Research Article

Circular RNA Profiling and Bioinformatic Modeling Identify Its Regulatory Role in Hepatic Steatosis

Xing-Ya Guo,1 Chong-Xin He,1 Yu-Qin Wang,1 Chao Sun,1 Guang-Ming Li,1 Qing Su,2 Qin Pan,1 and Jian-Gao Fan1,3

1Department of Gastroenterology, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200092, China
2Department of Endocrinology, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200092, China
3Shanghai Key Laboratory of Children's Digestion and Nutrition, Shanghai 200092, China

Correspondence should be addressed to Qin Pan; pan_qin@yeah.net and Jian-Gao Fan; fattyliver2004@126.com

Received 8 January 2017; Accepted 7 March 2017; Published 21 June 2017

Academic Editor: Elena Grasselli

Copyright © 2017 Xing-Ya Guo et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Circular RNAs (circRNAs) exhibit a wide range of physiological and pathological activities. To uncover their role in hepatic steatosis, we investigated the expression profile of circRNAs in HepG2-based hepatic steatosis induced by high-fat stimulation. Differentially expressed circRNAs were subjected to validation using QPCR and functional analyses using principal component analysis, hierarchical clustering, target prediction, gene ontology (GO), and pathway annotation, respectively. Bioinformatic integration established the circRNA-miRNA-mRNA regulatory network so as to identify the mechanisms underlying circRNAs’ metabolic effect. Here we reported that hepatic steatosis was associated with a total of 357 circRNAs. Enrichment of transcription-related GOs, especially GO: 0006355, GO: 004589, GO: 0045944, GO: 0045892, and GO: 0000122, demonstrated their specific actions in transcriptional regulation. Lipin 1 (LPIN1) was recognized to mediate the transcriptional regulatory effect of circRNAs on metabolic pathways. circRNA-miRNA-mRNA network further identified the signaling cascade of circRNA_021412/miR-1972/LPIN1, which was characterized by decreased level of circRNA_021412 and miR-1972-based inhibition of LPIN1. LPIN1-induced downregulation of long chain acyl-CoA synthetases (ACSLs) expression finally resulted in the hepatosteatosis. These findings identify circRNAs to be important regulators of hepatic steatosis. Transcription-dependent modulation of metabolic pathways may underlie their effects, partially by the circRNA_021412/miR-1972/LPIN1 signaling.

1. Introduction

Hepatic steatosis reflects a pathological disorder characterized by excess triglyceride (TG) accumulation (>5% of volume or weight) in the liver and demonstrates close association with obesity, type 2 diabetes, hyperlipidemia, and other components of metabolic syndrome with limited exceptions (i.e., alcohol abuse, chronic hepatitis C) [1–3]. Serving as the hallmark of nonalcoholic fatty liver disease (NAFLD), hepatic steatosis predisposes patients to nonalcoholic steatohepatitis (NASH), liver fibrosis/cirrhosis, and the final outcome of hepatocellular carcinoma (HCC) [4]. High incidence of extrahepatic death and disability (i.e., cardiovascular events, cerebral apoplexy, and cancers) also takes place in the population with hepatic steatosis [5–7]. Recent decades have witnessed a dramatic prevalence of hepatic steatosis increase worldwide, ranging in 24%–46% in the western countries and 7.9%–54% in the Asia-Pacific area [8–11], which suggests the coming emergency and serious burden of public health. For the sake of our limited understanding in its mechanisms, hepatic steatosis is still beyond the reach, to a large extent, of clinical interference.

Circular RNA (circRNA), a class of noncoding RNAs with the linking of 3’ and 5’ ends, used to be regarded as the nonfunctional byproducts of mRNA splicing [12]. However, characteristics of circRNA, including tissue- and development-specific expression, enrichment of miRNA response element (MRE), and resistance to both RNase R
and RNA exonuclease, highlight its potential role in the gene regulation of eukaryotes [13]. Studies indeed prove that circ-HIPK3 governs the proliferation of seven kinds of cancer cells via binding to miR-124 [14], while formation of circ-Foxo3-p21-CDK2 ternary complex induces the cell cycle arrest in noncancer cells [15]. Cdr1as and mmu-circRNA-015947 are well described to act as the regulator in insulin secretion and cerebral ischemia-reperfusion injury, respectively [16, 17]. Moreover, HRCR protects the heart from pathological hypertrophy and heart failure by targeting miR-223 [18]. Thus circRNAs are of physiological and pathological importance in various species, mainly on the basis of being complimentary to the “seed sequence” of miRNAs. But the effect of circRNAs on hepatic steatosis remains to be clarified until now.

We, therefore, investigated the expression profile of circRNAs in HepG2-based hepatic steatosis induced by high-fat stimulation. Targetome of these differentially expressed circRNAs was established at both miRNA and mRNA levels via complementation-dependent target prediction. Then gene ontology (GO) terms and pathway annotation were employed for functional analysis on the basis of circRNAs' targetome. Integrating significant GOs and the underlying pathways, circRNA-miRNA-mRNA network was constructed to identify the key circRNAs, and finally their action model, in steatogenesis.

### 2. Materials and Methods

#### 2.1. Establishment of Hepatic Steatosis by High-Fat Stimulation.

HepG2 cells (Cell Bank of Type Culture Collection, China), the cell line of human hepatocarcinoma, in the exponential phase were randomized into groups of control \((n = 3)\) and model \((n = 3)\), respectively, and seeded in the 96-well plate at \(4 \times 10^3\)/well. In contrast to the control group cultured by Dulbecco's modified Eagle's medium (DMEM, HyClone Laboratories, Inc., USA), penicillin-streptomycin, and 10% fetal bovine serum (FBS, Gibco BRL, USA), HepG2 cell in the model group were incubated additionally with 0.5 mM free fatty acid (FFA, oleate:palmitate = 2:1; Sigma-Aldrich, USA) dissolved in dimethyl sulfoxide (DMSO) [19].

#### 2.2. Oil Red-O Staining and Triglyceride Assay.

After the treatment of FFA for 24 hours, both control and model groups were evaluated by Oil Red-O staining. In this assay, formaldehyde-fixed HepG2 cells were administrated using 0.5% Oil Red-O in isopropyl alcohol for 20 minutes and then counterstained using hematoxylin for 1 minute. TG level was also enzymatically measured in the HepG2 cells of both groups using TG assay kit (Applygen Technologies Inc., China) according to the manufacturer's instructions.

#### 2.3. Microarray Hybridization for circRNA Profile.

Total RNA, derived from both control and model groups, was extracted by the TRIzol reagent. The RNA purity and integrity of each sample were checked using light absorbance (260 nm/280 nm) and agarose gel electrophoresis, respectively. To remove linear RNAs and enrich circRNA, total RNA from each sample was treated with Rnase R. circRNA was then transcribed into fluorescent cRNA by random primer according to the Arraystar Super RNA Labeling protocol (Arraystar, Inc., USA) and hybridized onto the Arraystar Human circRNA Arrays V2.0 (Arraystar, Inc., USA) at 65°C for 17 hours. After washing, the arrays were subjected to scanning by Agilent Scanner G2505C (Agilent, USA) and data extraction by Agilent Feature Extraction software (Agilent, USA). Finally, differentially expressed circRNAs between two groups were filtered using fold change cutoff (>2, or <0.5) and statistical significance. Both unsupervised hierarchical clustering (Cluster 3.0) and TreeView analysis (Stanford University, USA) were performed to these differentially expressed circRNAs.

#### 2.4. Quantitative Real-Time Polymerase Chain Reaction.

Six differentially expressed circRNAs were selected at random and verified by Mir-X miRNA First Strand Synthesis Kit (Takara, Da Lian, China) using Applied Biosystems 7500 Real-Time PCR Detection Systems (Bio-Rad, USA). The circRNA levels, being normalized against GAPDH [20, 21], were evaluated by the \(2^{-\Delta\Delta C_t}\) method [22]. Triplicates were performed for each sample in three independent experiments. Primers used for stem-loop RT-PCR are shown in Table 1.

#### 2.5. Principal Component Analysis.

Principal component analysis (PCA) was employed to investigate the overall relationships between circRNA expression and groups through dimensionality reduction and feature extraction. The analysis was conducted using R software 3.3.1 (R Development Core Team) [23–25].

### Table 1: Primers for qPCR.

| Gene                | Primer sequence (5’-3’) | Product length (nt) |
|---------------------|------------------------|---------------------|
| hsa_circ_002082     | F: CCAGCTGAGTGATAAAGGCTGA R: TCGTTCCTCCGCTCAATCTC | 158 |
| hsa_circ_004183     | F: CGTCCATTCCAGGAAGGTTCT | 113 |
| hsa_circ_000367     | F: CAATTGCAATTTCTCGACT | 114 |
| hsa_circ_007850     | F: CAGAAAGAAGGGGAAATTACAG | 106 |
| hsa_circ_004121     | F: CTTTGCTGGGAAATCAACAGA | 112 |
| hsa_circ_014724     | F: TCAGATATTCGCGGACCCAG | 144 |
| GAPDH               | F: TCAAGGGCTGAGACAGGAAG R: TGGACTCCACGAGTACTCA | 117 |
2.6. GO Terms and Signal Pathway Annotation. Target miRNAs of the differentially expressed circRNAs were predicted using Arraystar’s home-made miRNA target prediction software according to method of MRE-based circRNA/microRNA complementation (Arraystar Inc., USA) [26, 27]. miRNAs downstream to these miRNAs were pooled to construct the targetome by mirdb5.0 and targetscan71 databases [28]. Then, GO term analysis was applied to differentially expressed circRNAs on the basis of targetome using DAVID gene annotation tool (http://david.abcc.ncifcrf.gov/) [29]. In detail, two-side Fisher’s exact test was used to classify the GO category, while the false discovery rate (FDR) was calculated to correct the P value [30]. Those GOs with P value < 0.01 and FDR < 0.01 were filtered to be of statistical significance.

Similarly, pathway analysis uncovered the significant pathways related to differential expressed circRNAs according to the annotation of Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/) [31]. The threshold of significance was defined by P value (<0.01) and FDR (<0.01) as mentioned above. The gene interaction within GOs and pathways, respectively, was investigated by databases of Ingenuity® Pathway Analysis (IPA), KEGG, and PubMed [32].

2.7. Bioinformatics Modeling for the Regulatory Network of circRNAs. Both the circRNA-miRNA and miRNA-mRNA interactions, as previously described by Targetscan and miRanda algorithm [28], were integrated to delineate the regulatory network of steatosis-related circRNAs. First, the adjacency matrix of circRNA and miRNA was given by

\[
A = \begin{bmatrix}
a_{i,j}
\end{bmatrix}
\]

where \(a_{i,j}\) represents the weight of relation between miRNA (i) and circRNA (j) [33]. Similar matrix of mRNA and miRNA was also obtained by their weighted relation. Second, the circRNA-miRNA-mRNA network was established on the basis of both adjacency matrixes by Cytoscape 3.1.0 software [34]. Additionally, mRNA sets of the significant GOs and pathways were cross-intersected so as to identify the critical genes that mediate circRNAs’ pathway-dependent effects on biological processes. By the circRNA-miRNA-mRNA network, we uncovered the signaling cascade of circRNAs, miRNAs, and critical mRNAs with great importance to circRNAs’ regulatory role in hepatic steatosis.

2.8. Statistics. All the results were expressed as mean ± standard deviation (SD). Statistical analysis was performed by Student’s t-test for the comparison of different groups using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA) [35], whereas Fisher’s exact test was employed to filter the significant GOs and pathways using R software 3.3.1 (R Development Core Team). In both cases, P value of less than 0.05 (two-tailed) was considered statistically significant.

3. Results

3.1. Dysregulation of circRNA Profile in HepG2-Based Hepatic Steatosis. When compared to those of the normal group, HepG2 cells of the model group demonstrated plentiful lipid droplets positive to Oil Red-O staining after the high-fat stimulation for 24 hours (Figure 1(a)). In consistence to the phenotype alternation, significant upregulation in TG content characterized the model group (Figure 1(b)), reflecting the establishment of hepatic steatosis.

Using the Human circRNA Arrays platform with 13,617 labeled probes, we assessed the expression profiles of circRNA in normal and model groups (Figures 2(a) and 2(b)). A total of 357 circRNAs was determined to be dysregulated (Table 2), and distinctly separated samples into biologically interpretable groups on a basis of hierarchical clustering and volcano plot. Among these differentially expressed circRNAs, 154 circRNAs were identified to be upregulated more than 2-fold in steatotic HepG2 cells as compared to that in normal ones (Table 3) (Figures 2(c) and 2(d)). In contrast, 203 circRNAs exhibited the decreased level less than threshold (0.5-fold) during the progression of hepatic steatosis (Table 3) (Figures 2(c) and 2(d)).

Six circRNAs among these filtered ones were validated to be significantly different between the normal and model groups. In well consistence to the results of microarray hybridization, expression levels of hsa_circRNA_002082, hsa_circRNA_000367, and hsa_circRNA_004183 were down-regulated in the model group rather than the normal one (P < 0.01, Figures 2(f) and 2(g)), while hsa_circRNA_007850, hsa_circRNA_004121, and hsa_circRNA_014724 showed an opposite expression pattern (P < 0.01, Figures 2(e) and 2(g)).

3.2. Transcriptional Regulation Characterized the Effects of circRNAs. According to the complementary binding of microRNAs and MRE-containing sequences (Figure 3), 1,766 miRNAs and 17,876 mRNAs were predicted to be regulated by the differentially expressed circRNAs (Table 3). GO and pathway analyses further categorized these mRNAs depending on biological process and molecular function. After the filtration with thresholds of P value and FDR, 225 upregulated and 231 downregulated GOs revealed the functional effects of differentially expressed circRNAs. The top-ranking, downregulated GOs were regulation of transcription, DNA-dependent (GO: 0006355), multicellular organismal development (GO: 0007275), positive regulation of transcription, DNA-dependent (GO: 0045893), negative regulation of transcription, DNA-dependent (GO: 0045892), and positive regulation of transcription from RNA polymerase II promoter (GO: 0045944), and so forth (Figure 4(b)). On the other side, significantly upregulated GOs corresponding to circRNAs appeared to be multicellular organismal development (GO: 0007275), regulation of transcription, DNA-dependent (GO: 0006355), positive regulation of transcription, DNA-dependent (GO: 0045893), protein transport (GO: 0015031), negative regulation of transcription, DNA-dependent (GO: 0045892), and positive regulation of transcription from RNA polymerase II promoter (GO: 0045944), and so forth (Figure 4(a)). Transcriptional regulation, therefore, dominated both up- and downregulated GOs, suggesting a transcription-specific effect of steatosis-related circRNAs.
Pathway annotation provided us with another insight into the actions of circRNAs. As evaluated by the statistical significance and FDR, there were multiple up- and downregulated pathways with metabolic characteristics, including metabolic pathways (hsa01100), insulin signaling pathway (hsa04910), insulin resistance (hsa04931), and so forth (Figures 4(c) and 4(d)). Because of the HepG2 cells employed in our experiments, a lot of top-ranking pathways (i.e., pathways in cancer (hsa05200), proteoglycans in cancer (hsa05205), and transcriptional misregulation in cancer (hsa05202)) play essential roles in carcinogenesis, no matter in the up- or downregulated pathway sets (Figures 4(c) and 4(d)). Thus metabolic regulation was also indicated to underlie the effects of circRNAs.

Gene sets of GOs and pathways were then intersected for data mining. Interestingly, lipin 1 (LPIN1) was recognized to be the member of both downregulated transcription-regulating GO (regulation of transcription, DNA-dependent) and downregulated metabolic pathways. The bridging of GO and pathway qualified LPIN1 a critical part in circRNAs' regulation of hepatic steatosis.

3.3. circRNA_021412-miR-1972-LPIN1 Signaling Reflected the Regulatory Mechanisms Underlying Steatosis-Related circRNAs. In order to outline the global action of circRNAs in transcriptional regulation, we selected the differentially expressed circRNAs, target miRNAs with high-complementary activity (≤ 5 miRNAs for each circRNA), and gene sets of transcription-regulating GOs for bioinformatic modeling. Afterwards, delineation of the circRNA-miRNA and miRNA-mRNA interactions constructed a circRNA-miRNA-mRNA regulatory network comprising 357 circRNAs, 1,766 miRNAs, and 17,876 mRNAs (Figure 5(a)). Characteristically, this multilayer, complex network was featured by signaling cascade, to a large extent, in multi-to-multi-manner.

Taking advantage of the circRNA-miRNA-mRNA network, LPIN1 was found to be successively regulated by hsa-circRNA_021412 and hsa-miR-1972. LPIN1 also serves as the
Figure 2: Continued.
Figure 2: circRNA profiles differentiate the normal group from model group. (a) Principal component analysis (PCA) shows the difference between normal and model groups. (b) Scatter plots assess the variation of circRNA expression between two groups. The values plotted on x and y axes are the normalized signal values of each group (log2 scaled). Dots above the top green line and below the bottom green line represent differentially expressed circRNAs. (c, d) Unsupervised hierarchical clustering (c) and volcano plot (d) demonstrate the differential expression of circRNAs during hepatic steatosis. Both downregulated (green) and upregulated (red) circRNAs were visualized in the cluster. In similar, the red points in volcano plot represent the differentially expressed circRNAs with statistical significance. (e, f) Validation of the upregulated (e) and downregulated circRNAs (f) using QPCR. (g) The results of QPCR exhibit well consistence with those of microarray. Pairwise scatter plots reflect the fold changes (log2 transformed) of both microarray (horizontal axis) and the QPCR (vertical axis). The R stands for linear correlation coefficient. The presented results are expressed as means ± SD. "" P < 0.01.
pendent role of circRNA in metabolic regulation and steatosis important mechanism that underlay the transcription-de-

process of lipid degradation (Figure 5(c)). Resultantly, the

by KEGG database, ACSLs among these ones initiate the

SCP2 (Figure 5(b)), via PPARα, ACSL1, ACSL3, ACSL4, ACSL6, ACS22, G6PC, MEI, and SCP2 (Figure 5(b)), via PPARα activation [36]. As defined by KEGG database, ACSLs among these ones initiate the process of lipid degradation (Figure 5(c)). Resultantly, the circRNA_021412/miR-1972/LPIN1 signaling uncovered an important mechanism that underlay the transcription-dependent role of circRNA in metabolic regulation and steatosis induction (Figure 6).

4. Discussion

Mimicking high-fat diet that induces NAFLD, HepG2 cells were administrated using the mixture of saturated (palmitate) and unsaturated fatty acids (oleate) at a ratio of 1:2 [37]. Both the lipid droplets positive to Oil Red-O staining and the significant increase in TG content indicated the establishment of hepatic steatosis. When evaluated, by the microarray profiling, 154 up- and 203 downregulated circRNAs, representing 2.6% of the total amount, and characterized the model group as compared to those of the normal group. Moreover, two groups of HepG2 cells, with or without steatosis, were distinctly differentiated by these circRNAs on the basis of PCA, unsupervised hierarchical clustering, and volcano plot, respectively. circRNAs, therefore, are suggested to play a pathological role underlying the hepatic steatogenesis.

circRNAs have been widely accepted to function as the sponge for miRNAs and the competing endogenous RNAs for mRNAs, mainly on the basis of complementation between MRE and “seed sequence” [12]. Thus joint analysis of large-scale profiling coupled with computational prediction represents a powerful approach to elucidate the possible biological roles of circRNAs. Accordingly, differentially expressed circRNAs were subjected to target scanning by circRNA-miRNA, and miRNA-mRNA interaction. A total of 17,876 circRNAs were subjected to target scanning by circRNA-miRNA, and miRNA-mRNA interaction. A total of 17,876

circRNAs have been widely accepted to function as the sponge for miRNAs and the competing endogenous RNAs for mRNAs, mainly on the basis of complementation between MRE and “seed sequence” [12]. Thus joint analysis of large-scale profiling coupled with computational prediction represents a powerful approach to elucidate the possible biological roles of circRNAs. Accordingly, differentially expressed circRNAs were subjected to target scanning by circRNA-miRNA, and miRNA-mRNA interaction. A total of 17,876

circRNAs have been widely accepted to function as the sponge for miRNAs and the competing endogenous RNAs for mRNAs, mainly on the basis of complementation between MRE and “seed sequence” [12]. Thus joint analysis of large-scale profiling coupled with computational prediction represents a powerful approach to elucidate the possible biological roles of circRNAs. Accordingly, differentially expressed circRNAs were subjected to target scanning by circRNA-miRNA, and miRNA-mRNA interaction. A total of 17,876

Table 2: Predicted targets of top-20 differentially expressed circRNAs.

| Gene            | Regulation | Location | Target |
|-----------------|------------|----------|--------|
| hsa_circRNA_400019 | Up         | chr1     | hsa-miR-1298-3p hsa-miR-612 hsa-miR-204-3p hsa-miR-20b-3p hsa-miR-135a-3p |
| hsa_circRNA_006560 | Up         | chr3     | hsa-miR-6731-5p hsa-miR-3662 hsa-miR-4709-3p hsa-miR-6831-5p hsa-miR-98-5p |
| hsa_circRNA_010561 | Up         | chr3     | hsa-miR-888-3p hsa-miR-520c-3p hsa-miR-520b hsa-miR-373-3p hsa-miR-372-3p |
| hsa_circRNA_014724 | Up         | chr1     | hsa-miR-5009-5p hsa-miR-8058 hsa-miR-378h hsa-miR-2681-3p hsa-miR-378c |
| hsa_circRNA_014640 | Up         | chr8     | hsa-miR-193a-5p hsa-miR-520g-3p hsa-miR-520h hsa-miR-508-5p hsa-miR-519d-5p |
| hsa_circRNA_043044 | Up         | chr3     | hsa-miR-3121-5p hsa-miR-3692-3p hsa-miR-6504-3p hsa-miR-1324 hsa-miR-4436a |
| hsa_circRNA_004121 | Up         | chr2     | hsa-miR-1270 hsa-miR-4742-5p hsa-miR-4446-5p hsa-miR-6830-5p hsa-miR-4677-5p |
| hsa_circRNA_0100983 | Up         | chr1     | hsa-miR-376a-2-5p hsa-miR-873-5p hsa-miR-765 hsa-miR-576-5p hsa-miR-423-3p |
| hsa_circRNA_007850 | Up         | chr9     | hsa-miR-6758-5p hsa-miR-6856-5p hsa-miR-4668-5p hsa-miR-4773 hsa-miR-3976 |
| sa_circRNA_051778 | Up         | chr9     | hsa-miR-6762-5p hsa-miR-762 hsa-miR-4697-5p hsa-miR-4739 hsa-miR-4640-5p |
| hsa_circRNA_000317 | Down       | chr11    | hsa-miR-659-5p hsa-miR-3649 hsa-miR-365a-5p hsa-miR-6509-5p hsa-miR-6073 |
| hsa_circRNA_103117 | Down       | chr21    | hsa-miR-153-5p hsa-miR-152-5p hsa-miR-26b-3p hsa-miR-513a-3p hsa-miR-424-5p |
| hsa_circRNA_103829 | Down       | chr5     | hsa-miR-625-3p hsa-miR-129-5p hsa-miR-532-5p hsa-miR-548c-3p hsa-let-7c-5p |
| hsa_circRNA_001729 | Down       | chr16    | hsa-miR-367-3p hsa-miR-363-5p hsa-miR-323a-5p |
| hsa_circRNA_000367 | Down       | chr11    | hsa-miR-331-3p hsa-miR-4646-5p hsa-miR-4797-5p hsa-miR-3919 hsa-miR-3190-3p |
| hsa_circRNA_082680 | Down       | chr7     | hsa-miR-4518 hsa-miR-6851-5p hsa-miR-6863 hsa-miR-373-3p hsa-miR-4695-3p |
| hsa_circRNA_004183 | Down       | chr10    | hsa-miR-7162-5p hsa-miR-6875-3p hsa-miR-516b-3p hsa-miR-516a-3p hsa-miR-4678-3p |
| hsa_circRNA_101837 | Down       | chr16    | hsa-miR-766-5p hsa-miR-509-5p hsa-miR-18b-5p hsa-miR-18a-5p hsa-miR-380-3p |
| hsa_circRNA_103827 | Down       | chr5     | hsa-miR-411-5p hsa-miR-625-3p hsa-miR-129-5p hsa-miR-205-5p hsa-miR-532-5p |
| hsa_circRNA_002082 | Down       | chr11    | hsa-miR-512-5p hsa-miR-4773 hsa-miR-361I hsa-miR-4742-3p hsa-miR-6887-3p |

Table 3: Enumeration data of differentially expressed circRNAs and target miRNAs/mRNAs.

|                    | circRNA | miRNA | mRNA  |
|--------------------|---------|-------|-------|
| Up                 | 154     | 768   | 8448  |
| Down               | 203     | 1013  | 9428  |
Figure 3: Continued.
TG deposition (steatosis). Except for the signaling pathways related to its malignant origination (pathways in cancer, pathways in cancer, transcriptional misregulation in cancer, etc.), steatotic HepG2 cells were featured by dysregulation of metabolic pathways (hsa01100), insulin signaling pathway (hsa04910), and insulin resistance (hsa04931). Taken together, circRNAs are proposed to control metabolic pathways, and related ones, in hepatic steatosis by modulating the transcription of critical genes.

For the purpose of taking deep insight into circRNAs’ action model, differentially expressed circRNAs, target miRNAs, and downstream genes within the transcription-regulating GOs (up- and downregulated GOs of regulation of transcription, DNA-dependent) were integrated to construct a circRNA-miRNA-mRNA network. Briefly, the circRNA-miRNA-mRNA network was composed of 357 circRNAs, 1,766 miRNAs, and 17,876 mRNAs, indicating the steatogenic mechanisms with characteristics of signaling cascaded, multi-to-multi-regulation. LPIN1 among this network was identified to be the only member in both downregulated, transcription-regulating GO and downregulated metabolic pathways. Therefore, LPIN1 may serve as the critical agent that mediates the transcriptional regulatory effect of circRNAs on metabolic pathways. Has_circRNA_021412 and miR-1972 with LPIN1-regulating activity, and LPIN1 itself, were recognized to take the central place in circRNA-miRNA-mRNA network.

Interestingly, interaction of these circRNA, miRNA, and LPIN1 reveals some novel, yet important, mechanisms during hepatic steatosis. Being assessed by array hybridization and QPCR, has_circRNA_021412 experienced significant downregulation after high-fat stimulation. The decreased
Figure 4: Functional analysis reveals the effect of steatosis-related circRNAs. (a, b) Upregulated (a) and downregulated gene ontologies (GOs) (b) show the effects of differentially expressed circRNAs with specific actions in transcriptional regulation. (c, d) Upregulated (c) and downregulated pathways (d) exhibit the regulatory role of differentially expressed circRNAs in signaling transduction.
expression of circRNA attenuates its competitive inhibition to miRNA. As a result, reactivated miR-1972 induces the transcriptional and/or translational reduction in LPIN1 level. LPIN1 has been confirmed to play a dual function as phosphatidic acid phosphohydrolase (PAP) and transcriptional regulator, respectively, in hepatocytes [39, 40]. First, it suppresses the lipogenic program by catalyzing the penultimate step of TG synthesis in endoplasmic reticulum [34]. Second, direct interaction between LPIN1 and transcription factors (PPARα, PGC-1α) amplifies the hepatic PGC-1α/PPARα signaling in steatosis-related gene expression [40]. Indeed, 8 steatosis-related genes [41] of the metabolic pathways were recognized to be transcriptionally induced by LPIN1 in our experiments. Members of long chain acyl-CoA synthetase (ACSL) family (ACSL1, ACSL3, ACSL4, and ACSL6) dramatically prevailed in these genes. Because of ACSLs’ essential role in fatty acid oxidation [42, 43], LPIN1 is capable of selectively activating fatty acid oxidation and mitochondrial oxidative phosphorylation, which have already been proved by gain-of-function and loss-of-function strategies [40, 44–46]. Downregulated LPIN1, however, prevent the fatty acid degradation in an ACSL-dependent way. Furthermore, LPIN1 is verified to act as the key component of multiple signaling transductions that are deeply involved in lipid homeostasis, including SIRT1/AMPK signaling [47], mammalian target of rapamycin complex 1 (mTORC1)/SREBP signaling [48], NF-E2-related factor 1 (Nrf1) signaling [50], and hepatocyte nuclear factor 4 α (HNF4α) signaling [50]. Because of these reasons, abnormality in circRNA_021412-miR-1972/LPIN1 signaling cascade contributes to the hepatic steatosis via disrupting the balance of lipogenesis and catalytic separation.

In conclusion, genome-scale dysregulation of circRNAs is associated with hepatic steatosis. Transcriptional regulation characterizes their actions and is suggested to mediate the modulation of metabolic pathways. circRNA_021412/miR-1972/LPIN1 signaling may reflect the critical mechanism underlying circRNA-related fatty acid dysmetabolism, which results in the cellular steatogenesis.
Figure 6: Diagram of circRNA_021412-based regulation of hepatic steatosis. Arrow, blunt-headed line, and red cross represent effects of activation, inhibition, and blocking, respectively.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors’ Contributions

Xing-Ya Guo and Chong-Xin He contributed equally to this paper.

Acknowledgments

The work is supported by State Key Development Program for Basic Research of China (no. 2012CB827501); National Natural Science Foundation of China (81070322, 81270491, 81470859, and 81270492); 100 Talents Program (no. 81470840, 81470859, and 81270492); 100 Talents Program (no. 2012CB517501); National Natural Science Foundation of China (81070322, 81270491, 81470840, 81470859, and 81270492); 100 Talents Program (no. 2012CB517501); National Natural Science Foundation of China (81070322, 81270491, 81470840, 81470859, and 81270492); and Program of the Committee of Science and Technology (0914093500 and 13ZR14267).

References

[1] P.-L. Liew, W.-J. Lee, Y.-C. Lee, H.-H. Wang, W. Wang, and Y.-C. Lin, “Hepatic histopathology of morbid obesity: concurrence of other forms of chronic liver disease,” Obesity Surgery, vol. 16, no. 12, pp. 1584–1593, 2006.
[2] J. Richard and I. Lingvay, “Hepatic steatosis and type 2 diabetes: current and future treatment considerations,” Expert Review of Cardiovascular Therapy, vol. 9, no. 3, pp. 321–328, 2011.
[3] M. Gaggini, M. Morelli, E. Buzzigoli, R. A. DeFronzo, E. Bugianesi, and A. Gastaldelli, “Non-alcoholic fatty liver disease (NAFLD) and its connection with insulin resistance, dyslipidemia, atherosclerosis and coronary heart disease,” Nutrients, vol. 5, no. 5, pp. 1544–1560, 2013.
[4] L. C. Bertot and L. A. Adams, “The natural course of non-alcoholic fatty liver disease,” International Journal of Molecular Sciences, vol. 17, no. 5, article 774, 2016.
[5] A. Federico, M. Dallio, M. Masarone et al., “The epidemiology of non-alcoholic fatty liver disease and its connection with cardiovascular disease: role of endothelial dysfunction,” European Review for Medical and Pharmacological Sciences, vol. 20, no. 22, pp. 4731–4741, 2016.
[6] H. Moshayedi, R. Ahrabi, A. Mardani et al., “Association between non-alcoholic fatty liver disease and ischemic stroke,” Iranian Journal of Neurology, vol. 13, no. 3, pp. 144–148, 2014.
[7] I. Mikolasevic, L. Orlic, D. Stimac et al., “Non-alcoholic fatty liver disease and colorectal cancer,” Postgraduate Medical Journal, vol. 93, no. 1097, pp. 153–158, 2017.
[8] C. D. Williams, J. Stengel, M. I. Asike et al., “Prevalence of non-alcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study,” Gastroenterology, vol. 140, no. 1, pp. 124–131, 2011.
[9] L. Caballeria, G. Pera, M. A. Auladell et al., “Prevalence and factors associated with the presence of nonalcoholic fatty liver disease in an adult population in Spain,” European Journal of Gastroenterology and Hepatology, vol. 22, no. 1, pp. 24–32, 2010.
[10] J. Fung, C. K. Lee, M. Chan et al., “High prevalence of non-alcoholic fatty liver disease in the Chinese—results from the Hong Kong liver health census,” Liver International, vol. 35, no. 2, pp. 542–549, 2015.
[11] G. B. Goh, C. Kwan, S. Y. Lim et al., “Perceptions of non-alcoholic fatty liver disease—an Asian community-based study,” Gastroenterol Rep (Oxf), vol. 4, no. 2, pp. 131–135, 2016.
[12] T. B. Hansen, T. I. Jensen, B. H. Clausen et al., “Natural RNA circles function as efficient microRNA sponges,” Nature, vol. 495, no. 7441, pp. 384–388, 2013.
[13] S. Memczak, M. Jens, A. Elefsinioti et al., “Circular RNAs are a large class of animal RNAs with regulatory potency,” Nature, vol. 495, no. 7441, pp. 333–338, 2013.
[14] Q. Zheng, C. Bao, W. Guo et al., “Circular RNA profiling reveals an abundant circHIPK3 that regulates cell growth by sponging multiple miRNAs,” Nature Communications, vol. 7, article 11215, 2016.
[15] W. W. Du, W. Yang, E. Liu, Z. Yang, P. Dhaliwal, and B. B. Yang, “Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with p21 and CDK2,” Nucleic Acids Research, vol. 44, no. 6, pp. 2846–2858, 2016.
[16] H. Xu, S. Guo, W. Li, and P. Yu, “The circular RNA Cdrlas, via miR-7 and its targets, regulates insulin transcription and secretion in islet cells,” Scientific Reports, vol. 5, article 12453, 2015.
[17] S.-P. Lin, S. Ye, Y. Long et al., “Circular RNA expression alterations are involved in OGD/R-induced neuron injury,” Biochemical and Biophysical Research Communications, vol. 471, no. 1, pp. 52–56, 2016.
[18] K. Wang, B. Long, F. Liu et al., “A circular RNA protects the heart from pathological hypertrophy and heart failure by targeting miR-223,” European Heart Journal, vol. 37, no. 33, pp. 2602–2611, 2016.
[19] E. Grasselli, A. Voci, L. Canesi et al., “3,5-Diiodo-L-thyronine modifies the lipid droplet composition in a model of hepatosteatosis,” Cellular Physiology and Biochemistry, vol. 33, no. 2, pp. 344–356, 2014.
[20] X. Wang, Y. Zhang, L. Huang et al., “Decreased expression of hsa_circ_000988 in colorectal cancer and its clinical significance,” International Journal of Clinical and Experimental Pathology, vol. 8, no. 12, pp. 16020–16025, 2015.

[21] C. M. Tang, M. Zhang, L. Huang et al., “CircRNA_000203 enhances the expression of fibrosis-associated genes by derepressing targets of miR-26b-5p, Colla2 and CTGF, in cardiac fibroblasts,” Scientific Reports, vol. 7, article 40342, 2017.

[22] P. Li, S. Chen, H. Chen et al., “Using circular RNA as a novel type of biomarker in the screening of gastric cancer,” Clinica Chimica Acta, vol. 444, pp. 132–136, 2015.

[23] J. G. A. Whitehill, S. O. Opiyo, J. L. Koch, D. A. Herms, D. F. Cipollini, and P. Bonello, “Interspecific comparison of rarer and unique to manchurian ash, a species resistant to emerald ash borer,” Journal of Chemical Ecology, vol. 38, no. 5, pp. 499–511, 2012.

[24] F. Liu, J. Du, J. Liu, and B. Wen, “Identification of key target genes and pathways in laryngeal carcinoma,” Oncology Letters, vol. 12, no. 2, pp. 1279–1286, 2016.

[25] X. Yan, F. Yuan, X. Chen, and C. Dong, “Bioinformatics analysis to identify the differentially expressed genes of glaucoma,” Molecular Medicine Reports, vol. 12, no. 4, pp. 4829–4835, 2015.

[26] Y.-G. Zhang, H.-L. Yang, Y. Long, and W.-L. Li, “Circular RNA in blood corpuscles combined with plasma protein factor for early prediction of pre-eclampsia,” BioMed Research International, vol. 2017, Article ID e131, 2007.

[27] C. M. Tang, M. Zhang, L. Huang et al., “CircRNA_000203 enhances the expression of fibrosis-associated genes by derepressing targets of miR-26b-5p, Colla2 and CTGF, in cardiac fibroblasts,” International Journal of Clinical and Experimental Pathology, vol. 8, no. 12, pp. 16020–16025, 2015.

[28] Y. Pan, Y. Guo, Y. Luo, H. Li, and Y. Xu, “MicroRNA expression profiling of Chinese follicular lymphoma by microarray: a preliminary study,” International Immunopharmacology, vol. 39, pp. 41–47, 2016.

[29] G. Dennis Jr., B. T. Sherman, D. A. Hosack et al., “DAVID: database for annotation, visualization, and integrated discovery,” Genome Biol, vol. 4, no. 5, p. 3, 2003.

[30] D. Dupuy, N. Bertin, C. A. Hidalgo et al., “Genome-scale analysis of in vivo spatiotemporal promoter activity in Caenorhabditis elegans,” Nature Biotechnology, vol. 25, no. 6, pp. 663–668, 2007.

[31] M. Kanehisa, S. Goto, S. Kawashima et al., “The KEGG resource for deciphering the genome,” Nucleic Acids Research, vol. 32, supplement 1, pp. D277–D280, 2004.

[32] A. S. Marriott, O. Vasieva, and Y. Fang, “NUDT2 disruption elevates diadenosine tetraphosphate (Ap4A) and down-regulates immune response and cancer promotion genes,” PLoS One, vol. 11, no. 5, Article ID e0154674, 2016.

[33] J.-G. Joung, K.-B. Hwang, J.-W. Nam, S.-J. Kim, and B.-T. Zhang, “Discovery of microRNA-mRNA modules via population-based probabilistic learning,” Bioinformatics, vol. 23, no. 9, pp. 1141–1147, 2007.

[34] R. Shalgi, D. Lieber, M. Oren, and Y. Pilpel, “Global and local architecture of the mammalian microRNA-transcription factor regulatory network,” PLoS Computational Biology, vol. 3, no. 7, article e131, 2007.

[35] X. Zhang, D. Wu, M. Aldaroush et al., “ETS-1: a potential target of glycolysis for metabolic therapy by regulating glucose metabolism in pancreatic cancer,” International Journal of Oncology, vol. 50, no. 1, pp. 232–240, 2016.

[36] E. G. Cerami, B. E. Gross, E. Demir et al., “Pathway Commons, a web resource for biological pathway data,” Nucleic Acids Research, vol. 39, no. 1, pp. D685–D690, 2011.

[37] R. Cao, X. Zhao, S. Li et al., “Hypoxia induces dysregulation of lipid metabolism in HepG2 cells via activation of HIF-2α,” Cellular Physiology and Biochemistry, vol. 34, no. 5, pp. 1427–1441, 2014.

[38] D. Yang, L. Sun, Z. Li, and P. Gao, “Noncoding RNAs in regulation of cancer metabolic reprogramming,” Advances in Experimental Medicine and Biology, vol. 927, pp. 191–215, 2016.

[39] J. Donkor, M. Sariahmetoglu, J. Dewald, D. N. Brindley, and K. Reue, “Three mammalian lipins act as phosphatidate phosphatases with distinct tissue expression patterns,” Journal of Biological Chemistry, vol. 282, no. 6, pp. 3450–3457, 2007.

[40] B. N. Finck, M. C. Gropler, Z. Chen et al., “Lipin 1 is an inducible amplifier of the hepatic PGC-1α/PPARγ regulatory pathway,” Cell Metabolism, vol. 4, no. 3, pp. 199–210, 2006.

[41] C. T. Shearn, J. E. Mercer, D. J. Orlicky et al., “Short term feeding of a high fat diet exerts an additive effect on hepatocellular damage and steatosis in liver-specific PTEN knockout mice,” PLoS ONE, vol. 9, no. 5, Article ID e96553, 2014.

[42] H. I. Lee and M. K. Lee, “Coordinated regulation of scopolitin at adipose tissue-liver axis improved alcohol-induced lipid dysmetabolism and inflammation in rats,” Toxicol Lett, vol. 237, no. 3, pp. 210–218, 2015.

[43] Q. Deng, X. Li, S. Fu et al., “SREBP-1c gene silencing can decrease lipid deposits in bovine hepatocytes cultured in vitro,” Cell Physiol Biochem, vol. 33, no. 5, pp. 1568–1578, 2014.

[44] J. J. Lehman, P. M. Barger, A. Kovacs, J. E. Saffitz, D. M. Medeiros, and D. P. Kelly, “Peroxisome proliferator-activated receptor γ coactivator-1 promotes cardiac mitochondrial biogenesis,” Journal of Clinical Investigation, vol. 106, no. 7, pp. 847–856, 2000.

[45] J. M. Hess, R. P. Kopp, and D. P. Kelly, “Peroxisome proliferator-activated receptor coactivator-1 promotes cardiac mitochondrial biogenesis,” The Journal of Biological Chemistry, vol. 277, no. 43, pp. 40265–40274, 2002.

[46] R. B. Vega, J. M. Hess, and D. P. Kelly, “The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor α in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes,” Molecular and Cellular Biology, vol. 20, no. 5, pp. 1868–1876, 2000.

[47] H. Everitt, M. Hu, J. M. Ajmo et al., “Ethanol administration exacerbates the abnormalities in hepatic lipid oxidation in genetically obese mice,” American Journal of Physiology—Gastrointestinal and Liver Physiology, vol. 304, no. 1, pp. G38–G47, 2013.

[48] T. R. Peterson, S. S. Sengupta, T. E. Harris et al., “MTOR complex 1 regulates lipin 1 localization to control the srebp pathway,” Cell, vol. 146, no. 3, pp. 408–420, 2011.

[49] Y. Hirotsu, N. Hataya, F. Katsuo, and M. Yamamoto, “NF-E2-related factor 1 (Nrf1) serves as a novel regulator of hepatic lipid metabolism through regulation of the lipin1 and PGC-1α genes,” Molecular and Cellular Biology, vol. 32, no. 14, pp. 2760–2770, 2012.

[50] Z. Chen, M. C. Gropler, M. S. Mitra, and B. N. Finck, “Complex interplay between the lipin 1 and the hepatocyte nuclear factor 4α (HNF-4α) pathways to regulate liver lipid metabolism,” PLoS ONE, vol. 7, no. 12, Article ID e51320, 2012.