Transcriptome Analysis Illuminates a Hub Role of SREBP2 in Cholesterol Metabolism by α-Mangostin

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ABSTRACT: Whole-transcriptome analysis of α-mangostin-treated HepG2 cells revealed that genes relevant to lipid and cholesterol metabolic processes responded to α-mangostin treatment. α-Mangostin downregulated a series of cholesterol biosynthetic genes, including SQLE, HMGCR, and LSS, and controlled specific cholesterol trafficking-associated genes such as ABCA1, SOAT1, and PCSK9. In particular, the downregulation of SREBP2 expression highlighted SREBP2 as a key transcriptional factor controlling lipid or cholesterol metabolic processes. Gene network analysis of SREBP2 and responses of its target proteins demonstrated that the effect of α-mangostin on HepG2 cells was mediated by the downregulation of SREBP2 expression, which was further supported by the reduction of the amount of SREBP2−SCAP complex. In the presence of exogenous cholesterols, α-mangostin downregulated SREBP2 expression and suppressed PCSK9 synthesis, which might contribute to the increased cholesterol uptake in cells, in part explaining the cholesterol-lowering effect of α-mangostin.

■ INTRODUCTION

Mangosteen (Garcinia mangostana L., Clusiaceae) is a plant which produces fruit that is dark purple or reddish in color with a soft and juicy white edible pulp and a slightly acidic sweet flavor and pleasant aroma and is widely used as a dietary supplement.1,2 Mangosteen fruit contains a variety of xanthone derivatives as secondary metabolites.3,4 One of the major constituent xanthones in mangosteen is α-mangostin (Figure 1A), which exhibits various pharmacological properties, including antiallergic, antiasthmatic, antifungal, anti-inflammatory, and antitumor, and cytotoxic activities.5−9 Several recent studies have reported that α-mangostin also displays in vivo antiobesity and antiatherosclerotic effects in addition to reducing the plasma levels of low-density lipoprotein (LDL) cholesterol and total cholesterol.10−12 Moreover, α-mangostin could suppress the development of atherosclerotic lesions and lipogenesis in gallbladder cells and transgenic mice.11,13 However, the comprehensive transcriptional or post-transcriptional effect of α-mangostin relevant to hepatocellular cholesterol metabolism based on whole-transcriptomic analysis remains unexplored.

RNA-sequencing (RNA-seq) has been widely applied to understand and predict the transcriptional activity of chemical compounds14,15 since transcriptome profiling of individual compounds can provide a comprehensive picture of transcriptional responses. Accordingly, deciphering the transcriptome can facilitate the discovery of new biological pathways or processes that had not been previously identified.16 As controlling the cellular cholesterol metabolism involves complex biological processes,17,18 we employed whole-transcriptome sequencing of α-mangostin-treated and -untreated HepG2 cells to identify the transcriptional factors and relevant genes responsible for the cholesterol metabolism-regulating effects.

Whole-transcriptome sequencing techniques such as RNA-seq can reveal all of the genes activated by α-mangostin treatment and determine their transcriptional expression levels. In the current study, we aimed to identify candidate genes from RNA-seq data using differentially expressed gene (DEG) analysis, gene ontology (GO) analysis, and the related protein−protein interaction network. Candidate genes with a high probability of relevance were further investigated to verify their correlations with cellular cholesterol metabolism in the context of α-mangostin stimulation.

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Figure 1. (A) Structure of α-mangostin. Comprehensive gene profiling of HepG2 cells treated with α-mangostin for 24 h. (B) Protein–protein interaction network for the DEGs was analyzed with GeneMANIA (ver. 3.4.1) performed with the Cytoscape plugin of the Cytoscape (ver. 3.5.1, ACS Omega http://pubs.acs.org/journal/acsodf Article https://dx.doi.org/10.1021/acsomega.0c04282 ACS Omega 2020, 5, 31126−31136
RESULTS

α-Mangostin Inhibits Lipid Metabolic Pathways. To assess the transcriptional regulation of α-mangostin on HepG2 cells, an RNA-seq approach was adopted, in which the mRNAs were collected from HepG2 cells treated with α-mangostin at concentrations of 10 and 20 μM. A total of 68 DEGs with at least a 2-fold change in expression level were identified by comparing the group of cells treated with 10 μM α-mangostin with the mRNA levels of the untreated control group (Figure 2B, Table S1). Genes involved in the cholesterol biosynthesis process (16/33), sterol biosynthesis process (16/34), sterol metabolic process (17/80), and cholesterol metabolic process (16/69) were significantly affected by α-mangostin treatment (Tables S2 and S3). In particular, genes categorized into GO terms of “lipid metabolic process” (GO:0006629) or its children in GO.
among the identified DEGs (http://amigo1.geneontology.org/; Table S2). The predicted functional correlations of these genes were then visualized in a protein–protein interaction network (Figure 1A). Enrichment of the top five Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and GO biological processes in the metabolic gene profiling analysis were determined using DAVID software. The results highlighted steroid biosynthesis and metabolic pathways from the KEGG pathways and sterol biosynthesis, cholesterol biosynthesis, and sterol metabolic process in GO biological processes (Figure 1C,D; Benjamini–Hochberg-adjusted P-values are shown for each indicated bar).

Figure 3. Effect of α-mangostin on SREBP inhibition in the HepG2 human hepatocellular liver carcinoma cell line. (A) Protein expressions of SREBP1, SREBP2, and β-actin were assayed by western blotting in cells treated with α-mangostin for 24 h. (B) Regulation of SREBPs by α-mangostin. Cells were cultured for 24 h, fixed, permeabilized, and incubated with anti–SREBP1 and SREBP2 antibody followed by an Alexa 594 conjugated anti-rabbit IgG (red). The nuclei of the corresponding cells were visualized by DAPI staining (blue) (magnification: ×60, scale bars: 1 μm). (C) mRNA expression of SREBP1 was assayed by western blotting in HepG2 and Hur7 cells treated with α-mangostin for 24 h. (D) mRNA expression of SREBP2 was assayed by western blotting in HepG2 and Hur7 cells treated with α-mangostin for 24 h. (E) SREBP2 molecular network obtained with Cytoscape v. 3.5.1 using STRING database.
Principal component analysis (PCA) was first used to identify outliers among nontreated cells and those treated with different concentrations of α-mangostin. The PCA plot of the RNA-seq data showed clear segregation and clustering of α-mangostin-treated and nontreated groups (Figure 1E). Heat mapping confirmed the dose-dependent regulation of the gene expression levels altered by treatment with α-mangostin (Figure 1F).

**α-Mangostin Regulates Genes Involved in the Cholesterol Metabolism Pathway.** To select and investigate specific genes associated with cholesterol metabolism, the DEGs identified between nontreated (normal) and α-mangostin-treated HepG2 cells were further analyzed (Figure 2A-C). Treatment with α-mangostin led to changes in the signaling pathways related to steroid biosynthesis (hsa000100), as indicated by the KEGG pathway enrichment analysis (Figure 2A). De novo cholesterol production shares pathways with those used in steroid biosynthesis. Among these genes, α-mangostin treatment clearly downregulated the expressions of FDFT1, SQLE, LSS, CYP51A1, MSMO1, HSD17B7, and DHCR7 (Figure 2A), which are closely associated with de novo cholesterol synthesis. Furthermore, these DEGs fell into two categories: 16 genes dysregulated in the α-mangostin-treated cells are involved in cholesterol metabolic process (GO:0006695) and 19 genes differentially expressed in the α-mangostin-treated cells are involved in the cholesterol biosynthesis process (GO:0006704). Pathway analysis revealed that SREBP2, LSS, SQLE, HMGCR, 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCs1), isopentenyl-diphosphate delta isomerase 1 (ID11), cytochrome P450 family 51 subfamily A member 1 (CYP51A1), and farnesyl diphosphate synthase (FDP5) participate in the lipid metabolism (Figure 2B,C). These results were consistent with those of protein expression level analysis determined using immunoblotting, which validated the accuracy of the RNA-seq data.

**PCSK9, SQLE, HMGCR, and LSS are enzyme-encoding metabolic genes with significantly downregulated expression in HepG2 cells following α-mangostin treatment (Figures 2D and S2A). We selected 11 genes from the 2 clusters that had differential signals >6.0 in mRNA levels. The q value, representing higher expression, was validated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR; Figure 2E). Transcriptional expressions of ABCA1, ACSL6, DHCR7, FDFT1, FDPS, HMGCR, ID11, PCSK9, SOAT1, SQLE, SREBP1, and SREBP2, which are related to cholesterol synthesis, uptake, and efflux, were modulated by α-mangostin treatment. This suggested that SREBP2 is associated with cholesterol homeostasis.

**α-Mangostin Preferentially Downregulates SREBP2 over SREBP1 Expression.** To examine the effect and specificity of α-mangostin on SREBP1 and SREBP2 expressions, western blotting of HepG2 cells was performed, which indicated that the SREBP2 expression was apparently more downregulated than the SREBP1 expression (Figures 3A and S2B). Confocal microscopic observations showed that both SREBP1 and SREBP2 expressions were suppressed by α-mangostin treatment in the HepG2 cells (Figure 3B). Similar to the pattern observed in western blotting, the mRNA expression of SREBP1 and SREBP2 was observed in HepG2 and Huh7 cells treated by α-mangostin (Figure 3C,D). SREBP1 mRNA expression was not significantly suppressed by α-mangostin treatment compared with the vehicle-treated groups for both HepG2 and Huh7 cells, whereas SREBP2 mRNA expression was significantly downregulated in both HepG2 and Huh7 cells.

To investigate the plausible proteins with functions that interact with SREBPs, we used the STRING protein–protein interaction network database (www.string-db.org). The protein–protein interactions were predicted using version 9.1 of the STRING database with a combined score >0.9. Cytoscape software version 3.5 was used to visualize the protein–protein interaction network (www.cytoscape.org). A STRING interactive network was used to identify proteins that can interact with SREBPs. As shown in Figure 3E, SREBP2 has predominant connections to the proteins associated with cholesterol biological process in α-mangostin-treated hepatocytes.

**α-Mangostin Reduces the Interaction between SCAP and SREBPs.** Immunoprecipitation assays were used to determine whether α-mangostin could affect complex formation between SREBP cleavage activating protein (SCAP) and SREBP2. Cell lysates were subjected to western blotting using anti-SREBP2 or anti-SCAP antibodies. As shown in Figure 4, α-mangostin decreased the interaction between SCAP and SREBP2, suggesting that α-mangostin suppressed the formation of SCAP–SREBP2 complexes in the endoplasmic reticulum and Golgi.

**α-Mangostin Increases Cholesterol Uptake and Selectively Downregulates SREBP2 in the Presence of Exogenous Sterols.** To determine if α-mangostin could increase cholesterol uptake, we monitored intracellular cholesterol by staining the cells with filipin (Figure 5A). Since cholesterol synthesis was inhibited and cholesterol metabolism was elevated, we assessed the cellular regulation of cholesterol in HepG2 cells. Cells were incubated in media supplemented with cholesterol (10 μg/mL) and then exposed to α-mangostin or dimethyl sulfoxide (0.1%) as a vehicle control. As shown in Figure 5A, α-mangostin treatment increased cholesterol uptake, and quantification of the fluorescence showed significantly increased signal levels of filipin in the HepG2 cells (Figure 5B). To determine if α-mangostin could improve LDL-cholesterol uptake, we monitored intracellular LDL-cholesterol by staining the cells with 1,1′-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeled LDL. HepG2 cells were incubated with DiI-LDL for 4 h in the absence (control) or presence of α-mangostin (10 μM) and then examined by microscopy. As shown in Figure 5C, α-mangostin treatment increased DiI-labeled LDL.
Figure 5. Effect of α-mangostin on cholesterol uptake in the HepG2 human hepatocellular liver carcinoma cell line. (A) Filipin staining of HepG2 cells cultured for 24 h in cells treated with α-mangostin and cholesterol (10 μg/mL) as indicated. After 24 h, the cells were washed, fixed in

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paraformaldehyde, and stained with filipin. Intracellular filipin-cholesterol complexes were visualized by fluorescence microscopy, and images were captured with a fluorescence microscope. Representative images are shown. (Magnification: ×40; scale bars: 30 μm). (B) Relative fluorescence of filipin and PI-stained cells treated as described in A that were fluorescence detected using UV excitation around 340 nm and emission around 380 nm in a microplate reader. Statistical significance of the differences between each treatment group and the normal group (*p < 0.05) was determined. (C) Effect of α-mangostin on intracellular accumulation of DiI-LDL in HepG2 cells incubated with DiI-LDL (10 μg/mL) under control conditions or treated with 2 or 10 μM α-mangostin. Representative images are shown. (Magnification: ×40; scale bars: 30 μm). (D) Relative fluorescence of DiI-LDL and DAPI-stained cells treated as described in C that were fluorescence detected using UV excitation around 554 nm and emission around 571 nm in a microplate reader. Statistical significance of the differences between each treatment group and the normal group (p < 0.05) was determined. (E) HepG2 cells treated with hydroxysterol (1 μg/mL) and/or treated α-mangostin (10 μM) for 24 h. The expressions of SREBP1, SREBP2, and PCSK9 were assayed by western blot analysis. (F) HepG2 cells treated with hydroxysterol and/or treated with α-mangostin (10 μM) for 24 h. The expressions of LDL-R, ABCA1, SQLE, SREBP1, SREBP2, and PCSK9 were assayed by qRT-PCR.

labeled LDL uptake. Quantification of the fluorescence showed significantly increased signal levels (Figure 5D).

The formation of mature SREBP2 is prevented by 25-hydroxycholesterol (25-HC) via the suppression of SREBP2 cleavage, and 25-HC is also known to activate LXRs, which in turn upregulates SREBP1 transcription. Therefore, the effect of α-mangostin on SREBP1 and SREBP2 expressions was evaluated in HepG2 cells in the presence or absence of exogenous cholesterol such as 25-HC. Treatment of HepG2 cells with 25-HC suppressed SREBP2 expression compared with that detected in the nontreated group, whereas SREBP1 expression was not changed (Figures 5E and 5C). Furthermore, in the presence of 25-HC, α-mangostin treatment appeared to not enhance the suppression of SREBP1 and SREBP2 expressions. PCSK9 expression, which is regulated by SREBP2, appeared to be similar in HepG2 cells in both the presence and absence of 25-HC. PCSK9 expression was downregulated with α-mangostin treatment in both the absence and presence of 25-HC. In the qPCR analysis (Figure 5F), α-mangostin was found to control the expression of SREBP2, which is associated with cholesterol homeostasis, as well as the downstream target genes of SREBP2 involved in cholesterol synthesis (SQLE, IDI1) and cholesterol trafficking (ABCA1, PCSK9).

## DISCUSSION

As one of the major constituents in mangosteen fruit, α-mangostin is used as a popular dietary supplement and has been reported to possess lipid-lowering activities in both in vitro and in vivo studies. Despite extensive research on the properties of α-mangostin, transcriptional gene changes related to cellular cholesterol homeostasis regulated by α-mangostin have not been investigated. Owing to the complexity of cellular cholesterol homeostasis that is orchestrated by many interacting proteins and genes, it is not easy to determine the genes responsible for the effect of α-mangostin on cholesterol homeostasis.

By analyzing whole-transcriptome data using DEGs, GO, KEGG pathway, and genetic interactions, we found that some of the genes related to cholesterol synthesis (e.g., LSS, IDI1, DHCR7) and cholesterol trafficking (PCSK9) were downregulated or upregulated by α-mangostin treatment. Furthermore, specific genes, such as SQLE, DHCR7, and IDI1, and enzymes, such as HMGCR, SQLE, and LSS, that are involved in cholesterol synthesis were downregulated. Moreover, multiple genes closely associated with lipid metabolism were apparently downregulated following α-mangostin treatment. Members of the fatty acid desaturase (FADS) family, including FADS1 and FADS2, regulate fatty acid desaturation by introducing double bonds between defined carbons of fatty acyl chains, thereby playing an essential role in the lipid metabolic pathway. In addition, acetyl-CoA acetyltransferase 2 (ACAT2), also known as cytosolic acetoacetyl-CoA thiolase, plays a role in regulating lipid metabolism by catalyzing the synthesis of acetoacetyl-CoA from two acetyl-CoA molecules, which is later converted into steroids. We found that α-mangostin inhibited FADS1, FADS2, and ACAT2 expressions, implying the potential regulation of a lipid metabolic pathway.

Collectively, these observations suggest that α-mangostin might control the cholesterol metabolic process. In addition, among the α-mangostin-regulated genes, SQLE, LSS, HMGCR, and PCSK9 are well-known downstream targets of SREBPs. Therefore, SREBP1 and SREBP2 expressions in α-mangostin-treated cells were monitored in an effort to detect any dominant SREBP isoform. The genes encoding SREBP1 and SREBP2, the predominant SREBP isoforms expressed in the liver, contain sterol regulatory elements within their promoters that mediate feed-forward transcriptional regulation. SREBP2 expression was more downregulated by α-mangostin treatment than SREBP1 at both transcriptional and post-transcriptional levels. Moreover, predominant interactions of SREBP2 with proteins relevant to cholesterol metabolic processes in α-mangostin-treated hepatocytes were predicted based on analysis of the STRING protein—protein interaction network database. Previous reports also suggested that SREBP2 preferably modulates the expression of genes associated with cholesterol metabolism, whereas SREBP1 primarily regulates lipogenic genes. In line with these previous reports, we found that α-mangostin seemed to preferentially downregulate the expression of SREBP2 mRNA compared to SREBP1 mRNA in two independent hepatocyte cell lines (HepG2 and Huh7). Therefore, we concluded that the differential suppression of SREBP2 by α-mangostin treatment controlled the cholesterol metabolic process as a central factor of transcription.

Furthermore, downregulation of downstream targets of SREBPs is achieved through the translocation of SREBPs to the nucleus. Cholesterol, oxysterols, and fatty acids regulate the SREBP pathway through complex formation of SREBP with SCAP and the endoplasmic reticulum retention protein insulin-induced gene 1 protein (INSIG). The status of this complex controls the endoplasmic reticulum-to-Golgi trafficking of the SCAP–SREBP complex and thereby modulates the proteolytic activation of SREBPs. When cholesterol is abundant in a cell, SREBP remains in the endoplasmic reticulum in the form of the SREBP–SCAP–INSIG complex.
complex. In contrast, depletion of intracellular sterols enhances SREBP translocation from the endoplasmic reticulum to the Golgi apparatus, leading to subsequent cleavage by site-1 and site-2 proteases, thereby generating mature SREBP and leading to mature SREBP nuclear translocation.

Moreover, 25-HC is known to inhibit SREBP maturation by blocking the SCAP-mediated movement of SREBPs from the endoplasmic reticulum to the Golgi through the binding of INSIGs to SCAP as a consequence of binding of 25-HC to INSIGs. Treatment of cells with α and β-mangostin, although mRNA expression profi

LDLR degradation and thereby decreases LDL uptake.

which was in part explained by the downregulation of PCSK9, a downstream target protein of SREBP2. PCSK9 facilitates LDLR degradation and thereby decreases LDL uptake.

Collectively, the current study provides a comprehensive mRNA expression profile of hepatocytes treated with α-mangostin. Among the mRNAs regulated, SREBP2 was strongly associated with the effect of α-mangostin on the cholesterol biological process and appeared to function as an important hub gene in α-mangostin-treated hepatocytes for controlling the networks of cholesterol metabolism. The present study of mining the mRNA expression profile generated in response to an active component present in dietary supplements and edible fruit suggests that attempts to discover new potential of functional foods may be complemented by mining the whole transcriptome.

## MATERIALS AND METHODS

### Cell Culture, Drugs, and Chemicals.**

The HepG2 human hepatocellular liver cell line was obtained from the Korea Research Institute of Bioscience and Biotechnology (Daejeon, South Korea) and grown in Eagle’s minimum essential medium (EMEM) containing 10% fetal bovine serum and 100 U/mL penicillin/streptomycin. Cells were incubated in a humidified 5% CO₂ atmosphere at 37 °C. EMEM, penicillin, and streptomycin were purchased from HyClone (Logan, UT, USA). Bovine serum albumin (BSA) and 25-hydroxycholesterol were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4′,6-Diamidino-2-phenylindole (DAPI) and DiI-LDL were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Propidium iodide (PI) dye (50 μM) dissolved in phosphate-buffered saline (PBS) was then added and incubated for 5 min to stain the cells and nuclei acids. The cells were then incubated in 500 μL of a PBS-based filipin working solution (100 μg/mL; Sigma, St. Louis, MO, USA) for 2 h at room temperature. Filipin stained cells were imaged using a Nikon Eclipse Ti-U inverted microscope equipped with a S Plan Fluor 40× (N.A. 0.6) objective lens and a DS-Fi1 digital microscope camera in conjunction with NIS-Elements F software (Nikon, Tokyo, Japan).

### Measurement of DiI-LDL Uptake.

The amount of DiI-LDL uptake was measured according to the manufacturer’s instruction (Thermo Fisher Scientific). In brief, the culture medium of HepG2 cells was replaced with serum-free medium, and the cells were incubated with 10 μg/mL DiI-LDL at 37 °C for 24 h, followed by observation with a fluorescence Nikon Eclipse Ti-U inverted microscope (Nikon, Tokyo, Japan).

### Library Preparation and Sequencing.

Total RNA was isolated using a TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s protocols. The quantity and quality of the total RNA were tested using a model Agilent 2100 bioanalyzer system RNA kit (Agilent Technologies, Santa Clara, CA, USA). The total RNA was processed for preparing mRNA sequencing library using a TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer’s instructions. The quality and size of the libraries were assessed using an Agilent 2100 bioanalyzer DNA kit (Agilent Technologies). All libraries were quantified by qPCR using a CFX96 Real Time System (Bio-Rad Hercules, CA, USA) and sequenced on the NextSeq500 sequencers (Illumina) with a paired-end 75bp plus single 6bp index read run.

### Preprocessing and Genome Mapping.

To monitor the quality and soundness of the raw RNA sequence reads, base quality distribution and inclusion of the adapter sequences in the raw reads were evaluated using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) software. Potentially existing sequencing adapters and raw quality bases in the raw reads were trimmed by Cutadapt software. The options –q 0, -m 20, and -O 3 were used for trimming low-quality 5’ and 3’ ends of the raw reads. The cleaned high-quality reads after trimming the low-quality bases and sequencing adapters were mapped to the

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human reference genome mm10 of the UCSC genome (https://genome.ucsc.edu) by STAR software.\textsuperscript{37,38} Since the sequencing libraries were prepared strand-specifically by using Illumina’s strand-specific library preparation kit, the strand-specific library option, —library-type fr-firststrand, was applied in the mapping process.

**Quantifying Gene Expression and DEG Analysis.** Cufflinks software was used to quantify the mapped reads on the human reference genome into the gene expression values.\textsuperscript{8,39} The gene annotation of the human reference genome mm10 from UCSC genome (https://genome.ucsc.edu) in GTF format was used as the gene model, and the expression values were calculated in Fragments Per Kilobase of transcript per Million fragments mapped unit. The DEGs between the two selected biological conditions were analyzed by Cuffdiff software in Cufflinks package.\textsuperscript{40} To compare the expression profiles among the samples, the normalized expression values of the few hundred selected genes with the most variable expression were unsupervised clustered using MeV software of the TM4 microarray software suite.\textsuperscript{41} The scatter plots for the gene expression values and the volcano plots for the expression-fold changes and p-values between the two selected samples were drawn by in-house R scripts.

**Functional Category Analysis.** The biological functional role of the analyzed DEGs between the compared biological conditions was assessed using the gene set overlapping test. Functional categorized genes were determined using the DAVID tool and included the biological processes of GO, KEGG pathways, and transcription factor binding target gene sets.\textsuperscript{42}

**Immunofluorescence.** HepG2 cells cultured on slides were fixed with ethanol for 30 min at 4 °C. Following washing with PBS and blocking with 3% BSA in PBS for 30 min, the samples were incubated overnight at 4 °C with rabbit polyclonal anti-SREBP1 and mouse polyclonal anti-SREBP2 (1:500 dilution). The excess primary antibody was removed, and the samples were incubated with Alexa 488-conjugated and Alexa 594-conjugated secondary antibody (1:500 dilution). The excess primary antibody was removed, and the samples were incubated with Alexa 488-conjugated and Alexa 594-conjugated secondary antibody (Invitrogen Molecular Probes, Burlington, ON, Canada) for 2 h at room temperature. Following washing with PBS, the slides were mounted using ProLong Gold Antifade reagent containing DAPI (Thermo Scientific, Waltham, MA, USA) to visualize the nuclei. Specimens were covered with coverslips and evaluated under a confocal laser scanning microscope (Nikon Eclipse, Nikon, Japan).

**Quantitative Real-Time RT-PCR.** Total cellular RNA was isolated using a TRIzol RNA extraction kit according to the manufacturer’s instructions. The first-strand cDNA was synthesized with M-MLV reverse transcriptase (Promega, Madison, WI, USA). cDNA was then subjected to quantitative real-time PCR using a SYBR-Green PCR Master Mix (Bio-Rad) and the CFX384 Real-Time PCR Detection System (Bio-Rad). The specificity of the amplification was confirmed using a melting curve analysis. Data were collected and recorded by CFX Manager Software (Bio-Rad) and expressed as a function of the threshold cycle \((C_{T})\). The relative quantity of the gene of interest was then normalized to the relative quantity of GAPDH \((\Delta\Delta C_{T})\). The mRNA abundance in the sample was calculated using the equation \(2^{\Delta\Delta C_{T}}\). The following specific primer sets were used (5’ to 3’): human—GAPDH: GAAGGTGGAGGGTGGTCA (forward), AAT-GAAGGGGTTCATGATG (reverse); human—SREBF1: GGAGATTGGACTGACTTCCA (forward), GGCCCTTTCAACACAGGAA (reverse); human—SREBF2: ACCAG-CAGAGCAACCAAG (forward), GGAGGAGGGAGGAAGG (reverse); human—HMGCR: TGATGGACCTTTTCAGAGCAAG (forward), CTAAAATGCATTCCACCGAG (reverse); human—PCSK9: GTGATCAGCAACCCCAACCTG (forward), CCGAGTGTGCTGACCATACA (reverse); human—ID1: AACACGCCAAAATAGTCTCTGC (forward), GCCGACTCATTCCCAGCTGCT (reverse); human—SOLE: GGAAGACTGTTCTCCTCAAT (forward), GGAAGCTGTTCTCCTCAAT (reverse); human—FDFT1: ATAAACCAATGCACTGCACCA (forward), ATAAACCAATGCACTGCACCA (reverse); human—FDPT: GGAAGATGGGGAGGTTTCTTTTC (forward), GTCCCAAAAAGGTCAGAGGT (reverse); human—SOAT1: TTCTATCCGGTTGCTCCTCTG (forward), GACTC-CATTGCCCAAGAAA (reverse); Gene-specific primers were custom-synthesized by Bioneer.
Complete contact information is available at:

■ REFERENCES

Statistical Analysis. The experimental data are presented as the mean ± standard error of the mean. The level of statistical significance was obtained from analysis of variance (ANOVA) followed by Dunnett’s t-test for multiple comparisons. P values less than 0.05 were considered to be significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c04282.

RNA sequencing results (DEGs, full list of KEGG, and GO) (XLSX)

Metabolic gene expressions by qRT-PCR in Huh7 cells and quantitative analysis of the immunoblot (PDF)

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Notes

The authors declare no competing financial interest.

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