Supplementary materials

Perilipin 5 is a novel target of nuclear receptor LRH-1 to regulate hepatic triglycerides metabolism

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MATERIALS AND METHODS

Animal studies

Lrh−1fl/f and Lrh−1LKO mice were obtained by mating an Lrh−1 allele flanked by LoxP sites (Lrh−1fl/f) mice with albumin-Cre transgenic mice. Lrh−1fl/f mice were Kindly gifted by Prof. Timothy F. Osborne and albumin-Cre transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Eight-to-twelve-week-old Lrh−1fl/f and Lrh−1LKO male mice were used. Mice were housed in a specific pathogen-free facility and fed a standard chow diet and water ad libitum. All mice were kept under 12:12-h light-dark cycles (6 a.m.–6 p.m. light, 6 p.m.–6 a.m. dark) at 22–24°C and 60–70% humidity. All animal experiments were performed following the guidelines provided by the Institutional Animal Care and Use Committee of Keimyung University (KM-2020-12R1).

Cell culture

HepG2 immortalized human hepatocytes and human embryonic kidney (HEK)-293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 100 U/ml penicillin-streptomycin (P/S) as complete media. For LRH-1 agonist treatment, HepG2 cells were cultured in the presence or absence of 100 µM dialauroylphosphatidylcholine (DLPC, Sigma-Aldrich, Co., St. Louis, MO, USA) in DMEM complete media for 24 h. Primary hepatocytes were cultured in either William’s Medium E (Gibco, Grand Island, NY, USA) supplemented with 10% FBS, 1% Glutamax (Gibco), and 100 U/ml P/S or DMEM media (Hyclone) supplemented with 10% FBS and 100 U/ml P/S. Cells were incubated in a humidified atmosphere containing 5% CO2 at 37°C.

Quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from the liver samples of 24 h–fasted and fed Lrh−1fl/f and Lrh−1LKO mice, as well as from HepG2 cells treated with the LRH-1 agonist DLPC, using TriZol reagent (Life Technologies, Carlsbad, CA, USA). The qPCR was performed using a CFX96™ real time system (Bio-Rad, Hercules, CA, USA) to measure the expression level of various genes. Primer sequences used are listed in Supplementary Table 1. The relative mRNA expression level was normalized to ribosomal protein L32 or ribosomal phosphoprotein P0 by calculations based on the delta–delta threshold cycle method.
Western blot analysis

Western blot analysis was performed as described previously (1). Briefly, protein samples were collected from the liver tissues of 24 h-fasted and fed Lrh-1\(^{ff}\) and Lrh-1\(^{LKO}\) mice, as well as from LRH-1 agonist-treated HepG2 cells. Anti-LRH-1 from Aviva System Biology (San Diego, CA 92111 USA), anti-PLIN5 from Invitrogen (Carlsbad, California, USA), anti-CPT-1\(^{\alpha}\) from Santa Cruz Biotechnology (Dallas, Texas, USA), anti-PGC-1\(^{\alpha}\) from Abcam (Cambridge, MA, USA), anti-\(\beta\)-actin from Sigma Aldrich (Steinheim, Germany), and anti-GAPDH from Cell Signaling Technology (Danvers, MA, USA) antibodies were purchased. Antibody dilution and catalog number is listed in Supplementary Table 2. Horseradish peroxidase-conjugated mouse and rabbit secondary antibodies were used for the detection of protein bands. Antigen-antibody binding was detected using a chemiluminescent detection reagent (Bio-Rad).

Plasmid DNA design and transient transfection

The promoter region of mouse Plin5 gene from \(-1883\) to \(+93\) was synthesized by PCR, and the band size was confirmed by running it on an agarose gel. Then, the band was extracted by gel extraction and cloned into the pGL3 basic vector, which was designated as pmPLIN5\(-1883/+93\). In the promoter region, putative LRH-1-binding sequences were marked and deleted by site-directed mutagenesis using a Quick-change II Site-Directed Mutagenesis kit (Agilent Technologies Inc., Santa Clara, CA, USA). For transfection, HEK-293T cells were seeded on 6-well plates, and cells were transfected with pmPLIN5 with or without a murine LRH-1 and pCMV-\(\beta\)-galactosidase expression vector. The next day, 100 \(\mu\)M DLPC was administered to the cells, which were incubated for 24 h. Finally, cells were lysed in reporter lysis buffer (Promega corporation, Madison, WI 53711 USA) and luciferase assays were performed. The luciferase activity was normalized by \(\beta\)-galactosidase activity (2). For siPLIN5 overexpression, HepG2 cells were transfected with SMARTpool ON-TARGETplus Human PLIN5 siRNA (L-033568-01-0020, Dharmacon Inc., Colorado, USA). And as a control (D-001810-10-05, Dharmacon) were used. Next day, DLPC was treated to the cells and incubated for 24 h and total RNA was isolated.

Lipid analysis

For lipid extraction, livers were homogenized in 2:1 (v/v) chloroform:methanol three times followed by drying under a stream of nitrogen gas. These dried samples were resuspended in 1:1 (v/v) chloroform:methanol and 50 mM lithium chloride (LiCl) solution, and the lower
layer was collected after centrifugation at 1400 × g for 10 min. To the upper layer, chloroform was added, and the sample was re-centrifuged to collect the lower layer, which was repeated twice. Samples that were collected three times were pooled together and dried in a stream of nitrogen gas. Subsequently, dried samples were processed again and resuspended in 1:1 (v/v) chloroform:methanol maintaining the concentration of LiCl at 10 mM. After centrifugation, the lower layer was collected, and the aforementioned procedure was consecutively followed to collect the dried samples. To the dried sample, chloroform was added, and the sample was vortexed. Finally, TG and cholesterol were quantified with the TG-S and ASAN SET total-cholesterol kits (Asan pharm. Co., Gyeonggi-do, Korea) respectively, according to the manufacturer’s instructions. Lipid contents were normalized to the liver tissue weight. Similarly, serum TG and cholesterol were analyzed using the TG-S and ASAN SET total-cholesterol kit. Serum NEFA levels were determined utilizing the NEFA C kit (Wako, Osaka, Japan).

Isolation and culture of primary hepatocytes

Hepatocytes were isolated from the livers of Lrh−1f/f and Lrh−1LKO mice fed or fasted for 24 h using the perfusion method as described previously (3). Briefly, mice were anesthetized with isoflurane (Hana Pharm. Co., Gyeonggi-Do, Korea) and laparotomy was performed to uncover the portal vein. Then, the vein was catheterized and the liver was perfused with Earle’s balanced salt solution (EBSS: WELGENE Inc., Gyeongsan, Republic of Korea) and 0.5 M ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), which was followed by 40 µg/ml liberase perfusion (Roche Diagnostics, Indianapolis, IN, USA) containing EBSS and 2 M CaCl2·H2O. Next, the liver was instantly detached and gently minced with 1× EBSS and 2 M CaCl2·H2O. Then, cells were filtered through a 100-µm nylon cell strainer followed by centrifugation of the filtrate at 50 × g for 1 min at 4°C. Subsequently, the pellet was re-suspended in Percoll buffer (GE Healthcare, Uppsala, Sweden) and re-centrifuged at 100 × g for 10 min at 4°C to collect the pellet. Finally, the cell pellet was gently resuspended in William’s Medium E (Gibco) supplemented with 10% FBS, 1% Glutamax, and 100 U/ml P/S, successively plating the cells on collagen-coated culture dishes for 3~4 h.

LD staining

To mark LDs with Oil red-O, Lrh−1f/f and Lrh−1LKO mice were sacrificed after feeding or fasting for 24 h and livers were removed immediately. The liver samples were cut into 10-
μm sections and fixed with 4% paraformaldehyde for 10 min at room temperature. Similarly, for staining lipid droplets via BODIPY (Invitrogen), primary hepatocytes isolated from Lrh−1
\textsuperscript{ff} and Lrh−1\textsuperscript{LKO} mice were seeded on an 8-well chamber. Cells were grown overnight either in fed (DMEM, 10% FBS, 1% P/S) or in fasting (DMEM, 2% lipoprotein deficient serum, 1% P/S) media. The next day, hepatocytes were fixed with 4% paraformaldehyde for 10 min at room temperature. Then, cells were treated with blocking solution (1% bovine serum albumin in dulbecco’s phosphate-buffered saline) for 30 min followed by overnight incubation with a PLIN5 antibody (NB110-60509, Novus Biologicals, Centennial, CO, USA) at 4°C. The next day, cells were treated with a secondary antibody (Alexa Fluor 594, Invitrogen) and incubated for 1 h in the dark at room temperature. Subsequently, cells were stained with 2 μM BODIPY\textsuperscript{TM} 493/503 (D3922; Invitrogen) and incubated for 10 min at room temperature. Finally, cells were covered with mounting medium and then observed using confocal laser scanning microscopy (Carl Zeiss, Thornwood, NY, USA).

ChIP assay

ChIP assay was performed as described previously (4). Briefly, chromatin was prepared from liver tissue of Lrh−1\textsuperscript{ff} and Lrh−1\textsuperscript{LKO} mice fed or fasted for 24 h. Liver tissues were minced and crosslinked with 1% paraformaldehyde and rotated for 8 min at room temperature. Crosslinking was stopped by adding 0.125 M glycine, and then, samples were rotated for an additional 5 min at room temperature. Finally, total chromatin was extracted from liver tissues and sonicated enough to obtain DNA fragments of 200–500 bp. Next, DNA were subjected to ChIP using an anti-LRH−1 antibody (sc-393369X) from Santa Cruz Biotechnology (USA), and then qPCR was performed.

Liver-specific PLIN5 overexpression mice model

For overexpression of PLINS, Lrh−1\textsuperscript{ff} and Lrh−1\textsuperscript{LKO} mice were injected in tail vein with 200 μl of PLIN5 overexpression or pcDNA vector using in vivo-JetPEI\textsuperscript{®}-Gal (202–10G, Polyplus-transfection Inc., New York, NY, USA) transfection agent according to the instructions provided by the manufacturer. All mice were injected with pcDNA or PLIN5 overexpression vector for two days. In the subsequent day, all mice were fasted for 24 h and then livers were isolated to measure TGs levels.

Electrophoretic mobility shift assay

To prepare the LRH−1 enriched nuclear extract protein, Hek293T cells were transfected with
LRH-1 overexpression vector for 2 days. Cells were pelleted by centrifugation at 1000 × g for 5 min at 4 °C for and resuspended with buffer A (10 mM HEPES/KOH pH 7.6, 1.5 mM MgCl2, 10 mM KCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM EGTA, 250 mM sucrose) supplemented with protease inhibitors and incubated on ice for 15 min. By centrifuging at 1000 × g for 7 min at 4 °C, pellet was resuspended in buffer B (20 mM HEPES/KOH pH 7.6, 1.5 mM MgCl2, 0.42 M NaCl, 1 mM EDTA, 1 mM EGTA, 2.5% glycerol) supplemented with protease inhibitors. Finally, nuclear extract was separated via centrifugation at 55,000 rpm for 30 min at 4 °C.

The single stranded complementary oligonucleotides including −1620/−1614 region of the PLIN5 promoter sequence were annealed to obtain double stranded DNA fragments. These DNA fragments were labeled with ³²P radioisotopes and incubated with LRH-1 overexpressed nuclear extract protein. The DNA–nuclear extract protein complexes were resolved in 9% non-denaturing polyacrylamide gel. After electrophoresis, the gels were dried, and visualized DNA–LRH-1 complex by autoradiography. For competition experiment, unlabeled DNA fragments (Cold DNA x 50) were added to nuclear extract.

**Serum ketone bodies**

Serum was isolated from the blood samples of Lrh-1f/f and Lrh-1LKO mice fed or fasted for 24 h. To measure serum ketone bodies, serum β-hydroxybutyrate levels was analyzed using MAK041-1KT (Sigma-Aldrich) kit according to the manufacturer’s instructions.

**Statistical analysis**

Data were analyzed utilizing GraphPad Prism 8.4 software (GraphPad Software Inc., San Diego, CA, USA). Data are presented as the mean ± SEM. Statistical differences between groups were analyzed using a two-tailed student’s t-test. Differences with P-values < 0.05 were declared significant.
Supplementary Figure legends

**Supplementary Fig 1.** LRH-1 binds on mouse PLIN5 gene. (A) PLIN5 promoter sequence from −1 to −1883 from the transcriptional start site (+1). Arrows designates putative LRH-1 binding sites. (B) ChIP assay on putative LRE region of mouse PLIN5 exon was measured in Lrh-1<sup>f/f</sup> and Lrh-1<sup>LKO</sup> livers. (n=3−4/group). **p < 0.01, Lrh-1<sup>f/f</sup> vs. Lrh-1<sup>LKO</sup>.

**Supplementary Fig 2.** (A) Serum β-hydroxybutyrate levels in 24 h-fasted or fed Lrh-1<sup>f/f</sup> and Lrh-1<sup>LKO</sup> mice. (B) mRNA expression of Plin5 in PLIN5 overexpressed Lrh-1<sup>f/f</sup> and Lrh-1<sup>LKO</sup> mice.

**Supplementary Fig 3.** LRH-1 binds between the −1620 and −1614 region on the PLIN5 promoter sequence. (A) Oligonucleotides including −1620/−1614 region of LRH-1 binding sites on PLIN5 promoter sequence. (B) Annealing of complementary oligonucleotides and resolved in 9% non-denaturing gel. (C) EMSA of DNA–LRH-1 overexpressed nuclear extract complex in 2-fold serial dilution. (D) Competitive EMSA with cold DNA × 50. EMSA, electrophoretic mobility shift assay.

**Supplementary Fig 4.** siRNA of PLIN5 was transfected in HepG2 cells. (A) mRNA expression of PLIN5. (B, C) mRNA expression of PPAR<sub>α</sub> and PGC-1α. ***p < 0.001, **p < 0.01, siCont Mock vs. siCont DLPC, ###p < 0.01, ##p < 0.05, siCont Mock vs. siPLIN5 DLPC.
Supplementary Table legend

**Supplementary Table 1.** List of primers and their sequence used in qPCR. *ApoB*, apolipoprotein B; *Cpt-1α*, carnitine palmitoyltransferase 1 alpha; *Cyp8b1*, cytochrome p450 8b1; *Fgf21*, fibroblast growth factor 21; *Lrh-1*, liver receptor homolog-1; *L32*, ribosomal protein L32; *Mttp*, microsomal triglyceride transfer protein; *Pgc-1α*, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *Plin2*, perilipin 2; *Plin5*, perilipin 5; *Ppara*, peroxisome proliferator-activated receptor alpha; *RPLP0*, ribosomal protein lateral stalk subunit P0.

**Supplementary Table 2.** List of primary antibodies used in western blotting.
Supplementary Fig 2.

A

**β-Hydroxybutyrate**

|          | Fed       | Fast      |
|----------|-----------|-----------|
| Lrh-1^{ff} | ![Bar Chart](image) | ![Bar Chart](image) |
| Lrh-1^{LKO} | ![Bar Chart](image) | ![Bar Chart](image) |

B

**Plin5**

|          | pcDNA | PLIN5 |
|----------|-------|-------|
| Lrh-1^{ff} | ![Bar Chart](image) | ![Bar Chart](image) |
| Lrh-1^{LKO} | ![Bar Chart](image) | ![Bar Chart](image) |
Supplementary Fig 3.

A

PLIN5-LRE1:
TCTGCTTCTCTGCTTCTGCCCTCCAGAGCTGAGGTCACACACATGAGCCAGTGTCACACATGACAAATGTT

B

Single strand  double strand
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Double strand  Single strand
-----------  -----------

9% Non-denaturating PAGE gel

C

LRH-1 overexpression nuclear extract (2-fold serial dilution)

D

LRH-1 overexpression nuclear extract
Cold DNA X 50

↓ DNA-LRH1 complex

↓ Free DNA
Supplementary Fig 4.
**Supplementary Table 1.** List of primers and their sequence used in qPCR.

| Gene   | Species | Forward primers (5’→3’)  | Reverse primers (5’→3’) |
|--------|---------|--------------------------|-------------------------|
| L32    | Mouse   | ACATTGCGCTGAATGTGGTT     | ATCCCTTTGCCCTGATCCTT    |
| Lrh-1  | Mouse   | TCATGCTGCCAAAGTTGGAGA    | TGGTTTTGGACAGTTGCTT    |
| Plin5  | Mouse   | TGTCAGTGTACAACTCGG       | CAGGGGACAGGTAGTCACAC    |
| Plin2  | Mouse   | CCTAGCTTCTCTCTGTTAGGC    | CACTACTGCTGCTGCAATT    |
| Cyp8b1 | Mouse   | CAAAGCCCAGCGCCT          | TTCGACTTTCAAGCTGTCGA    |
| Cpt-1α | Mouse   | CTCCGCTGAGCCATGAAG       | CACCAGTGATGATGCAATT    |
| Fgf21  | Mouse   | CTGCTGGGGGGTCTACCAAG     | CTGCAGCTACCAGCTTCC      |
| Pparα  | Mouse   | AGAGCCCCATCTGTCTCCTCTC   | ACTGGTAGTCTGCAAAAACAAA   |
| Pgc-1α | Mouse   | CAAACCTGCCATTTGTAAG      | TGACAAATGCTCTTCGCTT    |
| Mttp   | Mouse   | CTCCGCTGAGGCTTTTTTCTCT   | GAGCTTGTATAAGCGCTCATT   |
| ApoB   | Mouse   | TTGGCAAACGTGAGCATGCACC   | TCAAAATTGGGACTCCCTTAAGC |
| RPLP0  | Human   | GTGCTGATGGGCAAGAAC       | AGGTCCCTCTTGGTGAAC      |
| LRH-1  | Human   | CTTTGTCGCCGTGTGGAGAT     | GTCGGCCCTTACAGCTCTTA    |
| PLIN5  | Human   | AGGCTGACGCAAGAAATTG      | AACAGAAGGCTTGGGAAA      |
| PPARα  | Human   | TTCGCAATCCATCGGCGAG      | CCACAGGATAAGTCACCAGG    |
| PGC-1α | Human   | TGAAGACGGATTGCCCTCATT    | GCTGGTGCCAGTAAGAGCTT    |
**Supplementary Table 2.** List of primary antibodies used in western blotting.

| Primary antibody | Catalog number   | Dilution |
|------------------|------------------|----------|
| LRH-1            | ARP37407_P050    | 1:1000   |
| PLIN5            | PA1-46215        | 1:1000   |
| CPT-1α           | sc-393070        | 1:1000   |
| PGC-1α           | ab54481          | 1:1000   |
| GAPDH            | 2118             | 1:1000   |
| β-actin          | A5441            | 1:1000   |
References

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