Resveratrol suppresses hepatic fatty acid synthesis and increases fatty acid β-oxidation via the microRNA-33/SIRT6 signaling pathway

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Abstract. Hyperlipidemia is a strong risk factor for numerous diseases. Resveratrol (Res) is a non-flavonoid polyphenol organic compound with multiple biological functions. However, the specific molecular mechanism and its role in hepatic lipid metabolism remain unclear. Therefore, the aim of the present study was to elucidate the mechanism underlying how Res improves hepatic lipid metabolism by decreasing microRNA-33 (miR-33) levels. First, blood miR-33 expression in participants with hyperlipidemia was detected by reverse transcription-quantitative PCR, and the results revealed significant upregulation of miR-33 expression in hyperlipidemia. Additionally, after transfection of HepG2 cells with miR-33 mimics or inhibitor, western blot analysis indicated downregulation and upregulation, respectively, of the mRNA and protein expression levels of sirtuin 6 (SIRT6). Luciferase reporter analysis provided further evidence for binding of miR-33 with the SIRT6 3’-untranslated region. Furthermore, the levels of peroxisome proliferator-activated receptor-γ (PPARγ), PPARγ-coactivator 1α and carnitine palmitoyl transferase 1 were increased, while the concentration levels of acetyl-CoA carboxylase, fatty acid synthase and sterol regulatory element-binding protein 1 were decreased when SIRT6 was overexpressed. Notably, Res improved the basic metabolic parameters of mice fed a high-fat diet by regulating the miR-33/SIRT6 signaling pathway. Thus, it was demonstrated that the dysregulation of miR-33 could lead to lipid metabolism disorders, while Res improved lipid metabolism by regulating the expression of miR-33 and its target gene, SIRT6. Thus, Res can be used to prevent or treat hyperlipidemia and associated diseases clinically by suppressing hepatic fatty acid synthesis and increasing fatty acid β-oxidation.

Introduction

Hyperlipidemia is also referred to as lipid metabolism disorder or lipid metabolism abnormality. Hyperlipidemia is a systemic disorder of lipid metabolism caused by various factors, such as elevated triglycerides (TG), total cholesterol (TC) and/or low-density lipoprotein cholesterol (LDL-C), and the reduction of high-density lipoprotein cholesterol (HDL-C) (1). Unhealthy diet and excessive energy intake make hyperlipidemia a chronic disease with an increasing incidence worldwide (1,2). Hyperlipidemia is a strong risk factor for numerous diseases, such as diabetes, atherosclerosis and cardiovascular disease (3-5). Therefore, preventing and treating hyperlipidemia are effective and common methods to reduce the incidence of cardiovascular disease and other chronic diseases (6). Hyperlipidemia should be prevented and treated as early as possible to reduce the incidence of associated diseases (7).

MicroRNAs (miRNAs/miRs) have emerged as critically important post-transcriptional regulators of disease pathogenesis. A number of miRNAs have been identified as critical regulators of cellular lipid and lipoprotein metabolism (8), including the miR-33 family (9). The miRNAs of this family
compromise miR-33a and miR-33b, which are encoded within the introns of the sterol regulatory element-binding protein (SREBP)2 and 1 genes, respectively (10,11). Although the miR-33 isoforms differ in two nucleotides in their mature forms, they share the same seed sequence and repress the same target genes (12). The miR-33 family is one of the most well-studied miRNA families as a potential therapeutic target to treat numerous diseases, including atherosclerosis, obesity and diabetes (13-15). Specifically, the miR-33 family serves key roles in regulating cholesterol and fatty acid homeostasis, controlling HDL-C biogenesis and cholesterol efflux by regulating ATP binding cassette subfamily A member 1 (ABCA1) gene expression, and regulating cellular functions, such as macrophage activation, mitochondrial biogenesis and autophagy (16). Furthermore, in the liver, miR-33 regulates reverse cholesterol transport by targeting factors involved in HDL-C biogenesis (ABCA1) and the cholesterol reverse transport process, and bile acid secretion and synthesis (17,18). Hepatic miR-33 deficiency not only improves regulation of glucose homeostasis but also prevents the development of fibrosis and inflammation (19,20). Thus, miR-33 deficiency can attenuate non-alcoholic fatty liver disease-non-alcoholic steatohepatitis-hepatocellular carcinoma progression (21,22).

Recent research has revealed that dietary polyphenols, including curcumin (23), resveratrol (Res) (24) and epigallocatechin gallate (25), modulate miRNA expression. Among these, Res is a non-flavonoid polyphenol organic compound and has now been identified in >70 plants, including grapes, Polygonum cuspidatum and Veratrum nigrum (26,27). Multiple studies have confirmed that Res has multiple biological functions, including regulating lipid metabolism, anti-inflammatory effects, mitochondrial protection and/or autophagy induction, and anti-oxidation (28-35).

The liver is essential for energy homeostasis and serves an active role in synthesis, storage and redistribution of glucose and free fatty acids (36). Res and atorvastatin have been used to treat high-fat diet (HFD) intake-induced non-alcoholic fatty liver disease by targeting genes involved in cholesterol metabolism and miR-33 (37). Additionally, Res and epigallocatechin gallate bind directly and distictively to miR-33a and miR-122, and modulate their levels in hepatocytes (38). Therefore, the specific molecular mechanism underlying the effects of miR-33 and its role in hepatic lipid metabolism are unclear. Hence, the aim of the present study was to elucidate how Res improves hepatic lipid metabolism by targeting miR-33.

**Materials and methods**

**Study subjects.** The present study was performed at the Physical Examination Center of Hebei General Hospital (Shijiazhuang, China) and was approved by the Hebei General Hospital Ethics Committee (2018 Scientific Research Ethics Review; approval no. 39; Shijiazhuang, China). All of the clinical samples were obtained from the Physical Examination Center of Hebei General Hospital. A total of 36 subjects with elevated blood lipids in the physical examination population between May 1, 2021 and November 30, 2021 were randomly selected as the hyperlipidemia group (27 men, 9 women; mean age, 65.50±7.71 years; age range, 50-83 years). Another 36 healthy subjects matched for age and sex with the subjects in the hyperlipidemia group were randomly selected during the same period from the Physical Examination Center of Hebei General Hospital as the normal control group (CG group; 27 men, 9 women; mean age, 69.11±8.81 years; age range, 43-83 years). All participants provided written informed consent. The diagnostic criteria for hyperlipidemia were in accordance with the Guidelines for the Prevention and Treatment of Dyslipidemia in Chinese Adults (Revised Edition 2016) (2), which are as follows: Plasma TC ≥6.2 mmol/l (240 mg/dl), TG ≥2.3 mmol/l (200 mg/dl), LDL-C ≥4.1 mmol/l (160 mg/dl) or HDL-C <1.0 mmol/l (40 mg/dl) in adults after a 12-h fast. Hyperlipidemia was diagnosed if any one criterion was met. Participants taking aspirin, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers or statins within the previous 2 months were excluded from the study. Participants with chronic liver disease (including hepatitis B virus carriers), kidney disease, thyroid insufficiency or abnormalities, hypertension, diabetes, blood system disorders, mental disorders, acute and chronic infectious diseases, autoimmune diseases, tumors, pregnancy, lactation, long-term oral contraceptive use, and/or recent surgical history were excluded. The inclusion criteria for the CG group were as follows: No history of hypertension, diabetes mellitus and other chronic diseases; blood glucose 3.9-6.1 mmol/l; and the following blood lipid concentration levels: TC <5.2 mmol/l, TG <1.7 mmol/l and LDL-C <3.4 mmol/l.

**Blood samples.** Fasting blood samples (5 ml) were collected from each participant and placed in a BD Vacutainer SST tube (Becton, Dickinson and Company). Peripheral blood mononuclear cells (PBMCs) were isolated from fasting blood samples by Ficoll-Paque density gradient centrifugation (20°C; 500 x g; 25 min) for miR-33 and sirtuin 6 (SIRT6) detection by reverse transcription-quantitative PCR (RT-qPCR). Biochemical tests [TC, TG, HDL-C, LDL-C, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and fasting blood glucose (FBG)] were performed using an automatic biochemical detection instrument at the Clinical Laboratory of Hebei General Hospital. Glycated hemoglobin (HbA1c) was analyzed at Hebei Key Laboratory of Metabolic Diseases (Shijiazhuang, China) using an automatic glycohemoglobin analyzer (ADAMS Alc HA-8180; ARKRAY, Inc.) at 25°C.

**Animal experiments.** A total of 24 C57BL/6J mice (male; age, 8 weeks; weight, 22.0±2.0 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The mice were housed in the animal laboratory at the Hebei Key Laboratory of Metabolic Diseases (temperature, 21-23°C; humidity, 40-60%; and 12/12-h light/dark cycle) with constant access to food and water. All experimental procedures were approved (2022 Scientific Research Ethics Review; approval no. 217) by the Animal Care and Use Committee of Hebei General Hospital (Shijiazhuang, China) and complied with the Animal (Scientific Procedures) Act 1986 and associated guidelines (39).

After 1 week of adaptive feeding, the mice were randomly divided into three groups, with 8 mice in each group. The diet for the normal diet (ND) group was an ordinary diet (D12450J formula, consisting of 20% protein, 70% carbohydrate, 10%...
tissues were embedded in paraffin wax, cut into 5-µm-thick sections, deparaffinized in xylene at 25°C and rehydrated in a reverse-gradient series of ethanol alcohol (100, 95, 80 and 75%). The sections were stained with hematoxylin at 25°C for 10 min and stained with eosin at 25°C for 3 min, and visualized under a light microscope.

**Oil Red O staining.** Parts of the fresh liver tissues were taken and embedded in optimum cutting temperature compound, quickly frozen, and then sliced into 6-µm tissue sections. The sections were washed with PBS, stained with Oil red O working solution (6:4, oil red stock solution:distilled water; Oil red O: WSIG20100803; Sinopharm Chemical Reagent Co., Ltd.) at room temperature for 15 min and washed three times with PBS to remove the excess Oil red O dye. Subsequently, the sections were stained with Harris's hematoxylin (20151216; Nanjing Jiancheng Bioengineering Institute) for 3 min at 25°C. The morphological features of the liver sections were observed under a light microscope.

**Body weight and food intake measurement.** The body weight and food intake of the mice in each group were measured at baseline and weekly thereafter until 6 weeks after baseline.

**Detection of serum glucose and lipids in mice.** Serum glucose levels were determined using a glucose assay kit (cat. no. 60408ES60; Shanghai Yeasen Biotechnology Co., Ltd.). The TG content assay kit (cat. no. D799796-0100; Sangon Biotech Co., Ltd.) was used to detect TG levels.

**Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT).** After feeding for 6 weeks, glucose (1 g/kg) was given to each mouse via an orogastric tube for the OGTT. Blood glucose was measured immediately after glucose administration and 15, 30, 60 and 120 min after administration. A total of 24 h after the OGTT, the ITT was performed after a 12-h fast. The mice were injected intraperitoneally with insulin (1.5 IU/40 g; Tonghua Dongbao Pharmaceutical Co., Ltd.) and glucose levels were determined using a glucose assay kit (cat. no. L3000015; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

**Histomorphometric comparison of mouse liver tissues**

H&E staining. Parts of the liver tissues were taken and fixed in 4% paraformaldehyde at 25°C for 24 h. Subsequently, the tissues were embedded in paraffin wax, cut into 5-µm-thick sections, deparaffinized in xylene at 25°C and rehydrated in a reverse-gradient series of ethanol alcohol (100, 95, 80 and 75%). The sections were stained with hematoxylin at 25°C for 10 min and stained with eosin at 25°C for 3 min, and visualized under a light microscope.

**Cell culture.** HepG2 cells (human liver cancer cells) were purchased from Procell Life Science & Technology Co., Ltd., and cultured in complete DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Sangon Biotech Co., Ltd.) and 1% penicillin/streptomycin (Sangon Biotech Co., Ltd.) at 37°C with 5% CO₂. HepG2 cells were immersed in normal medium and medium containing 0.25 mmol/l palmitate (PA) for 24 h. At the end of the stimulation period, the cells were washed three times with PBS and fixed with 4% paraformaldehyde for 10 min at 37°C. Subsequently, cells were washed twice with PBS, then stained with 0.5% Oil red O for 30 min at 37°C. After staining, the cells were washed once with 60% isopropanol, washed with PBS until a colorless solution was obtained, and observed under a fluorescence inverted microscope at a magnification of x50. Short tandem repeat profiling was used for authentication of HepG2 cells. HepG2 cells cultured in normal medium and transfected with miR-33 mimics, and HepG2 cells cultured in medium containing 0.25 mmol/l PA for 24 h after transfection with miR-33 inhibitor or SIRT6-pcDNA 3.1 were used to analyze the effect of transfection on lipid metabolism-related genes and lipid deposition.

**Cell transfection.** miR-33 mimics, inhibitor and the corresponding controls were synthesized by Shanghai GenePharma Co., Ltd. For miR-33 mimics transfection, the HepG2 cells were seeded in 6-well plates at a density of 5x10⁵ cells/well. When 70-80% confluence was reached, cells were divided into three groups: CON (liposome), NC mimics (liposome + mimics control sequence) and miR-33 mimics (liposome + miR-33 mimics). The CON group was the control group, in which cells were transfected without any sequence. The NC mimics group was the scrambled negative control. The sequence of the corresponding controls (100 nmol/l) was 5'-GGUCUUCAGUCAGUCACAAUUCUG-3'. Cells were transfected using Lipofectamine® 3000 Transfection Reagent (cat. no. L3000015; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The cells were transfected for 6 h at 37°C in a cell incubator with 5% CO₂, and then the medium was replaced with fresh DMEM. In the miR-33 mimics group, the cells were transfected with 100 nmol/l miR-33 mimics (5'-GUGCAUUGAUUGCAUUGCA-3')
using Lipofectamine® 3000 Transfection Reagent (cat. no. L3000015; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. Cells were transfected for 6 h at 37˚C in a cell incubator with 5% CO₂, and then the medium was replaced with fresh DMEM. Subsequently, cells were incubated for 24 h in an incubator with 5% CO₂ at 37˚C, and the cells were collected for subsequent experiments.

To investigate the effect of miR-33 inhibitor transfection, HepG2 cells were divided into three groups: CON (liposome), NC inhibitor (liposome + inhibitor control sequence) and miR-33 inhibitor (liposome + miR-33 inhibitor). The CON group was the control group, in which cells were transfected without any sequence. The NC inhibitor group was transfected with 100 nmol/l scrambled negative controls (5'-UGGCGUCAUCAGUCAGCAUUAUCUG-3’) using Lipofectamine® 3000 Transfection Reagent (cat. no. L3000015; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. Cells were transfected for 6 h at 37˚C in a cell incubator with 5% CO₂, and then the medium was replaced with fresh DMEM. In the miR-33 inhibitor group, cells were transfected with 100 nmol/l inhibitor (5’-UGCAUGCAUCACAAUGAC-3’) using Lipofectamine® 3000 Transfection Reagent (cat. no. L3000015; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. Cells were transfected for 6 h at 37˚C in a cell incubator with 5% CO₂, and then the medium was replaced with fresh DMEM. Subsequently, cells were transfected for 48 h in an incubator with 5% CO₂ at 37˚C, and the cells were collected for subsequent experiments.

To investigate the effect of miR-33 inhibitor on intracellular lipid metabolism, cells were divided into three groups: PA + lipo (liposome), PA + NC inhibitor (liposome + inhibitor control sequence) and PA + miR-33 inhibitor (liposome + miR-33 inhibitor). miR-33 inhibitor or NC inhibitor (scramble control sequence) and PA + miR-33 inhibitor (liposome + miR-33 inhibitor) were transfected with Lipofectamine® 3000 Transfection Reagent (cat. no. L3000015; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. Cells were transfected for 6 h at 37˚C in a cell incubator with 5% CO₂, and then the medium was replaced with fresh DMEM. Subsequently, cells were transfected for 48 h in an incubator with 5% CO₂ at 37˚C, and the cells were collected for subsequent experiments.

To investigate the effect of SIRT6 overexpression, HepG2 cells were divided into the pcDNA 3.1 group (transfected with 500 ng pcDNA 3.1) and the SIRT6-pcDNA 3.1 group (transfected with 500 ng SIRT6-pcDNA 3.1). Cells were transfected in an incubator with 5% CO₂ at 37˚C for 6 h with the aforementioned plasmids using Lipofectamine® 3000 Transfection Reagent (cat. no. L3000015; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol, and then the medium was replaced with fresh DMEM. Subsequently, cells were transfected for 24 h in an incubator with 5% CO₂ at 37˚C, and then collected for the subsequent experiments.

To investigate the effect of SIRT6 overexpression on lipid metabolism, HepG2 cells were divided into three groups: PA + lipo (liposome), PA + pcDNA 3.1 (liposome + 500 ng pcDNA 3.1) and PA + SIRT6-pcDNA 3.1 (liposome + 500 ng SIRT6-pcDNA 3.1). SIRT6-pcDNA 3.1 or pcDNA 3.1 transfection was performed as aforementioned. After transfection for 6 h at 37˚C, cells were incubated with PA (0.25 mmol/l) for 24 h in an incubator with 5% CO₂ at 37˚C, and then collected for subsequent experiments. The human SIRT6-pcDNA 3.1 (cat. no. V38520) was purchased from Thermo Fisher Scientific, Inc.
sodium deoxycholate and 0.1% SDS), and the total soluble protein was quantified using a BCA Protein Assay kit (Beijing Solarbio Science & Technology Co., Ltd.). Protein (20 μg/lane) from cell lysates was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Following transfer of the proteins onto polyvinylidene fluoride membranes, the membranes were blocked at room temperature for 60 min in 5% skim milk and probed with the primary antibodies overnight at 4°C: Acetyl-CoA carboxylase (ACC; dilution, 1:2,000; cat. no. 3676; Cell Signaling Technology, Inc.), fatty acid synthase (FASN; dilution, 1:1,000; cat. no. ab128870; Abcam), SREBP1 (dilution, 1:2,000; cat. no. 557036; BD Biosciences), peroxisome proliferator-activated receptor-γ (PPARγ; dilution, 1:1,000; cat. no. 16,643-1-AP; Proteintech Group, Inc.), anti-PPARγ-coactivator 1 α (PGC1α; dilution 1:1,000; cat. no. 66,369-1-lg; Proteintech Group, Inc.), carnitine palmitoyltransferase 1 (CPT1; dilution 1:1,000; cat. no. AF5658; Beyotime Institute of Biotechnology), SIRT6 (dilution, 1:1,000; cat. no. ab191385; Abcam) and anti-β-actin (dilution, 1:1,000; cat. no. 60008-1; Proteintech Group, Inc.). The membranes were incubated with the secondary antibodies for 2 h at room temperature. The secondary antibodies included the HRP-conjugated goat anti-rabbit antibody (dilution, 1:5,000; cat. no. ZDR-5306; OriGene Technologies, Inc.) and the HRP-conjugated goat anti-mouse antibody (dilution, 1:10,000, cat. no. ZDR-5307; OriGene Technologies, Inc.). Protein bands were visualized using enhanced chemiluminescent substrate (Pierce ECL Western Blotting substrate; Thermo Fisher Scientific, Inc.), and the band intensities were evaluated using Image J software (V1.8; National Institutes of Health).

**Dual luciferase assay.** The synthesized SIRT6 3'-untranslated region (UTR) was inserted into the pmirGLO vector (Promega Corporation). The mutation in the miR-33 seed-matching sequences was designed using the SIRT6 wild-type (WT) sequence generated by overlap extension PCR. SIRT6 WT and SIRT6 mutant-type (MT) reporter plasmids were designed and constructed by Guangzhou Ribobio Co., Ltd. 293T cells (Shanghai GeneChem Co., Ltd.) were cultured in High Glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Sangon Biotech Co., Ltd.) and 1% penicillin/streptomycin at 37°C with 5% CO2. The WT and MT sequences were co-transfected with the miR-33 mimic (5'-GUGCAUGUAGUGCUAGCA-3'; 100 nM) or corresponding control (5'-GGUCUACGUCAGUCACAUAAUCUG-3'; 100 nM) into 293T cells using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 6 h. After transfection for 24 h, the cells were lysed and subjected to a Dual-Luciferase Reporter Assay (Promega Corporation). Luciferase activity was measured and calculated as the ratio of firefly luciferase activity to Renilla luciferase activity. The experiment was repeated three times.

**Statistical analysis.** All experimental data are presented as the mean ± SD. All experiments were repeated at least three times to verify the trends. One-way ANOVA with Tukey's post hoc test was used for comparisons among multiple groups. Comparisons between groups were performed using an unpaired Student's t-test. Sex differences were compared using the Pearson χ2 test. SPSS (version 25.0; IBM Corp.) was used for all analyses. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Clinical and metabolic characteristics of the participants.** Demographic, clinical and biochemical data were obtained from 36 participants with hyperlipidemia and 36 healthy control participants. As shown in Table I, BMI, weight, TC, TG, LDL-C, FBG, HbA1c, ALT and AST concentration levels were significantly higher, while HDL-C levels were significantly lower, in participants with hyperlipidemia compared with CG participants. No significant differences in sex, age or height were observed between the groups.

miR-33 and SIRT6 expression levels differ between participants with hyperlipidemia and CG participants. PBMCs from the hyperlipidemia group and CG were tested for miR-33 and SIRT6 expression levels and it was identified that miR-33 expression levels were significantly higher (Fig. 1A), and SIRT6 expression was significantly lower in the hyperlipidemia group compared with the CG (Fig. 1B).

**Res reverses the changes in lipid metabolism and expression of miR-33 and SIRT6 in the HFD mouse model.** Before investigating the underlying mechanism of Res in lipid metabolism, the mice in the HFD group were used to investigate lipid metabolism and miRNA expression in blood or liver tissues, respectively. After 6 weeks, body weights were significantly higher in the HFD group compared with the ND group. From 5 weeks, body weights were decreased significantly in the HFD + Res group compared with the HFD group (Fig. 2A). There was no significant difference in the daily food intake among the three groups (Fig. 2B). TC, TG, LDL-C, MDA, ALT and AST concentration levels were significantly higher in mice in the HFD group compared with mice in the ND group, and these levels were decreased significantly in the HFD + Res group compared with the HFD group (Fig. 2C and D). By contrast, HDL-C concentration levels were significantly lower in the HFD group compared with the ND group, and significantly increased in the HFD + Res group compared with the HFD group (Fig. 2C). Blood glucose levels were also recorded, and OGTT and ITT results are shown in Fig. 2E and G. In the OGTT, there was a significant decrease in the AUC in the HFD + Res group compared with the HFD group (Fig. 2F). Consistently, there was a statistically significant difference in QUICKI values between the HFD + Res and HFD groups (Fig. 2H).

To investigate the effect of Res on hepatic lipid deposition in mice, H&E staining and oil red O staining were performed using mouse liver tissues. H&E staining of mouse liver tissues revealed uniform cell cytoplasm in ND mice and fewer lipid droplets (Fig. 3A). In comparison, hepatocyte staining in the HFD group revealed disordered cellular structure and more lipid droplets (Fig. 3B). In comparison, hepatocyte staining in the HFD group revealed disordered cellular structure and more lipid droplets (Fig. 3B). In the HFD group, the morphology of the liver tissue and the number of lipid droplets were intermediate to those of the ND and HFD groups (Fig. 3C). Liver cell staining with oil red O showed that cells from the ND group contained blue nuclei with a small number of orange-red lipid droplets (Fig. 3D). The HFD group exhibited numerous...
orange-red lipid droplets (Fig. 3E), whereas after Res treatment, the number of lipid droplets decreased (Fig. 3F).

To gain further insights, the effect of Res on lipid metabolism and gene expression was investigated. The results revealed that miR-33 expression was significantly higher (Fig. 4A) and SIRT6 mRNA expression was significantly lower (Fig. 4B) in liver tissue of the HFD group compared with the ND group. Western blot analysis to assess SIRT6 expression in tissues revealed a significant decrease in the HFD group (Fig. 4C and D). In the HFD + Res group, Res reversed the increase in miR-33 expression and the decrease in SIRT6 expression. It was also found that mRNA expression levels (Fig. 4E) and protein expression levels (Fig. 4F and G) of ACC, FASN and SREBP1 were increased in the HFD group compared with the ND group, whereas PPARγ, PGC1α and CPT1 mRNA and protein expression levels were decreased. However, these changes in the expression levels of liver genes and proteins were reversed in the HFD + Res group (Fig. 4E-G). These findings indicated that Res improved basic metabolic parameters and changed the expression levels of metabolism-related genes in mice fed a HFD supplemented with Res.

Res reverses the changes in lipid metabolism and expression of miR-33 and SIRT6 in PA-induced HepG2 cells. To further examine the underlying mechanism, PA-induced HepG2 cells were used to investigate the effect of Res on lipid metabolism and expression levels of miR-33 and SIRT6 in vitro. First, a high-fat model was constructed by inducing HepG2 cells with...
PA, and changes after Res treatment were observed. It was found that lipid deposition in HepG2 cells improved after the addition of Res (Fig. 5A). Next, changes in miR-33 and SIRT6 mRNA expression were detected in PA-induced HepG2 cells. RT-qPCR analysis demonstrated that miR-33 expression was increased significantly (Fig. 5B) and mRNA levels of SIRT6 decreased significantly (Fig. 5C) in PA-induced HepG2 cells compared with CON cells. Additionally, treatment with Res decreased miR-33 expression and increased SIRT6 expression in PA-induced HepG2 cells (Fig. 5B and C). Furthermore, western blotting indicated that, with Res supplementation, protein expression levels of SIRT6, PPARγ, PGC1α and
LIU et al: RESVERATROL INCREASES FATTY ACID β-OXIDATION VIA miR-33/SIRT6

Figure 3. Histomorphological findings of hepatic lipid deposition. (A-C) H&E staining of liver tissues. (A) ND group; (B) HFD group; (C) HFD + Res group. (D-F) Oil Red O staining of liver tissues. (D) ND group; (E) HFD group; (F) HFD + Res group. Scale bar, 100 µm. HFD, high-fat diet; ND, normal diet; Res, resveratrol.

Figure 4. Effect of Res on the expression levels of miR-33, SIRT6 and genes involved in fatty acid synthesis and fatty acid β-oxidation in vivo. (A) Relative expression levels of miR-33 in liver tissues. (B) Relative expression levels of SIRT6 in liver tissue. (C) Protein levels of SIRT6. (D) Western blot analysis of SIRT6. (E) mRNA expression levels of genes involved in fatty acid synthesis and fatty acid β-oxidation in liver tissues. (F) Western blot analysis of ACC, FASN, SREBP1, PPARγ, PGC1α and CPT1. (G) Expression levels of proteins involved in fatty acid synthesis and fatty acid β-oxidation in liver tissues. β-actin was used as a control for the normalization of samples for western blotting. Data are presented as the mean ± SD (n=3). *P<0.05 and **P<0.001 vs. ND group; #P<0.05 and ##P<0.001 vs. HFD group (one-way ANOVA with Tukey's multiple comparison test). ACC, acetyl-CoA carboxylase; CPT1, carnitine palmitoyl transferase 1; FASN, fatty acid synthase; HFD, high-fat diet; HFD + Res, HFD supplemented with Res; miR, microRNA; ND, normal diet; PGC1α, PPARγ-coactivator 1α; PPARγ, peroxisome proliferator-activated receptor-γ; Res, resveratrol; SIRT6, sirtuin 6; SREBP1, sterol regulatory element-binding protein 1.
Figure 5. Effect of Res on the expression levels of miR-33, SIRT6 and genes involved in fatty acid synthesis and fatty acid β-oxidation in vitro. (A) Oil Red O staining of HepG2 cells. Pale blue cytosol and a small amount of orange lipid droplets were visible in CON cells. Numerous orange lipid droplets were visible in PA-treated cells. After Res treatment, the numbers of lipid droplets were decreased compared with those in PA-treated cells (Scale bar, 100 µm). (B) Relative expression levels of miR-33. (C) Relative expression level of SIRT6. (D) Western blot analysis of SIRT6. (E) Protein levels of SIRT6. (F) Western blot analysis of ACC, FASN, SREBP1, PPARγ, PGC1α and CPT1. (G) Expression levels of proteins involved in fatty acid synthesis and fatty acid β-oxidation. (H) Genes involved in fatty acid synthesis and fatty acid β-oxidation. β-actin was used as a control for the normalization of samples for western blotting. Data are presented as the mean ± SD (n=3). *P<0.001 vs. CON group; **P<0.001 vs. the PA group (one-way ANOVA with Tukey’s multiple comparison test). ACC, acetyl-CoA carboxylase; CON, control; CPT1, carnitine palmitoyl transferase 1; FASN, fatty acid synthase; miR, microRNA; PA, palmitate; PGC1α, PPARγ-coactivator 1α; PPARγ, peroxisome proliferator-activated receptor-γ; Res, resveratrol; SIRT6, sirtuin 6; SREBP1, sterol regulatory element-binding protein 1.
LIU et al.: RESVERATROL INCREASES FATTY ACID β-OXIDATION VIA miR-33/SIRT6

CPT1 (Fig. 5F and G) were increased compared with those in PA-induced HepG2 cells, whereas the expression levels of ACC, FASN and SREBP1 were decreased (Fig. 5F and G). Additionally, RT-qPCR results revealed that incubation with Res reversed mRNA expression levels of the aforementioned genes in PA-induced HepG2 cells (Fig. 5H). These results indicated that Res significantly changed the expression of metabolism-related genes in vitro.

**miR-33 mimics transfection affects the expression of SIRT6 and lipid metabolism-related genes.** 100 nmol/l miR-33 mimics or mimic controls were transfected into HepG2 cells. The results demonstrated that, after transfection with miR-33 mimic, the expression levels of miR-33 in cells were significantly increased (Fig. 6A). Transfection of miR-33 mimics (but not a negative control miRNA) led to a significant decrease in SIRT6 mRNA (Fig. 6B) and protein expression levels (Fig. 6C and D), and promoted lipid deposition in HepG2 cells (Fig. 6E).

**miR-33 inhibitor transfection affects the expression of SIRT6 and lipid metabolism-related genes.** miR-33 inhibitor or inhibitor controls (100 nmol/l) were transfected into HepG2 cells. The results demonstrated that, after transfection with miR-33 inhibitor, the expression levels of miR-33 in cells were significantly decreased (Fig. 7A). Similarly, miR-33 inhibition significantly increased SIRT6 mRNA expression (Fig. 7B) and protein expression compared with those in the inhibitor control group (Fig. 7C and D). HepG2 cells were induced by PA for 24 h after transfection, and then RT-qPCR was used to analyze the effect of miR-33 inhibitor transfection on lipid metabolism-related genes, and Oil Red O staining.

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Figure 6. Effects of miR-33 mimics transfection on the expression levels of SIRT6 and lipid deposition. HepG2 cells were transfected with NC mimics or miR-33 mimics for 24 h before harvesting. (A) Expression levels of miR-33 after transfection with NC mimics or miR-33 mimics. (B) mRNA levels of SIRT6. (C) Western blot analysis of SIRT6. (D) Protein levels of SIRT6. (E) Lipid deposition in HepG2 cells after miR-33 mimics transfection. β-actin was used as a control for the normalization of samples for western blotting. Scale bar, 100 µm. Data are presented as the mean ± SD (n=3). **P<0.01 vs. NC mimics group (one-way ANOVA with Tukey’s multiple comparison test). CON, liposome; NC mimics, liposome + mimics control sequence; miR-33 mimics, liposome + miR-33 mimics; miR, microRNA; SIRT6, sirtuin 6.
staining was used to analyze the effect of miR-33 inhibitor on lipid deposition. RT-qPCR results revealed that transfection with the miR-33 inhibitor significantly increased the mRNA expression levels of PPARγ, PGC1α and CPT1, decreased the mRNA expression levels of ACC, FASN and SREBP1 (Fig. 7E). Simultaneously, Oil Red O staining revealed decreased lipid deposition in HepG2 cells after miR-33 inhibitor transfection (Fig. 7F).
SIRT6 overexpression affects the expression of lipid metabolism-related genes. To further analyze the effect of SIRT6 overexpression on intracellular lipid metabolism, SIRT6-pcDNA 3.1 was transfected into HepG2 cells. The results demonstrated that, after transfection with SIRT6-pcDNA 3.1, the expression levels of SIRT6 mRNA were
significantly increased (Fig. 8A) and the protein expression levels of SIRT6 were also significantly increased compared with those in the pcDNA 3.1 group (Fig. 8B and C). HepG2 cells were induced by PA for 24 h after transfection, and RT-qPCR and western blotting were used to analyze the effect of SIRT6 overexpression on its downstream lipid metabolism-related genes, and Oil Red O staining was used to analyze the effect of SIRT6 overexpression on lipid deposition. The results revealed that transfection of HepG2 cells with SIRT6 overexpression vector increased PPARγ, PGC1α and CPT1 expression, and decreased ACC, FASN and SREBP1 mRNA (Fig. 8D) and protein expression levels (Fig. 8E and F). Simultaneously, Oil Red O staining revealed decreased lipid deposition in HepG2 cells after SIRT6 overexpression (Fig. 8G).

miR-33 binds to the 3′-UTR of SIRT6 mRNA and inhibits SIRT6 expression. To confirm the direct binding of miR-33 to the 3′-UTR of SIRT6 mRNA, luciferase reporter constructs were generated containing the miR-33 binding site (SIRT6-WT) and its mutant sequence (SIRT6-MT). The results of the luciferase reporter assay demonstrated that the miR-33 mimic significantly decreased the luciferase activity in the SIRT6-WT group (Fig. 9A and B) and had no obvious effect in the SIRT6-MT group (Fig. 9B), indicating that SIRT6 was a direct target of miR-33.

Discussion

miRNAs represent a novel level of regulation that could provide novel therapeutic targets for the treatment of numerous human diseases (11,43–47). Manipulating the expression of miRNAs has good potential for treating lipid metabolism (48). miR-33 is one of the most well-studied miRNAs and regulates hepatic lipoprotein metabolism, fibrosis and regeneration (49). Previous studies have reported that short-term treatment with miR-33 inhibitors markedly increased plasma HDL-C levels (50–52). Numerous studies (53–57) have confirmed that Chinese herbal medicines or their active components can target miRNAs in the treatment of diseases. However, few studies (58–60) have focused on the treatment of lipid metabolism disorders with traditional Chinese medicines or their active components targeting miR-33. To this end, in the current study, the in vivo and in vitro effects of Res were investigated in a HFD mouse model and PA-induced HepG2 cells. The results indicated that Res antagonized abnormal lipid metabolism by targeting miR-33. A further search for downstream genes found that Res inhibited miR-33 expression in the liver and upregulated SIRT6, a key regulator of hepatic lipid metabolism and liver health (61,62). Res also altered the expression levels of genes involved in fatty acid synthesis and fatty acid β-oxidation. Thus, the present study indicated that Res should be further studied for its potential clinical use to prevent or treat hyperlipidemia and associated diseases.

Although multiple animal experiments have confirmed that miR-33 is an important small RNA in regulating lipid metabolism, few studies have been conducted on its expression levels in circulating blood in individuals with hyperlipidemia (20,58,63–67). A total of two studies have confirmed upregulation of miR-33 expression in circulating blood using different methods in participants with hyperlipidemia compared with participants without hyperlipidemia (68,69). Therefore, in the present study, the serum levels of miR-33 were detected in participants with hyperlipidemia. The results showed significant upregulation of miR-33 expression in participants with hyperlipidemia, consistent with previous research findings.

The expression levels of SIRT6, a known target gene of miR-33 (62), were lower in participants with hyperlipidemia...
levels of PGC1α, PPARγ and CPT1 in the livers of mice fed a HFD. The aforementioned genes are involved in fatty acid β-oxidation. For example, CPT1 participates in hepatic lipid metabolism and adipocyte differentiation (85,86). CPT1 activation can reduce the number of adipocytes, facilitate adipocyte differentiation and control lipid peroxidation (87). Upregulation of PPARγ in subcutaneous adipose tissue can combat HFD-induced obesity and promote β-oxidation of fatty acids (85,88). PGC1α, as a key PPARγ coactivator, regulates fatty acid catabolism (89). The present results indicated a direct interaction between miR-33 and the SIRT6-3'-UTR. It was also demonstrated that miR-33 negatively regulated SIRT6 protein expression at the post-translational level in vitro, and SIRT6 overexpression changed the expression levels of genes involved in fatty acid synthesis and fatty acid β-oxidation. The present results suggested that Res improved lipid metabolism by regulating the miR-33/SIRT6 signaling pathway.

In conclusion, the present study revealed a negative association between miR-33 and SIRT6 expression in hyperlipidemia. miR-33 negatively regulated lipid metabolism by targeting SIRT6. Res improved lipid metabolism by regulating the miR-33/SIRT6 signaling pathway. However, Res regulated miR-33 expression remains uncertain and requires further investigation in subsequent studies. It is expected that further research can provide additional insights into potential therapeutics for lipid metabolism disorders, such as miR-33 antagonists to reduce the harm caused by elevated blood lipids.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CL and GS conceived and designed the study. CL, XH, XW and CW acquired and analyzed the data. CL, CW and GS confirmed the authenticity of all the raw data. CL prepared the draft of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was provided by all participants. Patient studies (2018 Scientific Research Ethics Review; approval no. 39) and animal experiments (2022 Scientific Research Ethics Review; approval no. 217) were approved by the Hebei General Hospital Ethics Committee (Shijiazhuang, China). Animal experiments complied with the Animal (Scientific Procedures) Act 1986 and associated guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

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