg/L MS-222. Liver tissues collected from the sampled fish were frozen in liquid nitrogen and then stored at  $-80$  °C until further molecular experiments. On day 56, liver tissue from another GIFT from each tank was collected for histological analyses. The use of fish in these experiments was approved by the Bioethical Committee of Freshwater Fisheries Research Center (2013863BCE) and all experimental manipulations complied with animal welfare requirements.

### 2.2. Hepatic Histological Analysis

The samples were fixed in 4% paraformaldehyde for 24 h and then dehydrated overnight in a 30% sucrose solution at 4 °C. Each sample was embedded in OCT compound and cut into 8- $\mu$ m sections using a freezing microtome (Leica 3050S, Wetzlar, Germany). After rewarming to room temperature, the slices were immersed in oil red O staining

solution for 10 min, immersed in 60% isopropyl alcohol for 30 s to remove the background color, and cleaned with pure water. After redyeing with Mayer's hematoxylin for 3 min, the slices were sealed with glycerin gelatin and observed under a CX31 microscope (Olympus, Tokyo, Japan). The area of lipid droplets in six digital fields per group was analyzed using Image-Pro plus v6.0.0.260 [22].

### 2.3. RNA Extraction and Sequencing

Total RNA was isolated from liver tissues sampled at day 56 using TRIZOL reagent (Invitrogen, Carlsbad, USA) and genomic DNA was removed by DNase I (Promega, Madison, USA). RNA quality was assessed using a Bioanalyzer 2100 (Agilent, Palo Alto, USA) [23]. The sequencing was performed with three bio-replicates per group (RNA from three samples per group was mixed). Thus, six cDNA libraries (HFD\_1, HFD\_2, HFD\_3, NFD\_1, NFD\_2, NFD\_3) were constructed using an mRNA-Seq sample preparation kit (Illumina, San Diego, USA) after rRNA removal with the Epicentre Ribo-Zero Gold Kit (Illumina), following the manufacturer's instructions. The library was sequenced on the Illumina Novaseq™ 6000 platform.

### 2.4. Data Filtering and lncRNA Identification

Raw reads were trimmed by Cutadapt v1.10 to eliminate low-quality reads and adaptor sequences. Sequence quality was checked by FastQC v0.10.1. HISAT2 v2.0.4 was used to map reads to the Nile tilapia genome (<https://www.ncbi.nlm.nih.gov/genome/?term=Oreochromis+niloticus>), accessed on 20 July 2018 [24]. The alignment results were then assembled into transcripts using StringTie v1.3.0 and classified with Gffcompare v0.11.2. Among them, transcripts with the class code J, I, O, U, or X were further screened to identify lncRNAs. Among them, transcripts with the class code J, I, O, U, or X were further screened to identify lncRNAs.

To identify lncRNAs, we removed the following sequences: (1) those shorter than 200 bp or containing less than one exon; (2) those with reads coverage <3; and (3) those with known non-lncRNA annotations. Then, Coding Potential Calculator (CPC) v0.9-r2 [25] and Coding-Non-Coding Index (CNCI) v2.0 [26] were used to estimate coding potential of each remaining transcript. Transcripts with CPC score < 0 and CNCI score < 0 were considered to be lncRNAs [27].

### 2.5. Differential Expression Analyses of lncRNAs and mRNAs

StringTie v1.3.0 was used to standardize the expression levels of lncRNA and mRNA in each sample by calculating the FPKM (fragments per kilobase per million mapped reads) values. Then, EdgeR v3.22.3 was used to identify the differentially expressed (DE)-mRNAs and DE-lncRNAs between the HFD and NFD groups [28]. The criteria for differential expression between the two groups were  $|\log_2 \text{fold change}| > 1$  and  $p < 0.05$  [29].

### 2.6. Target Gene Prediction

Next, we predicted the potential *cis* and *trans* targets of DE-lncRNAs. *Cis*-acting lncRNAs target neighboring genes. In this study, genes located within 100 kb upstream or downstream of the de-lncRNA were selected as *cis*-regulated genes using Bedtools v2.17.0 [30]. lncRNAs can also regulate genes across chromosomes in a *trans*-acting manner. This interaction mainly depends on free energy demands for the formation of a secondary structure between the lncRNA and the target mRNA. Rsearch v2.0 was used to investigate the relationships between lncRNAs and mRNAs [31]. The thresholds for screening *trans*-target genes were as follows:  $\geq 10$  directly interacting bases, and minimum free energy  $< -50$  [32].

### 2.7. Pathway Enrichment Analysis

To explore the function of De-lncRNAs, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were conducted for the *cis*- and *trans*-target genes. The threshold for identifying significantly enriched GO terms and KEGG pathways was  $p < 0.05$ .

### 2.8. qRT-PCR Analyses

Total RNA was extracted from liver tissues sampled at days 14, 35, and 56 using the method described in 2.3. The cDNA was synthesized using PrimeScript RT Master Mix (Takara, Dalian, China) according to the manufacturer's instructions. qPCR was performed on an ABI QuantStudio 5 instrument (ABI, Foster City, USA) with a SYBR® Premix Ex Taq kit (Takara). The reaction mixture (25 µL) contained 12.5 µL SYBR Premix Ex Taq II (2×), 0.5 µL ROX Dye II, 1 µL forward and reverse primers (10 µM), 2 µL cDNA template, and 8 µL RNase-free water. All reactions were performed in triplicate with the following thermal cycling program: 95 °C, for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Elongation factor 1α (*ef1α*) and β-actin were used as reference genes. Relative gene transcript levels were calculated by the  $2^{-\Delta\Delta Ct}$  method and  $\Delta Ct = Ct_{target} - (Ct_{EF1\alpha} + Ct_{\beta-actin})/2$ . The primer sequences are presented in Table 1. Data were expressed as the mean ± standard error and were analyzed using SPSS 22.0 (SPSS Inc., Chicago, USA). Significance ( $p < 0.05$ ) was detected by one-way ANOVA followed by Duncan's test.

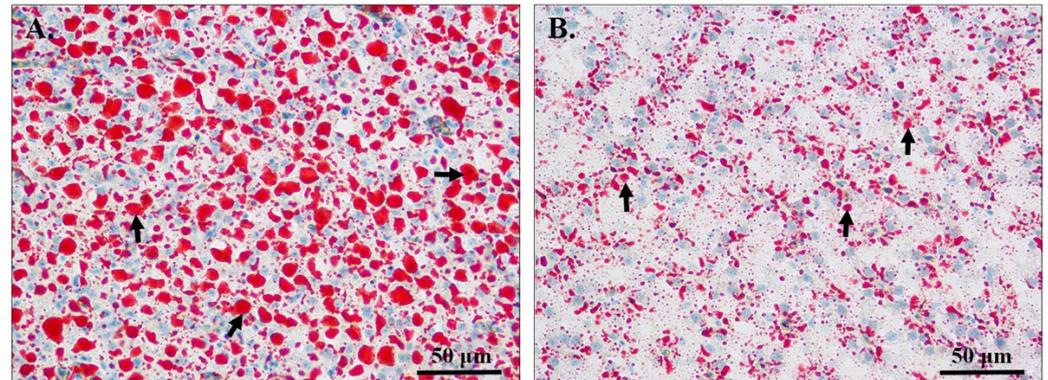
**Table 1.** Primer sequences used for qRT-PCR of DE-mRNAs and DE-lncRNAs.

Gene	Primer Sequence (5'-3')	Amplicon Size	Efficiency%
MSTRG.6725.3	F: CGTGCTTTAGACGGGCCTAT R: TGGATGTCACAGAACCACCG	167	102.51
MSTRG.14598.1	F: AGCCAATCAGAAGAGAGGTGAC R: CAGAAACCTTCAGCAACCACTT	196	100.97
MSTRG.32774.1	F: TTCACCGAGGAGAGAACCCT R: TCTCACACAGAGGATGCGTG	195	101.14
MSTRG.13364.2	F: ACCTAAAAGGCCAGTCCGTG R: CCTCTGGGTTTATGGGTAGCAT	200	105.49
MSTRG.43073.2	F: CTGGGACTCTCCATCTTTTGGT R: TACGGGTAAGCTTCTCCACAC	150	100.90
MSTRG.24713.1	F: GTGTCGATCATTGCCCCAT R: TCCAGCACAGTCTTCGCTTC	192	103.44
MSTRG.17221.1	F: AGCAACCGTGTGTGAAAAGTG R: AAAAACAAACACCGCTCCCG	198	96.20
<i>slc25a48</i>	F: AATGGCACCAACATCCGTCT R: GCAGAAGATGGCGTAAGGGA	192	97.06
<i>faldh</i>	F: CAACAGCACGGTGGGAAAAG R: TGTAGTCTGGGGCAATGCAG	185	95.51
<i>vlacs</i>	F: GCAGAGTCCAGCAGCCTAC R: GTGATAAAGCGGGAGGGCAT	157	95.90
<i>fads2</i>	F: TATCAACACGCTGGACACCT R: GCCGGTTATCATGGTGTG	176	101.86
<i>pck1</i>	F: GAACTGACCCGAGGGATGTG R: GTCCTACCTCTCATGCAGCC	181	101.78
<i>ehhadh</i>	F: GCTCCGTGGGCTTGATTACT R: CTTGATGTCTGCTCCCCAG	167	100.74
<i>fabp7a</i>	F: TGCAGAAGTGGGATGGCAA R: GGCTTTCTCATACGTGCGGA	113	97.62
<i>ef1α</i>	F: ATCAAGAAGATCGGCTACAACCCT R: ATCCCTTGAACCAGCTCATCTTGT	109	96.55
<i>β-actin</i>	F: CCACACAGTGCCCATCTACGA R: CCACGCTCTGTCAGGATCTTCA	111	99.27

### 3. Results

#### 3.1. Hepatic Histological Analysis

Hepatic lipid deposition was observed by oil red O staining (Figure 1). Compared with the liver of fish from the NFD group (Figure 1B), the liver of fish from the HFD group had a much larger area of lipid droplets (Figure 1A, Table 2). This extensive lipid accumulation may have impaired normal liver function.



**Figure 1.** Oil red O staining of liver tissues in genetically improved farmed tilapia (GIFT) fed a high-fat diet (HFD) or normal-fat diet (NFD) for 56 days. Bar = 50 µm. (A) Liver status of GIFT in the HFD group; (B) Liver status of GIFT in the NFD group. Black arrows indicate lipid droplets.

**Table 2.** Area of lipid droplets in the liver of GIFT fed a HFD or NFD for 56 days.

	HFD Group	NFD Group
lipid droplet (object/total, %)	0.28 ± 0.01 *	0.10 ± 0.01

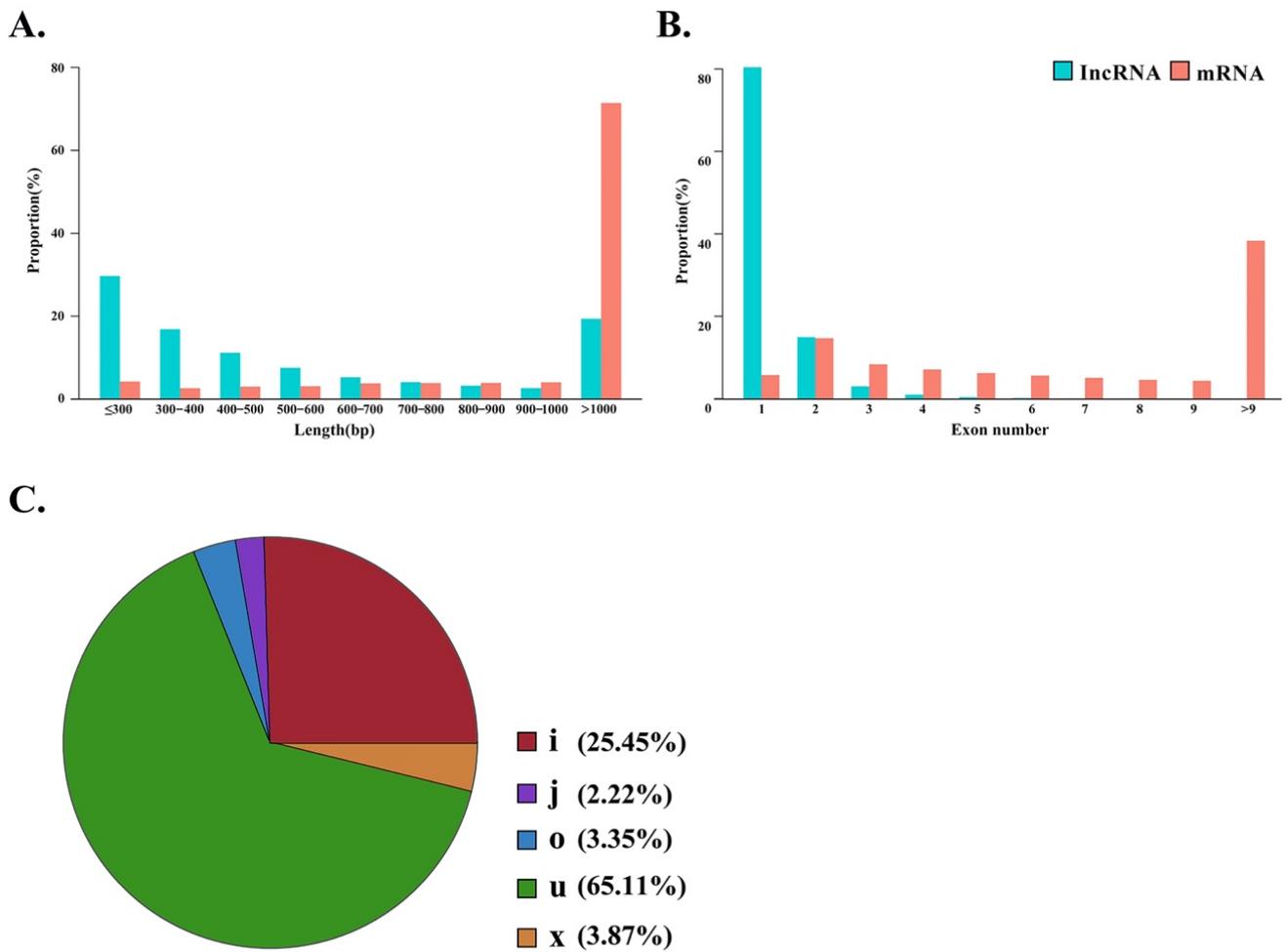
\* means significant difference ( $p < 0.05$ ) between HFD and NFD group. ( $n = 6$ ).

#### 3.2. Overview of Liver RNA Sequencing

Liver tissues from fish fed a HFD or NFD for 56 days were used to construct cDNA libraries for sequencing. The number of clean reads in each library ranged from 60,134,656 to 86,665,870 after filtering. The Q30 values of these libraries ranged from 96.77% to 97.91% (Table S2). Comparisons of the expression density of all transcripts and overall gene transcript levels among the six libraries (Figure S1A,B) confirmed the reliability of the Illumina sequencing data. For the HFD\_1, HFD\_2, HFD\_3, NFD\_1, NFD\_2, and NFD\_3 libraries, 87.11%, 86.96%, 87.10%, 87.32%, 86.80%, and 87.43% of the reads were mapped to the Nile tilapia genome, respectively (Table S2).

#### 3.3. Identification and Quantification of lncRNAs

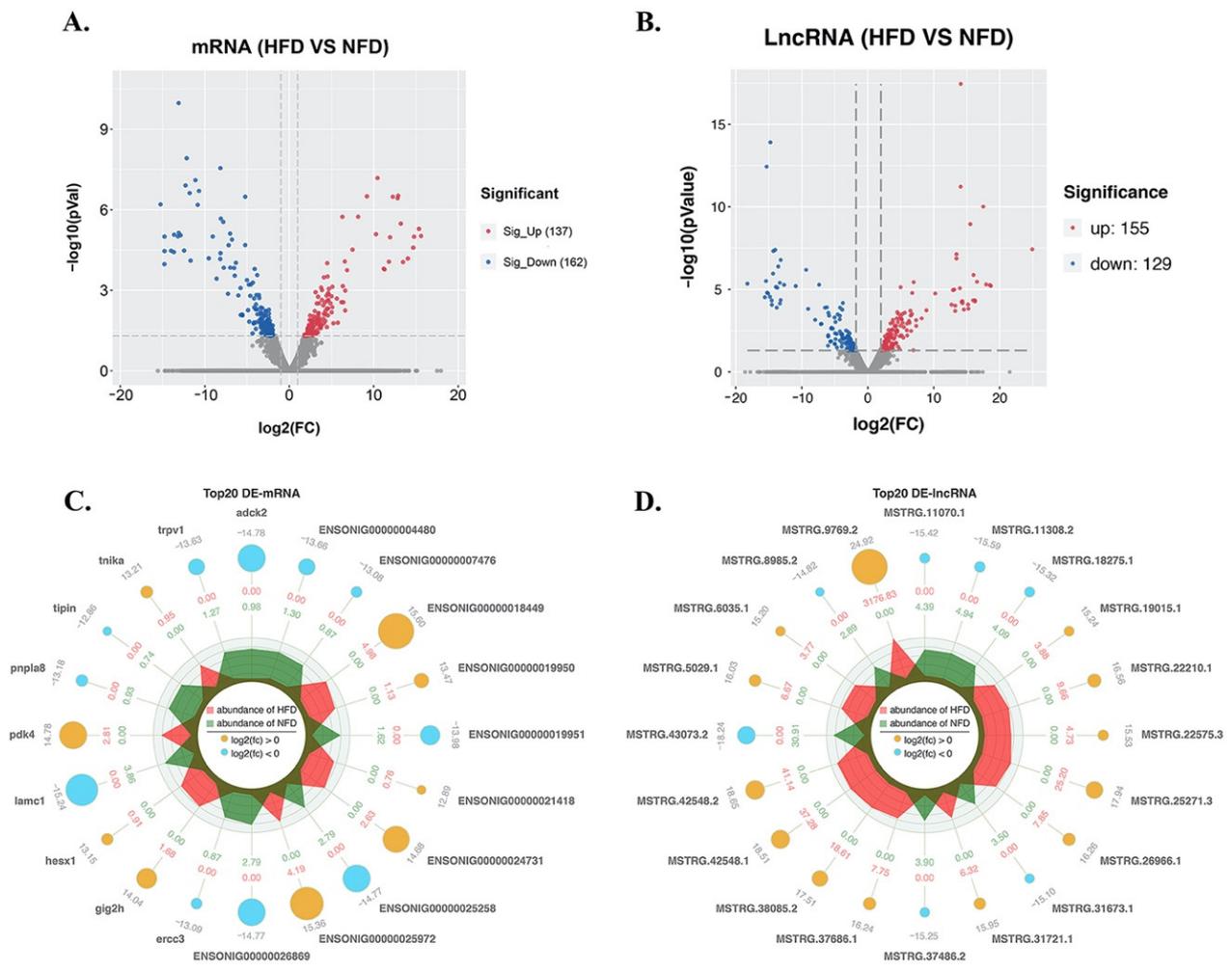
Several filtering steps were performed to identify lncRNAs from total transcripts, yielding 19,928 candidate lncRNAs. The full length of most lncRNAs ranged from 200 to 700 bp, while most full-length mRNAs were longer than 1000 bp (Figure 2A). A large number of lncRNAs contained only one or two exons (95.38%), while the number of exons in mRNAs was more variable (Figure 2B). The candidate lncRNAs were classified into five types (U, I, X, J, and O) by Gffcompare software. Most lncRNAs belonged to the U (intergenic) and I (intronic) classes, accounting for 65.11% and 25.45% of total lncRNAs, respectively (Figure 2C).



**Figure 2.** Comparison of lncRNAs and mRNAs in GIFT. (A) Length distribution of mRNAs and lncRNAs. (B) Number of exons in mRNAs and lncRNAs. (C) Pie chart of lncRNA classification groups.

### 3.4. Screening of DE-lncRNAs and DE-mRNAs in Response to a High-Fat Diet

After 56 days of a HFD, 299 mRNAs and 284 lncRNAs in the GIFT liver showed significant changes in abundance (Table S3), including 137 up-regulated and 162 down-regulated mRNAs (Figure 3A), and 155 up-regulated and 129 down-regulated lncRNAs (Figure 3B). Radar plots were constructed to show the top 20 DE-mRNAs (nine up-regulated and 11 down-regulated mRNAs) (Figure 3C) and the top 20 DE-lncRNAs (13 up-regulated and seven down-regulated lncRNAs) (Figure 3D).

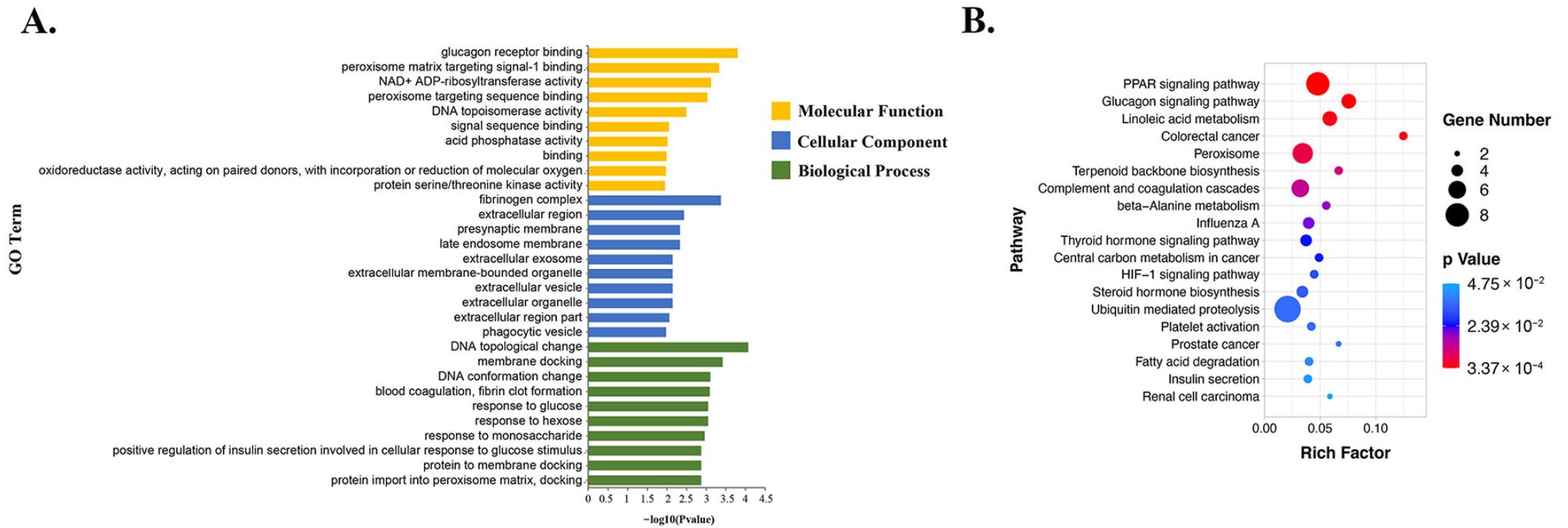


**Figure 3.** Differential expression analysis of lncRNAs and mRNAs. Volcano plot of differentially expressed (DE)-mRNAs (A) and -lncRNAs (B); Radar plots of the top 20 DE-mRNAs (C) and DE-lncRNAs (D).

### 3.5. Functional Enrichment Analysis of DE-mRNAs

A GO enrichment analysis was conducted to investigate the biological functions of DE-mRNAs after 56 days of a HFD (Figure 4A and Figure S2). In the biological process category, DE-mRNAs were mainly enriched in the “cellular process”, “single-organism process” and “metabolic process” subcategories, among which “DNA topological change” and “membrane docking” were the most significantly enriched terms. In the cellular component category, DE-mRNAs were mainly enriched in the “cell”, “cell part”, and “organelle” subcategories, among which “fibrinogen complex” and “extracellular region” were the most significantly enriched terms. In the molecular function category, DE-mRNAs were mainly enriched in the “binding” and “catalytic activity” subcategories, with the top two GO terms being “glucagon receptor binding” and “peroxisome matrix targeting signal-1 binding”.

The DE-mRNAs were further annotated at the KEGG database to identify crucial signaling pathways (Figure 4B). The top 20 KEGG pathways enriched with DE-mRNAs included glycolipid metabolism-related pathways, such as “PPAR signaling pathway”, “glucagon signaling pathway”, and “linoleic acid metabolism”.



**Figure 4.** GO and KEGG enrichment analysis of DE-mRNAs in the liver of the HFD group compared with the NFD group. **(A)** Top 10 GO terms in three categories: biological process, molecular function, and cellular component. **(B)** Top 20 enriched KEGG pathways.

### 3.6. Data Confirmation by qRT-PCR

In order to evaluate the results of the RNA-seq, we selected seven DE-lncRNAs and seven DE-mRNAs related to lipid metabolism for qRT-PCR analyses. The qRT-PCR results were consistent with the RNA-seq data. (Table 3).

**Table 3.** Comparison of qRT-PCR and RNA-seq results of 14 candidates (7 lncRNAs and 7 mRNAs).

Type	Name	qRT-PCR Results (Relative Ratio)		qRT-PCR Results	RNA-Seq Results	Up/Down
		HFD Group	NFD Group	Log <sub>2</sub> Fold Change	Log <sub>2</sub> Fold Change	
mRNA	<i>slc25a48</i>	1.58 ± 0.13 *	0.71 ± 0.10	1.15	3.12	Up
mRNA	<i>faldh</i>	0.29 ± 0.05 *	0.93 ± 0.08	−1.68	−2.75	Down
mRNA	<i>vlacs</i>	1.17 ± 0.12 *	2.51 ± 0.30	−1.10	−3.18	Down
mRNA	<i>fads2</i>	0.29 ± 0.04 *	0.77 ± 0.07	−1.41	−2.30	Down
mRNA	<i>pck1</i>	1.33 ± 0.15 *	0.53 ± 0.13	1.33	4.29	Up
mRNA	<i>ehhadh</i>	0.32 ± 0.04 *	0.90 ± 0.05	−1.49	−2.28	Down
mRNA	<i>fabp7a</i>	0.24 ± 0.04 *	1.23 ± 0.27	−2.36	−5.22	Down
LncRNA	MSTRG.6725.3	4.09 ± 0.26 *	0.53 ± 0.09	2.95	Inf	Up
LncRNA	MSTRG.14598.1	1.83 ± 0.10 *	0.74 ± 0.06	1.31	Inf	Up
LncRNA	MSTRG.32774.1	0.26 ± 0.05 *	0.89 ± 0.15	−1.78	Nam	Down
LncRNA	MSTRG.13364.2	0.34 ± 0.05 *	0.92 ± 0.09	−1.44	Nam	Down
LncRNA	MSTRG.43073.2	0.11 ± 0.02 *	0.93 ± 0.10	−3.08	Nam	Down
LncRNA	MSTRG.24713.1	0.34 ± 0.05 *	1.39 ± 0.12	−2.03	Nam	Down
LncRNA	MSTRG.17221.1	2.50 ± 0.36 *	0.94 ± 0.06	1.41	4.98	Up

\* means significant difference ( $p < 0.05$ ) between HFD and NFD group based on qRT-PCR analysis. *ef1a* and  $\beta$ -actin were used as reference genes. ‘Inf’ means that the lncRNAs were only expressed in HFD group; Compared with that in NFD group, the transcript levels of the selected candidates in HFD group were up-regulated. ‘Nam’ means that the lncRNAs were only expressed in NFD group; Compared with that in NFD group, the transcript levels of the selected candidates in HFD group were down-regulated.

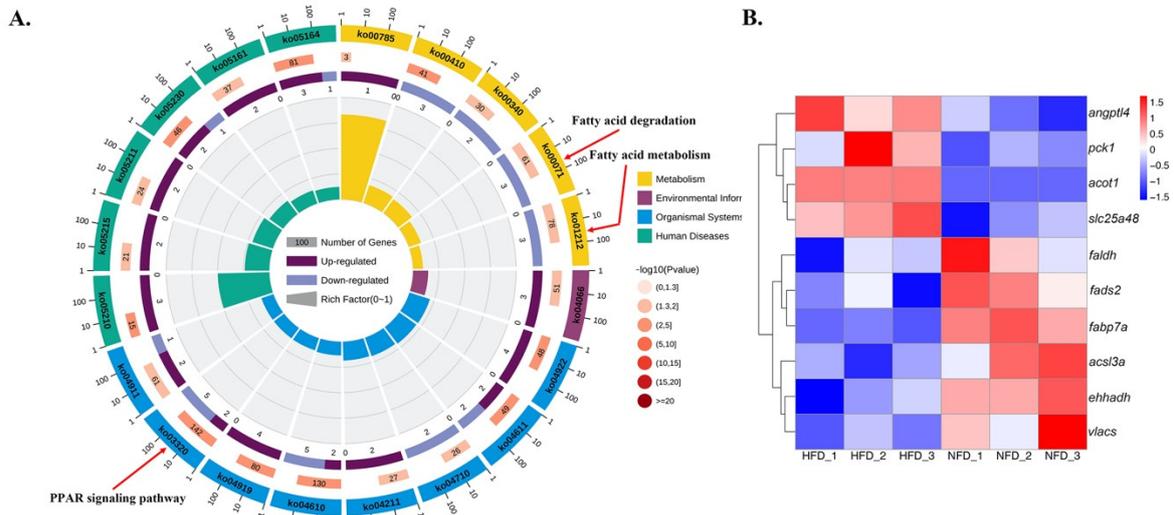
### 3.7. Cis and Trans Roles of DE-lncRNAs

The potential functions of the 284 DE-lncRNAs were further explored by predicting their *cis* and *trans* targets. We found that 39 DE-lncRNAs were located adjacent to 65 protein-coding genes (Table S4), indicating that these lncRNAs might be associated with *cis* regulation of these genes. GO and KEGG enrichment analyses were conducted for these *cis* targets to better understand their functions and those of the *cis*-acting lncRNAs (Table S5). Regarding *trans* action, 3610 interactions were predicted to exist between 237 DE-lncRNAs and protein-coding genes (Table S4). Functional enrichment analysis found that these *trans* targets were significantly enriched in 147 GO terms and 20 KEGG pathways (Table S6), including several GO terms related to lipid metabolism, such as “lipid metabolic process”, “linoleic acid metabolic process”, and “unsaturated fatty acid biosynthetic process”. The pathways related to lipid metabolism included PPAR signaling, fatty acid degradation, and fatty acid metabolism pathways (Figure 5A). A heat map was constructed to highlight 10 selected target DE-mRNAs involved in these three pathways (Figure 5B), and an interaction network between these 10 lipid metabolism-related DE-mRNAs and DE-lncRNAs was generated using Cytoscape software (Figure 6). As shown in Figure 6, the *trans*-regulatory interaction network was quite complex. Some mRNAs (e.g., *ehhadh* encoding peroxisomal bifunctional enzyme, and *slc25a48* encoding solute carrier family 25 member 48) were regulated by multiple lncRNAs (MSTRG.24713.1, MSTRG.7400.1, and others), and one lncRNA (MSTRG.30660.2) regulated multiple mRNAs (*fabp7a* encoding fatty acid-binding protein7a, *faldh* encoding fatty aldehyde dehydrogenase, and *acsl3a* encoding long chain fatty acid-CoA ligase 3a).

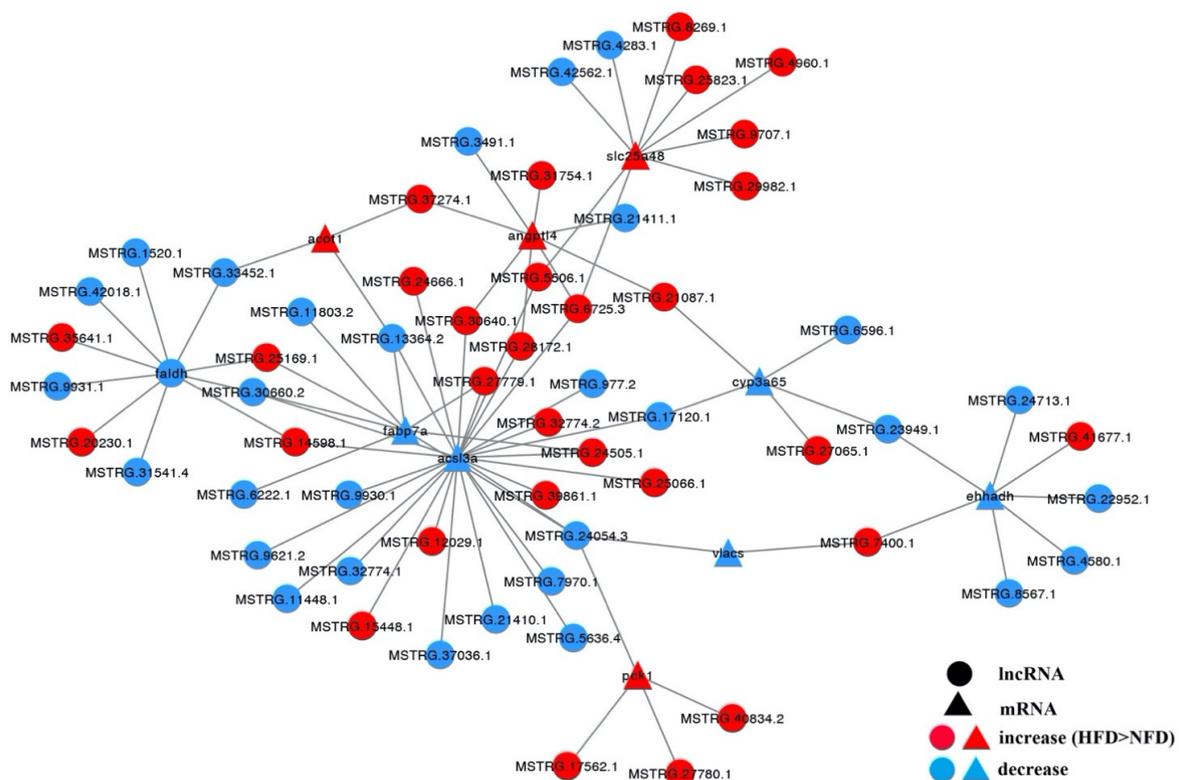
### 3.8. Expression Patterns of Several DE-lncRNAs and Their Lipid Metabolism-Related Target mRNAs in Response to a High-Fat Diet

On the basis of the results of the *cis* and *trans* action analyses, three lncRNAs (MSTRG.14598.1, MSTRG.6725.3, and MSTRG.13364.2) and their corresponding target mRNAs (*faldh*, *slc25a48*, and *fabp7a*) were selected for further analyses. Their expression pat-

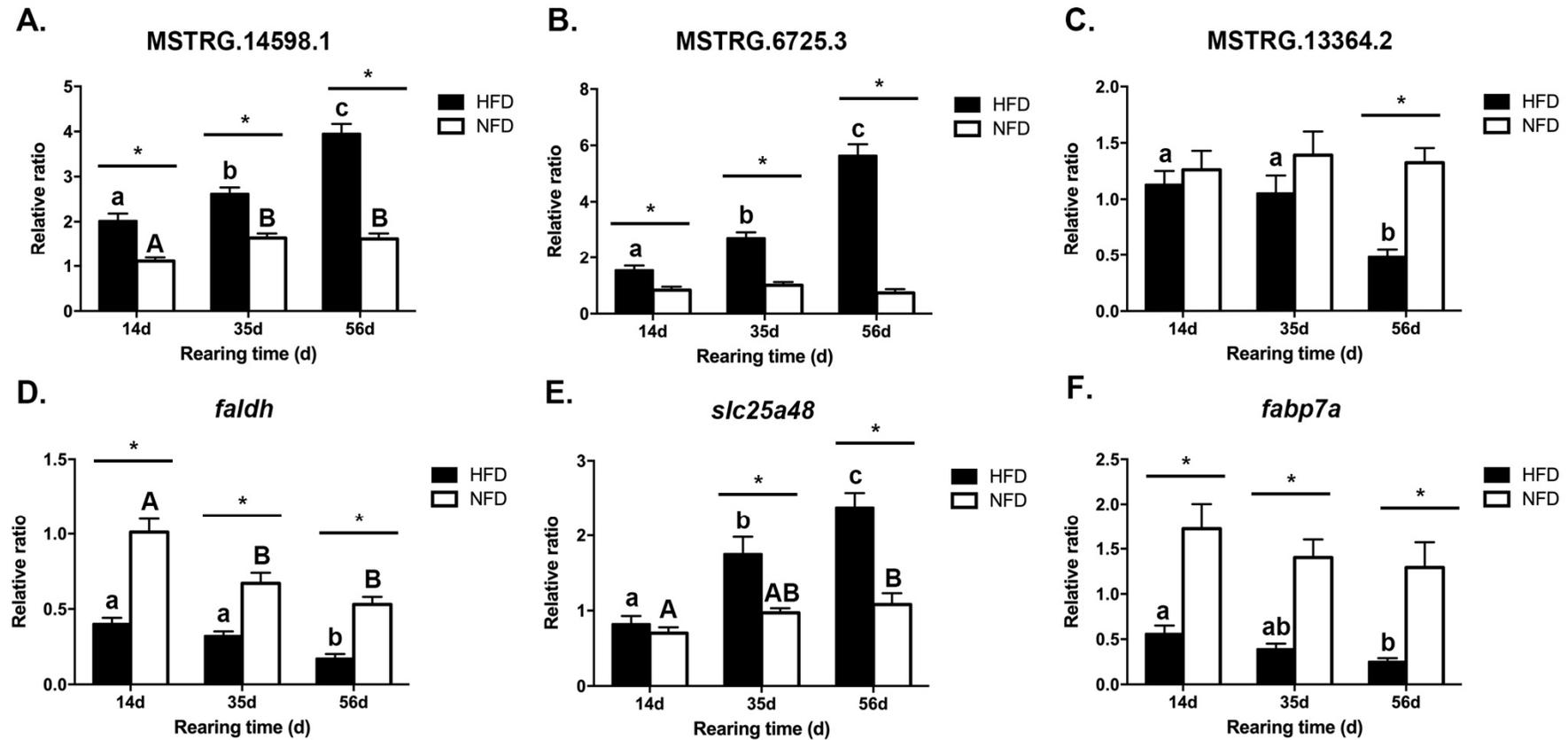
terns on days 14, 35, and 56 were determined (Figure 7). HFD exposure showed significantly up-regulated levels of MSTRG.14598.1 on day 14, day 35, and day 56 ( $p < 0.05$ ) (Figure 7A), and similar trends in MSTRG.6725.3 (Figure 7B). The transcript level of MSTRG.13364.2 was not significantly different on days 14 and 35 ( $p > 0.05$ ), but was significantly lower on day 56 after high-fat feeding ( $p < 0.05$ ) (Figure 7C).



**Figure 5.** (A) Results of KEGG enrichment analysis showing lipid metabolism-related pathways enriched with genes that are potentially *trans*-regulated by lncRNAs. (B) Heatmaps and hierarchical clustering of 10 selected mRNAs. Red, high expression; blue, low expression.



**Figure 6.** LncRNA–mRNA interaction network in HFD vs. NFD groups. Ellipses represent lncRNAs, triangles represent mRNAs; red represents up-regulation (HFD vs. NFD) and blue represents down-regulation.



**Figure 7.** Expression patterns of selected DE-lncRNAs and their lipid metabolism-related target mRNAs in the liver in the HFD group on days 14, 35, and 56. (*n* = 9). (A) MSTRG.14598.1; (B) MSTRG.6725.3; (C) MSTRG.13364.2; (D) *faldh*; (E) *slc25a48*; (F) *fabp7a*. \* indicates significant difference ( $p < 0.05$ ) between HFD and NFD groups at the same sampling time; different lowercase letters indicate significant difference ( $p < 0.05$ ) in the HFD group among different sampling times. Different capital letters indicate significant difference ( $p < 0.05$ ) in the NFD group among different sampling times.

The transcript levels of *faldh* and *fabp7a* were significantly lower in the HFD group than in the NFD group from day 14 onward ( $p < 0.05$ ) (Figure 7D,F). However, the transcript level of *slc25a48* was significantly higher in the HFD group than in the NFD group ( $p < 0.05$ ) from day 35 onward (Figure 7E).

#### 4. Discussion

China is the world's largest producer and trader of tilapia, although it is farmed worldwide [33]. Intensively farmed tilapia frequently suffer from fatty liver disease, which negatively affects their health and growth [4,5]. Previous studies have explored the regulatory mechanism of hepatic lipid metabolism in tilapia. For example, some key lipid metabolism genes have been functionally analyzed [34,35]. Our previous research suggested that miRNAs (e.g., miR-122, miR-29a, miR-145-5p) may be regulators of lipid metabolism in tilapia [4]. However, the functional roles of lncRNAs in tilapia were largely unknown. In this study, in an effort to better understand the roles of lncRNAs in lipid metabolism, we explored the hepatic lncRNA profiles of GIFT fed diets containing normal (8%) or high (18.5%) lipid levels using RNA-seq.

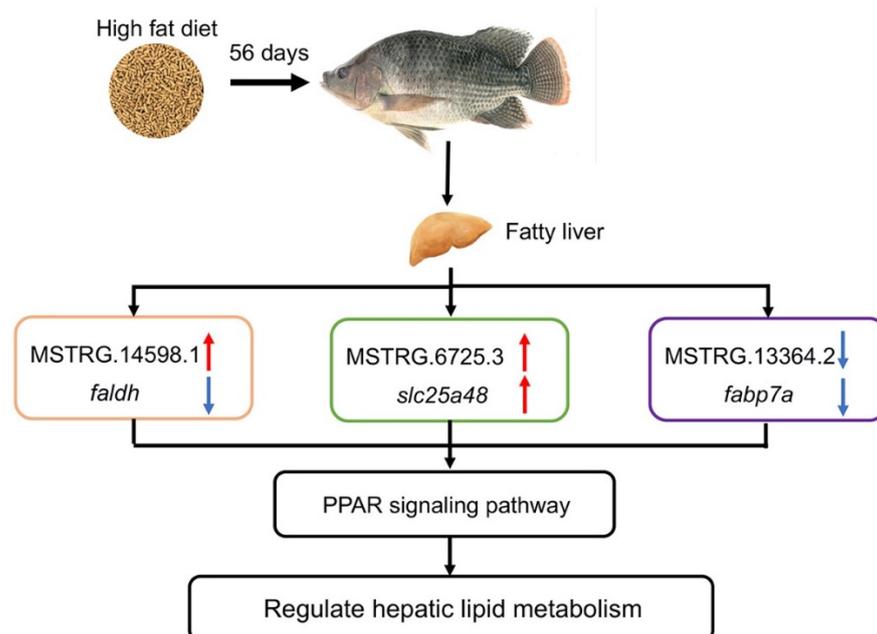
We used a rigorous filtering process to obtain 19,928 high-confidence lncRNAs from the livers of tilapia fed a HFD and an NFD. These lncRNAs had shorter transcript lengths and fewer exons compared with protein-coding mRNAs, consistent with the results of other studies on the lncRNAs of different freshwater fish [36,37]. We also identified 299 DE-mRNAs and 284 DE-lncRNAs in the liver between the HFD group and the NFD group. The reliability of RNA-seq data was confirmed by qRT-PCR. We found that the expression trend of all candidates was consistent with the RNA-seq results. However, there is a certain discrepancy between fold difference measured by qRT-PCR and RNA-seq analyses, and some reports found that these differences are normal and reasonable [38,39], which may be due to the different detection method between of sequencing and qRT-PCR and the relative low expression of candidate DE-lncRNAs in this study. Similar results have been reported previously [40,41], indicating that the identified candidates in the present study were reliable. Studies in non-fish animals have clearly demonstrated that some lncRNAs are critical regulators of lipid biosynthesis, lipid catabolism, and lipid transport to modulate lipid homeostasis [42,43]. Therefore, we speculate that these DE-lncRNAs identified here also affect liver lipid metabolism in tilapia.

lncRNAs can act as *cis*-regulators by targeting nearby protein-encoding genes [44]. Previous reports have documented some of the lncRNAs that play *cis* regulatory roles in hepatic lipid metabolism. For example, in chicken (*Gallus domesticus*), lncRNA-FNIP2 *cis*-regulates *LPIN1*, thereby regulating hepatic adipogenesis [45]. In blunt snout bream (*Megalobrama amblycephala*), lncRNA-MSTRG.6100 regulates two upstream *cis* targets, the genes encoding histone-lysine *N*-methyltransferase SET and MYND domain-containing protein 3, to regulate hepatic lipid metabolism [46]. Our analyses predicted 39 DE-lncRNAs with *cis*-acting regulation of 65 protein-coding genes. Identification of the functions of the targeted *cis*-regulated genes can provide clues about the regulatory functions of the DE-lncRNAs. lncRNAs can also regulate hepatic lipid metabolism via a *trans*-acting mechanism. For example, lncRNA-FLRL2 down-regulates the gene encoding the aryl hydrocarbon receptor nuclear translocator-like protein by a *trans*-acting mechanism, thereby participating in non-alcoholic fatty liver disease pathogenesis [47]. lncRNA-XLOC\_001424 *trans*-regulates the expression of the gene encoding fatty acid desaturase 2 to promote non-alcoholic steatohepatitis in bama minipig (*Sus scrofa domestica*) [48]. In the present study, we identified 3610 pairs of DE-lncRNAs and *trans*-regulated targets. Thus, our data indicate that lncRNAs may participate in hepatic lipid metabolism in GIFT through *cis*- or *trans*-acting mechanisms.

Many pathways are related to lipid metabolism, such as the PPAR signaling pathway [49], the SREBP signaling pathway [50], the AMPK signaling pathway [51], and the fatty acid metabolism signaling pathway [52]. However, little was known about the roles of lncRNAs in regulating lipid metabolism in fish. The functions of lncRNAs are predicted

mainly based on their target genes and related pathways [53]. In our analyses, we found that seven KEGG pathways were enriched with *cis* target genes and 20 KEGG pathways were enriched with *trans* target genes of the DE-lncRNAs. Several crucial pathways related to lipid metabolism were enriched with targets of DE-lncRNAs, including the PPAR signaling pathway, the fatty acid metabolism signaling pathway, the fatty acid degradation signaling pathway, and the alpha-linolenic acid metabolism signaling pathway. These findings strongly suggest that these are the key pathways by which DE-lncRNAs participate in the regulation of lipid metabolism.

To further investigate the specific roles of three lncRNAs (MSTRG.14598.1, MSTRG.6725.3 and MSTRG.13364.2) in lipid metabolism, we analyzed their expression patterns and those of their potential targets (*faldh*, *slc25a48*, and *fabp7a*), all of which are involved in key lipid metabolism signaling pathways, on day 14, day 35, and day 56. *fabp7a* and *slc25a48* are two important regulators of the PPAR signaling pathway (Figure 8), and *fabp7* is involved in fatty acid uptake and intracellular transport (Liu et al., 2021). Silencing of FABP7 was shown to relieve stearic acid-induced lipid accumulation in Hepa 16 cells [54]. *slc25a48* encodes a member of the *slc25* family that transports fatty acids across the mitochondrial membrane [55]. In this study, *fabp7* was down-regulated and *slc25a48* was up-regulated in the HFD group compared with the NFD group. Studies have shown that fish can change their lipid metabolism strategies, namely reduce lipogenesis and increase  $\beta$ -oxidation of fatty acids, to adapt to excessive lipid intake [56,57]. Therefore, in the HFD group, the down-regulation of *fabp7* may have helped to reduce fatty acid uptake into hepatocytes, while the up-regulation of *slc25a48* may have enhanced fatty acid transport to the mitochondria for  $\beta$ -oxidation. Interestingly, we detected positive relationships between the levels of the lncRNAs MSTRG.6725.3 or MSTRG.13364.2 and those of *slc25a48* and *fabp7a* transcripts, respectively, in the HFD group. LncRNAs bind to the 3'-untranslated (UTR) region of the target gene's mRNA and block a large number of miRNA target sites, thereby maintaining the stability of the target mRNA [58]. LncRNA-PXN-AS1-L was suggested to bind to the 3'-UTR of *PXN* mRNA, thereby stabilizing it and increasing *PXN* expression, ultimately promoting the growth of tumor cells [59]. We speculate that MSTRG.6725.3 and MSTRG.13364.2 may stabilize transcripts of their target genes *slc25a48* and *fabp7a*, respectively, thereby positively regulating their expression and maintaining lipid homeostasis.



**Figure 8.** Diagram of lncRNA-mediated regulated pathway in the liver of GIFT fed a HFD for 56 days.

FALDH is a crucial enzyme for phytic acid decomposition [60]. Activation of FALDH can protect cells from oxidative stress associated with lipid peroxidation [60]. The expression of *FALDH* is regulated by the PPAR $\alpha$  signaling pathway [60]. In the present study, *faldh* was down-regulated in the HFD group, which had disrupted hepatic lipid metabolism. This may have led to the accumulation of lipid peroxides and, consequently, oxidative stress injury. LncRNAs can form complementary double strands with transcripts of protein-encoding genes, and produce endogenous siRNAs under the action of Dicer enzymes. In this way, they can silence gene expression [58]. In this study, MSTRG.14598.1 was up-regulated and *faldh* was down-regulated in the HFD group, indicating that the interaction between MSTRG.14598.1 and *faldh* might play a crucial role in hepatic fatty acid metabolism.

## 5. Conclusions

To our knowledge, this is the first report on the differences in hepatic lncRNA-mRNA expression profiles between GIFT fed a NFD and those fed a HFD. A total of 299 DE-mRNAs and 284 DE-lncRNAs were identified based on RNA-seq data analyses. The transcript profiles of seven DE-lncRNAs and seven DE-mRNAs related to lipid metabolism were verified by qRT-PCR. Functional enrichment analyses of *cis*- and *trans*-targets of DE-lncRNAs revealed that these DE-lncRNAs were involved in regulating key signaling pathways in lipid metabolism, such as PPAR signaling, fatty acid degradation, and fatty acid metabolism. Analyses of the expression patterns of three lncRNAs (MSTRG.14598.1, MSTRG.6725.3, and MSTRG.13364.2) and their potential targets (*faldh*, *slc25a48*, and *fabp7a*) revealed that these lncRNAs might participate in lipid metabolism by post-transcriptionally regulating their respective target genes. Although further experimental analyses are required to test the interactions between these lncRNAs and mRNAs, the information gained in this study provides new insights into the molecular mechanism of lncRNA-mediated lipid metabolism in tilapia. The lncRNAs identified in this study will serve as a reference for further research on the regulation of lipid metabolism in tilapia.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fishes7060332/s1>, Figure S1: Density plot (A) showing the distribution of expression density of each sample. Box plot (B) showing the distribution of maximum, minimum and percentile values for normalized signals of each sample; Figure S2: DE-mRNAs enriched in three categories: biological process, molecular function and cellular component, and their main level 2 GO terms. Table S1: Ingredients and composition of NFD and HFD; Table S2: Overview of hepatic sequencing data and quality filtering.

**Author Contributions:** P.X. and J.Q. conceived and designed the experiments, S.L. performed the experiments. T.Z. analyzed the RNA-seq data. M.L. conducted qRT-PCR experiments. Y.T. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** All raw sequencing data have been deposited in NCBI and Sequence Read Archive databases under the accession number GSE 211712 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE211712>) (accessed on 21 August 2022) with secure token "gxyrougctb0fhcj".

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**Conflicts of Interest:** The authors declare that they have no competing interest.

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