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Canagliflozin Ameliorates Nonalcoholic Fatty Liver Disease by Regulating Lipid Metabolism and Inhibiting Inflammation through Induction of Autophagy

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Purpose: Nonalcoholic fatty liver disease (NAFLD) is closely associated with metabolic diseases, including obesity and diabetes, and has gradually become the most common cause of chronic liver disease. We investigated the effects of sodium glucose cotransporter 2 (SGLT2) inhibitor canagliflozin on NAFLD in high-fat diet (HFD)-induced obese mice and possible underlying mechanisms.

Materials and Methods: Male C57BL/6 mice were fed a normal-diet, HFD, or HFD with canagliflozin for 14 weeks. AML-12 hepatocytes were treated with canagliflozin. Expression of related pathways was assessed.

Results: Canagliflozin administration reduced body weight and fat mass, compared with HFD alone. Canagliflozin improved glucose and lipid metabolic disorders. Compared with HFD-fed mice, liver weight, serum alanine transaminase (ALT) levels, and hepatic lipid accumulation were decreased after canagliflozin administration. Additionally, canagliflozin upregulated lipolysis markers (CPT1a, ACOX1, and ACADM), downregulated lipogenesis markers (SREBP-1c and FASN), and suppressed the production of inflammatory cytokines (TNFα, MCP1, IL-1β, and IL-6), consistent with significantly increased LC3 II/I and Atg7 levels in the liver following canagliflozin treatment. In vitro, canagliflozin increased CPT1a, ACOX1, and ACADM expression, decreased SREBP-1c and FASN protein expression, and reduced TNFα, MCP1, IL-1β, and IL-6 mRNA levels in lipid mixture (LM)-induced hepatocytes in a dose-dependent manner. These changes were reversed by 3-MA, an autophagy inhibitor.

Conclusion: Our findings suggest that canagliflozin ameliorates the pathogenesis of NAFLD by regulating lipid metabolism and inhibiting inflammation, which may be associated with its promotion of autophagy.

Key Words: Canagliflozin, nonalcoholic fatty liver disease, high-fat diet, lipid metabolism, inflammation, autophagy

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INTRODUCTION

In parallel with a global increase in the incidence of obesity and type 2 diabetes mellitus (T2DM), the prevalence of nonalcoholic fatty liver disease (NAFLD) is rapidly increasing and gradually becoming the most common form of chronic liver disease. According to a recent study, the global incidence of NAFLD is estimated to be 25.24%.[1] This increasing prevalence of NAFLD will be accompanied by an increasing number of patients with liver fibrosis, cirrhosis and hepatocellular carcinoma.[2,3] Although the global health and economic burden of NAFLD is in-

creasing, no FDA-approved medications specifically targeting NAFLD are available.[4,5] Hence, the development of effective drugs for NAFLD is urgently needed.

The pathogenesis of NAFLD is multifactorial, and its mechanisms are not completely understood. Insulin resistance is one of the important mechanisms in NAFLD pathogenesis, similar to T2DM.[6] A meta-analysis showed that patients with NAFLD face almost twice the risk of T2DM than those without NAFLD,[7] and NAFLD occurs in up to 70%–75% of patients with T2DM.[8] Because a link between NAFLD and T2DM is very clear and the two diseases share multiple pathophysiological processes and risk factors, many antidiabetic drugs, such as pioglitazone or liraglutide, may be effective at improving NAFLD.

Sodium glucose cotransporter 2 (SGLT2) inhibitors are a new class of oral antidiabetic agents that exert their hypoglycemic effects by increasing urinary glucose excretion.[9] In addition to their glucose lowering effects, accumulating evidence has shown that they exert many other beneficial metabolic effects.[10,11] An animal study showed that canagliflozin decreases the hepatic lipid content and protects against NAFLD in diabetic high-fat diet (HFD)-fed rats through the upregulation of α-2 glycoprotein.[12] Another study showed that canagliflozin reduces liver fat, triglyceride (TG), and glycogen contents and subsequently ameliorates hepatic steatosis.[13] In a human study, canagliflozin improved serum liver enzymes and type IV collagen concentrations and reduced hepatic fat content.[14] However, the mechanism by which canagliflozin ameliorates NAFLD remains unclear.

In this study, we explored the role of canagliflozin in improving NAFLD and metabolic diseases in diet-induced obese mice and evaluated the effects of canagliflozin on lipid metabolism and inflammation in hepatocytes. Moreover, we sought to investigate the potential mechanisms underlying its effects.

MATERIALS AND METHODS

Reagents and antibodies

Primary antibodies against CPT1a (cat. No. 12252), FASN (cat. No. 3180), NF-κB p65 (cat. No. 4764), phosph-NF-κB p65 (cat. No. 3033), LC3 II/I (cat. No. 4108), and Atg7 (cat. No. 8558), and horseradish peroxidase-conjugated anti-rabbit IgG (cat. No. 7074) and anti-mouse IgG (cat. No. 7076) were obtained from Cell Signaling Technology (Boston, MA, USA). Antibodies against SREBP-1c (cat. No. ab191857) and SGLT2 (cat. No. ab37296) were purchased from Abcam (Shanghai, China). Antibodies against ACOX1 (cat. No. sc-517306) and ACADM (cat. No. sc-365109) were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Canagliflozin was provided by Selleckchem (Houston, TX, USA). All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless indicated otherwise.

Mice and canagliflozin treatment

Male C57BL/6 mice were obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd. Diets were provided by Beijing Whitby Technology Development Co. Ltd. All animals were maintained under the following standard conditions: 12 h light/dark cycle, 21°C–25°C temperature, and 50%–60% humidity. The mice had free access to dieted food and water. After two weeks of acclimation, mice were randomly divided into three groups and fed the respective diet for 14 consecutive weeks: a normal-diet+vehicle group (ND, D12450B, Research Diets, Brunswick, NJ, USA), HFD+vehicle group (60% kcal from fat, D12492, Research Diets), or HFD+canagliflozin group (HFD+Cana, 60 mg/kg/d). Each group consisted of six mice. Body weight and food and water intake were measured weekly. The mice were placed in metabolic cages and allowed to adapt for 1 day, and 24-h urine was collected every week. Dual-energy X-ray absorptiometry (DEXA, Ge Lunar Prodigy, Madison, WI, USA) was applied to detect the body composition of mice before they were sacrificed. Blood samples were collected after a 12-h fast. Liver tissues were quickly weighed, frozen in liquid nitrogen, and stored at -80°C. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Shandong First Medical University (IRB Number: S304).

Biochemical measurement

Serum levels of lipids, such as serum total cholesterol (TC), TG, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C), and liver function biomarkers, such as alanine transaminase (ALT) and aspartate transaminase (AST), were determined after the intervention using enzymatic assay kits (Applygen Technologies Inc., Shanghai, China). Insulin levels in plasma were determined using an ELISA kit (ALPCO, Salem, NH, USA).

Glucose tolerance test and insulin tolerance test

For glucose tolerance test (GTT), mice fasted overnight were injected intraperitoneally with a glucose solution (1 g/kg body weight). For insulin tolerance test (ITT), the mice were fasted for 4 h and injected intraperitoneally with insulin (1 U/kg body weight). All mice were fasted with access to water. Blood samples were collected at 0, 30, 60, 90, and 120 min after glucose or insulin injection. Glucose concentrations were determined using a One-Touch Glucometer (Ascensia Breeze, Bayer, Germany).

Hepatic TG measurement

According to the manufacturer's recommended protocols, TG content in the liver tissues was measured using a TG quantitation assay kit (Abcam).

Hematoxylin and eosin staining

Liver tissue was fixed with formalin, embedded in paraffin, and sectioned at a 5 μm thickness. Tissue sections were stained

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with hematoxylin and eosin (H&E) as described previously.[15]

Immunofluorescence staining

Liver sections were fixed with fresh 4% paraformaldehyde for 10 min followed by three washes with phosphate buffer saline (PBS) for 10 min each. Afterwards, liver sections were blocked in PBS containing 3% bovine serum albumin (BSA) for 1 h at room temperature and then incubated with a primary antibody against Atg7 diluted in 3% BSA-PBS at 4°C overnight. Sections were washed twice with PBS and incubated with the secondary antibody for 1 h at room temperature. Then, sections were washed two times with PBS for 10 min each and incubated at ambient temperature in PBS containing Bodipy (1:100 dilution, Sigma Aldrich, St. Louis, MO, USA) for 15 min. PBS was added to the sections with Hoechst (1:100 diluted, Sigma Aldrich) for 2 min, followed by two washes with PBS for 10 min each. Photographs were captured using a Zeiss Axiovert 3.0 microscope. (Carl Zeiss, Gottingen, Germany). The images were processed using Axiovision Rel.4.7 software (Carl Zeiss).

Cell culture and treatment

The AML-12 mouse hepatocyte cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM/F-12 medium (Gibco BRL, Carlsbad, CA, USA) with 10% fetal bovine serum, 0.1 μM dexamethasone, and 1% insulin-transferrin-selenium. AML-12 hepatocytes were pretreated with or without lipid mixture (LM, 2.5%) for 24 h and then incubated with different concentrations of canagliflozin (0, 5, 10, or 50 μM) for another 24 h. 3-MA (5 mM) or rapamycin (100 nM) was used to treat AML-12 hepatocytes to investigate underlying mechanisms.

Adenovirus transfection and immunofluorescent cytochemistry

AML-12 cells were seeded into 24-well plates at 40%–50% confluency and transfected with red fluorescent protein (RFP)-green fluorescent protein (GFP)-LC3 adenovirus at a multiplicity of infection of 50. After culturing for 6 h, the medium was replaced, and the cells were incubated with or without LM and different concentrations of canagliflozin. The treated cells were washed three times with PBS and fixed with 4% paraformaldehyde for 10 min. The transfected cells were observed under a laser confocal microscope. The number of autophagosomes (yellow due to merging of the red and green puncta) and autolysosomes (red puncta) were counted.

Cell viability analysis

Cell viability was evaluated using the CCK8 assay. AML-12 hepatocytes were seeded in a 96-well plate at a density of 2×10^5 cells per well and incubated overnight. Then, the cells were treated with various concentrations of canagliflozin (0, 1, 5, 10, 50, or 100 μM) for 24 h. Subsequently, 10 μL of CCK8 mixed with 100 μL of medium were added to each well. After 2 h of incubation at 37°C, the absorbance of each well was measured at a wavelength of 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

RNA isolation and RT-PCR

After treatment, TRIzol reagent (Takara, Dalian, China) was used to extract the total RNA from the liver or hepatocytes according to the manufacturer's protocol. The PrimeScript RT reagent Kit (Takara) was used to carry out reverse transcription reactions. Quantitative real-time PCR (qPCR) was performed using SYBR Premix Ex Taq (Takara). The PCR products were verified with an ABI 7500 sequencer (Applied Biosystems, Foster City, CA, USA) as described previously.[16] The primer sequences are listed in Table 1.

Western blot analysis

As described previously,[17] total protein was extracted from the liver and hepatocytes using RIPA lysis buffer (Beyotime, Jiangsu, China). Protein concentrations were measured using a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). 15 μg protein from each sample were separated on SDS-PAGE gels and electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad Corp., Hercules, CA, USA). The membranes were blocked with 5% skimmed milk powder for 1 h and then incubated with primary antibodies against CPT1a, ACOX1, ACADM, FASN, SREBP-1c, p65, p-p65, LC3 II/I, and Atg7. Then, HRP-conjugated anti-rabbit IgG or anti-mouse IgG was applied as the secondary antibody. Immunoreactive proteins were revealed using ECL Plus Western blotting detection reagents (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

Statistical analysis

Data were analyzed with GraphPad Prism software, version 6.0 (La Jolla, CA, USA). Data are presented as the mean±standard error of the mean after testing for assumptions of normality distribution and homogeneity of variance. Two-way repeated measures ANOVA followed by Tukey HSD post hoc comparison were used for comparison of body weight, GTT, and ITT. One-way ANOVA followed by Tukey HSD post hoc comparison were performed for all other parameters. Representative

Table 1. Primer Sequences Used for RT-PCR

Gene	Primer sequence
TNFα	F: 5'-AAGCCTGTAGCCCACGTCGTA-3' R: 5'-GGCACCACTAGTTGGTTGTCTTTG-3'
MCP1	F: 5'-TTCCTCCACCACCATGCAG R: 5'-CCAGCCGGCAACTGTGA-3'
IL-1β	F: 5'-TCCAGGATGAGGACATGAGCAC R: 5'-GAACGTCACACACCAGCAGGTTA
IL-6	F: 5'-CCACTTCACAAGTCGGAGGCTTA R: 5'-GCAAGTGCATCATCGTTGTTCATAC

blots from at least three experiments are presented. Two-tailed p values<0.05 were considered statistically significant.

RESULTS

Canagliflozin reduces body weight and mitigates hyperglycemia and dyslipidemia in HFD-induced obese mice

During the 14-week feeding period, a significantly higher body weight was observed in HFD-fed mice than in ND-fed mice. Compared to the HFD-fed mice, daily canagliflozin administration significantly decreased body weight, despite almost equal food intake was observed (Fig. 1A and B). Water intake was significantly increased in the HFD+Cana-fed mice (Fig. 1C). In addition, urine volume and urinary glucose levels were also increased in the HFD+Cana-fed mice (Fig. 1D). Canagliflozin administration significantly reduced both fat mass and fat mass to body weight ratio, compared with the HFD-fed mice (Fig. 1E). Although canagliflozin treatment did not affect lean mass, it increased the lean mass to body weight ratio (Fig. 1F).

Considering the beneficial effect of canagliflozin on body weight and body composition, we further investigated its effect on glucose and lipid metabolism. GTT and ITT were car-

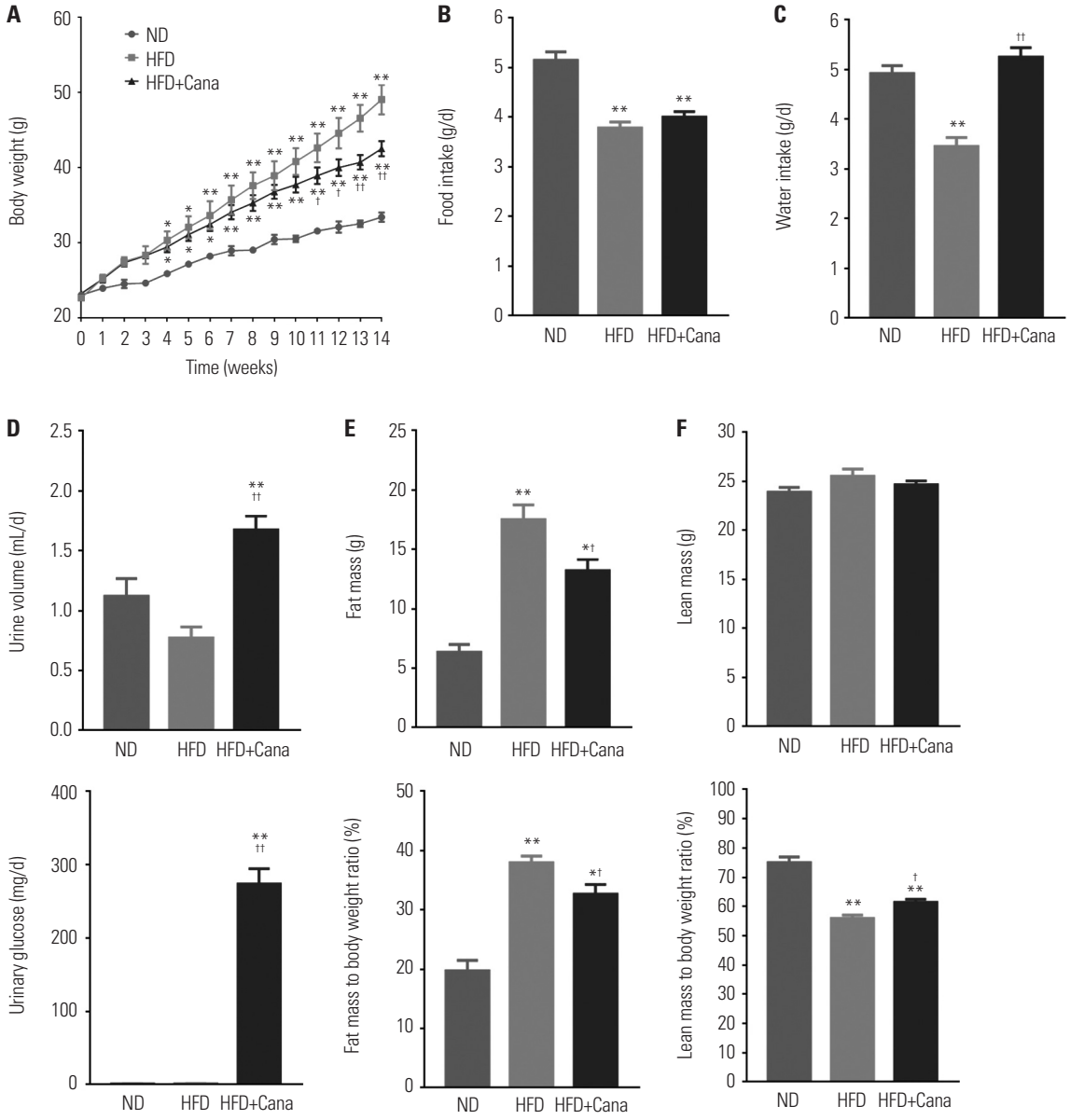

Fig. 1. Canagliflozin reduces body weight and changes the body composition of HFD-induced obese mice. (A) Body weight during the 14-week treatment, (B) food intake, (C) water intake, (D) urine volume and urinary glucose levels, (E) fat mass and fat mass to body weight ratio, and (F) lean mass and lean mass to body weight ratio at week 14 of treatment. The results are presented as means±SEM (n=6). *p<0.05 and **p<0.01 compared with the ND group; †p<0.05 and ††p<0.01 compared with the HFD group. ND, normal-diet; HFD, high-fat-diet.

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ried out after 14 weeks to evaluate the effects of canagliflozin on insulin sensitivity. HFD-fed mice exhibited a significant increase in blood glucose levels in both tests, compared to ND-fed mice, indicating that these mice developed obesity-induced glucose intolerance. Canagliflozin administration significantly decreased blood glucose levels in obese mice (Fig. 2A and B). In addition, plasma insulin levels and homeostasis model assessment of insulin resistance (HOMA-IR) were also significantly reduced after canagliflozin treatment (Fig. 2C and D). Lipid profiles were measured to assess whether canagliflozin also exerted beneficial effects on lipid metabolism. Although canagliflozin did not exert an obvious effect on HDL-C and LDL-C levels, canagliflozin administration significantly reduced serum TG and TC levels (Fig. 2E). Based on these results, canagliflozin was deemed to mitigate hyperglycemia and dyslipidemia in HFD-induced obese mice.

Canagliflozin alleviates liver injury and hepatic lipid accumulation in HFD-induced obese mice

As illustrated in Fig. 3A, a significantly higher liver weight was observed in the HFD-fed mice than in the ND-fed mice. After canagliflozin treatment for 14 weeks, liver weights significantly decreased in the HFD-fed mice. No significant difference in liver to body weight ratio was observed between HFD-fed mice and HFD+Cana-fed mice (Fig. 3A). ALT and AST are two important markers of liver injury. Serum ALT levels were dramatically increased in HFD-fed mice, which were reversed after canagliflozin treatment. Canagliflozin-treated mice also showed

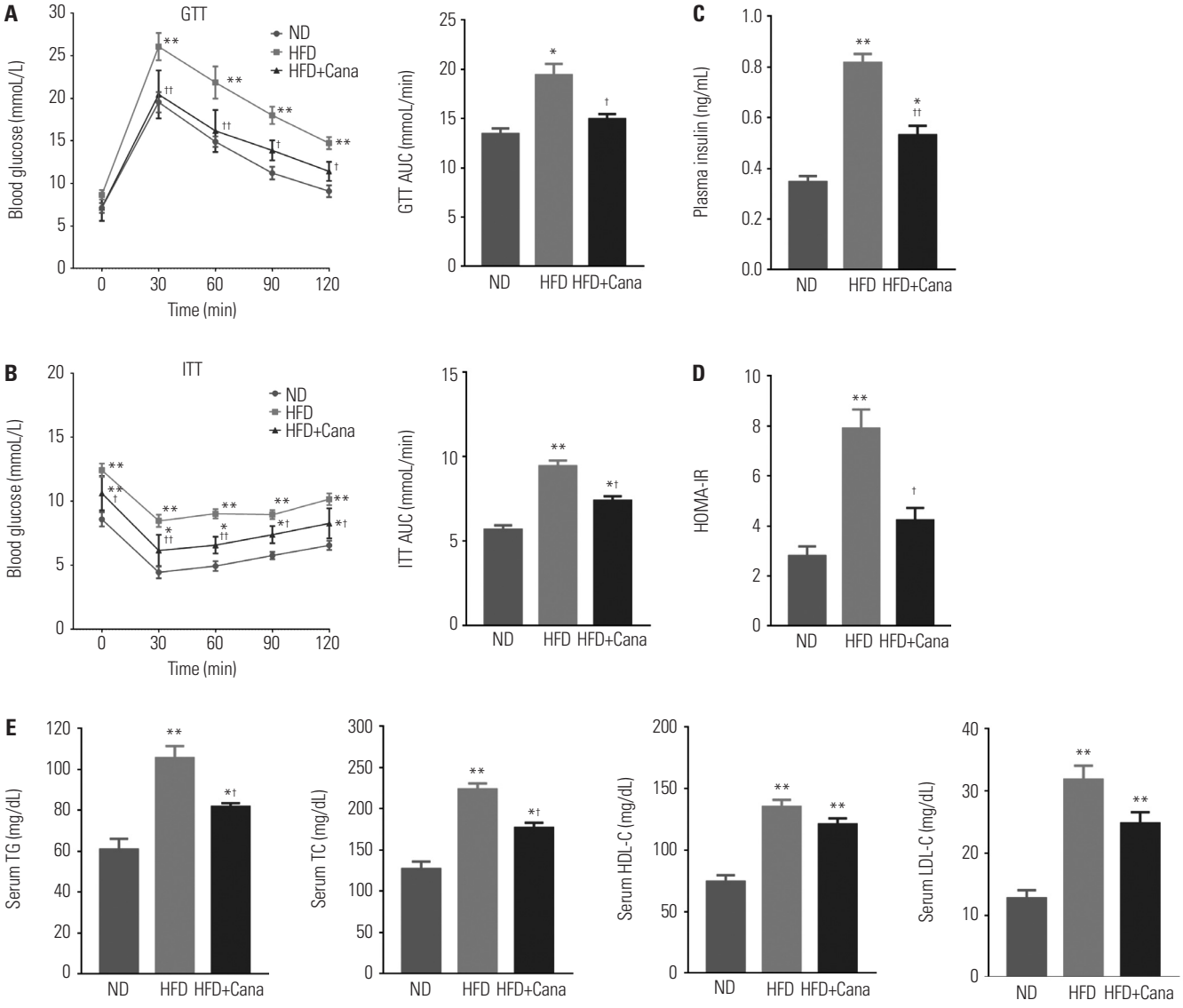

Fig. 2. Canagliflozin mitigates hyperglycemia and dyslipidemia in HFD-induced obese mice. (A) GTT and GTT AUC, (B) ITT and ITT AUC, (C) plasma insulin levels, and (D) HOMA-IR, (E) serum TG, TC, HDL-C, and LDL-C levels at week 14 of treatment. Data are presented as means±SEM (n=6). *p<0.05 and **p<0.01 compared with the ND group; †p<0.05 and ††p<0.01 compared with the HFD group. GTT, glucose tolerance test; ITT, insulin tolerance test; AUC, area under the curve; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; ND, normal-diet; HFD, high-fat-diet; HOMA-IR, homeostasis model assessment of insulin resistance.

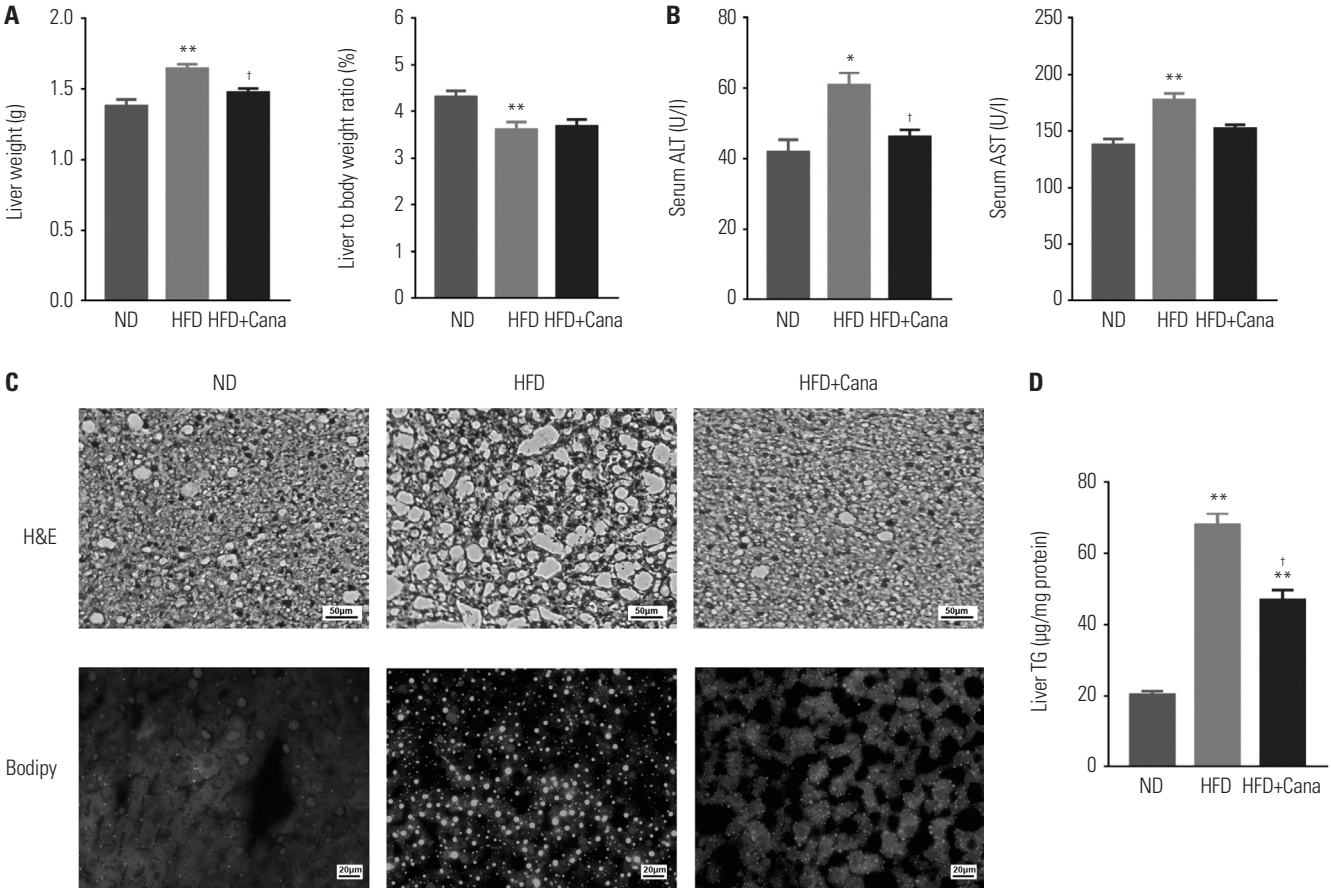

Fig. 3. Canagliflozin alleviates liver injury and hepatic lipid accumulation in HFD-induced obese mice. (A) Liver weight and liver to body weight ratio, (B) serum ALT and AST levels, and (C) H&E and Bodipy fluorescence staining of liver tissue sections. (D) Hepatic TG levels. Data are reported as means±SEM (n=6). *p<0.05 and **p<0.01 compared with the ND group; †p<0.05 compared with the HFD group. ALT, alanine transaminase; AST, aspartate transaminase; TG, triglyceride; ND, normal-diet; HFD, high-fat-diet.

a decreasing trend in serum AST levels (Fig. 3B). Hepatic lipid accumulation is a hallmark of NAFLD. As observed in Fig. 3C. Bodipy fluorescence staining showed that mice in the HFD group presented more prominent lipid droplets than those in the ND group, while canagliflozin significantly decreased lipid droplets in the liver. Furthermore, large areas of macrovesicular steatosis and hepatocellular ballooning were observed in H&E-stained liver tissues from HFD-fed mice, both of which were alleviated by canagliflozin treatment (Fig. 3C). Consistent with histopathological findings, the HFD-fed mice displayed higher TG content in the liver, and canagliflozin administration was effective at attenuating hepatic TG levels (Fig. 3D). These results suggested that canagliflozin alleviates liver injury and hepatic lipid accumulation in HFD-induced obese mice.

Canagliflozin promotes lipolysis and suppresses lipogenesis and inflammation in the liver of HFD-induced obese mice and hepatocytes

We analyzed the expression of proteins involved in lipid metabolism in the liver to investigate whether canagliflozin alleviates hepatic steatosis in HFD-induced obese mice by regulating lipid metabolism. The expression of lipogenic proteins, including SREBP-1c and FASN, was significantly increased in HFD-fed mice, compared with ND-fed mice, which was partially reversed by canagliflozin administration (Fig. 4A). Moreover, the expression of proteins associated with lipolysis, such as CPT1a, ACOX1, and ACADM, was upregulated by canagliflozin (Fig. 4A). Inflammatory processes participate in the development of liver steatosis. We examined the levels of inflammatory cytokines in the liver to determine whether canagliflozin suppresses hepatic inflammatory responses in NAFLD. The mRNA levels of TNFα, MCP1, IL-1β, and IL-6 in the liver were significantly higher in HFD-fed mice than in ND-fed mice. Canagliflozin administration, however, reversed the upregulation of the TNFα, MCP1, IL-1β, and IL-6 mRNA (Fig. 4B).

We established a cell steatosis model by exposing AML-12 hepatocytes to LM to further explore whether canagliflozin regulates lipid metabolism and inflammation in vitro. First, we examined whether canagliflozin altered the proliferation of AML-12 hepatocytes by performing a CCK8 assay. No obvious cytotoxicity to the cells was observed, even at a concentration of 100 μM (Fig. 5A). Compared to the control group, LM sig-

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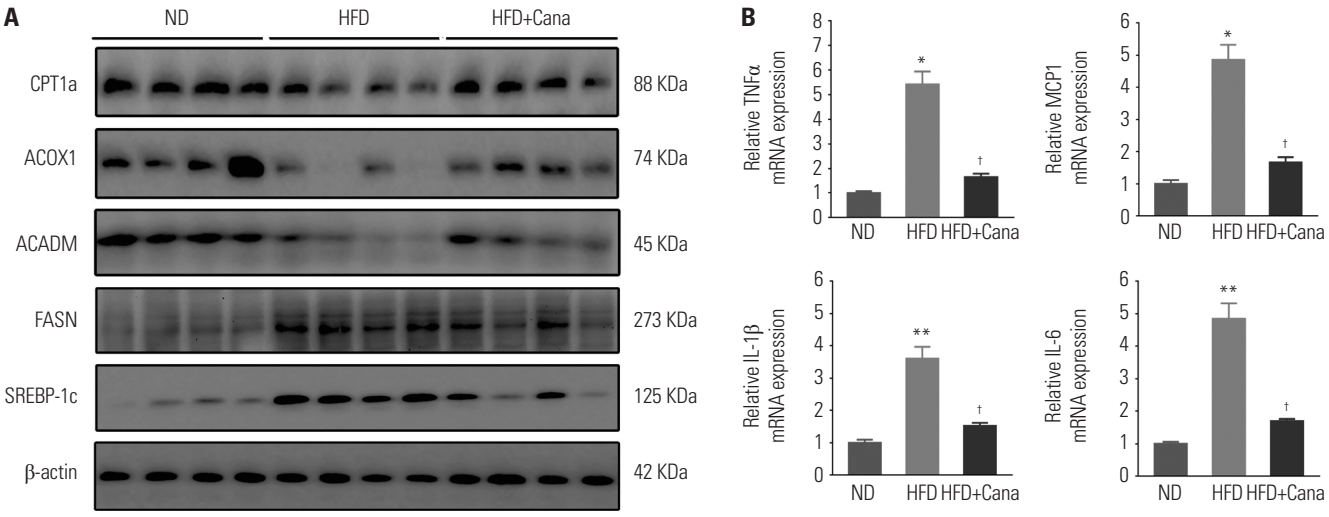

Fig. 4. Canagliflozin promotes lipolysis and suppressed lipogenesis and inflammation in the liver of HFD-induced obese mice. (A) Western blots showing the levels of CPT1a, ACOX1, ACADM, FASN, and SREBP-1c proteins in the liver. (B) RT-PCR results for the expression of the TNFα, MCP1, IL-1β, and IL-6 mRNAs in the liver. The results are described as means±SEM (n=6). *p<0.05 and **p<0.01 compared with the ND group; †p<0.05 compared with the HFD group. ND, normal-diet; HFD, high-fat-diet.

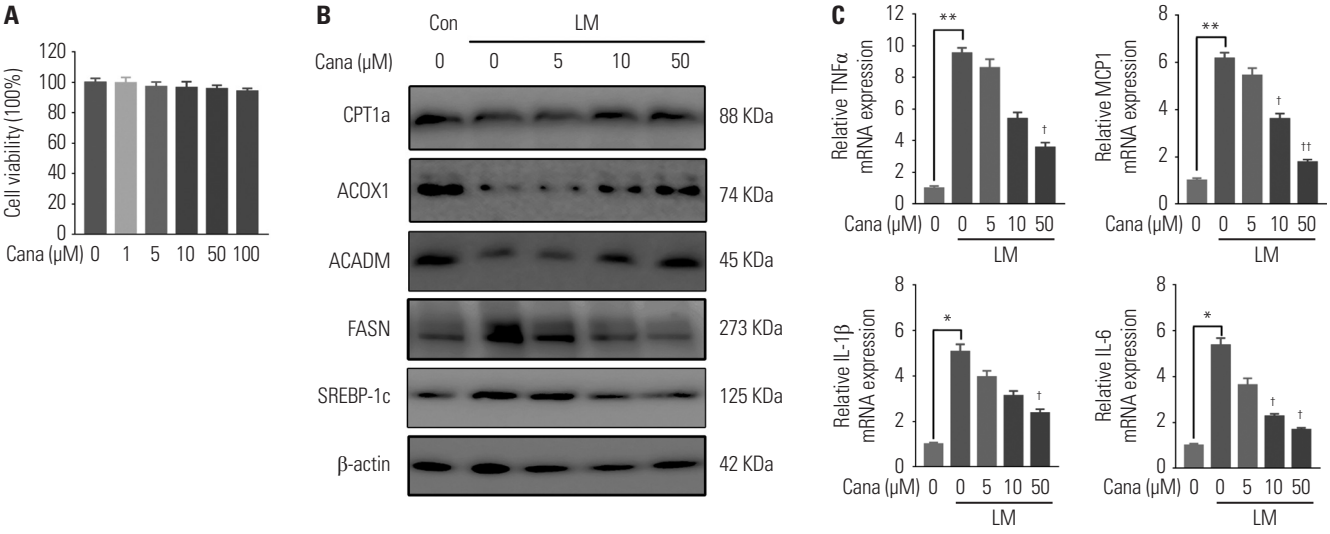

Fig. 5. Canagliflozin promotes lipolysis and suppresses lipogenesis and inflammation in hepatocytes. AML-12 hepatocytes were incubated with LM and different concentrations of canagliflozin for 24 h. (A) Cell viability of AML-12 hepatocytes treated with canagliflozin. (B) The expression of CPT1a, ACOX1, ACADM, FASN, and SREBP-1c proteins in the hepatocytes was measured using Western blots. (C) The expression of TNFα, MCP1, IL-1β, and IL-6 mRNA in the hepatocytes was tested by RT-PCR. Data are presented as means±SEM (n=3). *p<0.05 and **p<0.01 compared with control group; †p<0.05 and ††p<0.01 compared with the LM group. LM, lipid mixture.

nificantly decreased CPT1a, ACOX1, and ACADM protein levels. Interestingly, the reduction in the expression of these protein was reversed by canagliflozin in a dose-dependent manner (Fig. 5B). In addition, canagliflozin induced a dose-dependent decrease in SREBP-1c and FASN protein expression in LM-treated AML-12 hepatocytes (Fig. 5B). We analyzed the levels of inflammatory cytokines in AML-12 hepatocytes to further determine the anti-inflammatory effect of canagliflozin on NAFLD. LM treatment significantly increased TNFα, MCP1, IL-1β, and IL-6 mRNA levels; however, their levels were reduced with high doses of canagliflozin (Fig. 5C). Altogether, these data indicated that canagliflozin promotes lipolysis and suppresses lipogenesis and inflammation in the livers of HFD-induced obese mice and hepatocytes.

Canagliflozin induces autophagy in the liver of HFD-induced obese mice and hepatocytes

To explore the effects of canagliflozin on lipid metabolism and inflammation in hepatocytes, we initially evaluated the expression levels of SGLT2 protein in the liver and AML-12 hepatocytes. SGLT2 protein was detected in the kidneys and HK-2 cells, but not in the liver and AML-12 hepatocytes, which indicated

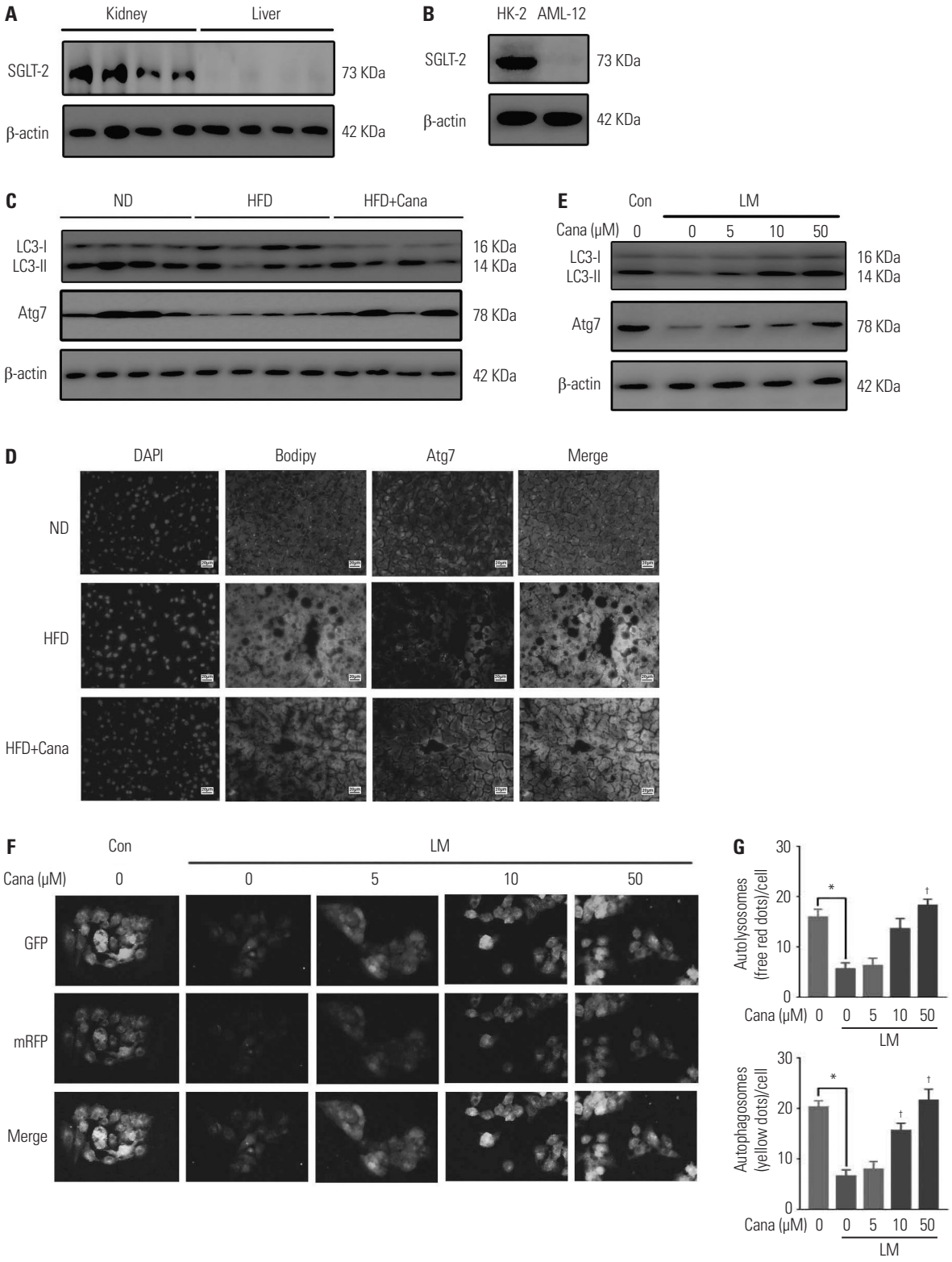

Fig. 6. Canagliflozin induces autophagy in the livers of HFD-induced obese mice and hepatocytes. (A) SGLT2 protein levels in the kidneys and liver were measured using Western blots. (B) SGLT2 protein levels in HK-2 cells and AML-12 cells were measured using Western blots. (C) LC3 II/I and Atg7 protein levels in the liver were measured using Western blots. (D) Immunofluorescence staining for Bodipy and Atg7 in the liver. (E) LC3 II/I and Atg7 protein levels in the hepatocytes were measured using Western blots. (F) Representative confocal microscopic images showing LC3 staining in different groups of AML-12 cells transfected with GFP-mRFP-LC3 adenovirus. (G) Quantification of the number of autophagosomes (yellow dots) and autolysosomes (free red dots) in merged images. *p<0.05 compared with the control group; $^{\dagger}p$<0.05 compared with the LM group. LM, lipid mixture; ND, normal-diet; HFD, high-fat-diet.

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that the effect of canagliflozin on NAFLD is likely SGLT2 independent (Fig. 6A and B). Emerging studies have reported a relationship between the regulation of autophagy and NAFLD. In the present study, low LC3 II/I and Atg7 protein levels were observed in the livers of HFD-fed mice, while treatment with canagliflozin significantly increased the levels of these proteins (Fig. 6C). Next, we evaluated the effect of canagliflozin on autophagy using IF staining for Atg7 and microscopy. Similar to the Western blot results, Atg7 levels were significantly increased after canagliflozin administration (Fig. 6D). Consistent with the downregulation of LC3 II/I and Atg7 expression in the liver of HFD-fed mice, we also observed reductions in LC3 II/I and Atg7 protein levels in LM-treated AML-12 hepatocytes, and the levels of both of these proteins were increased by canagliflozin treatment in a dose-dependent manner (Fig. 6E). Moreover, we tracked autophagy flux in hepatocytes using mRFP-GFP-LC3 adenovirus. The number of autolysosomes decreased significantly in LM-treated AML-12 cells, while canagliflozin significantly increased the number of autolysosomes in LM-treated AML-12 cells. Canagliflozin also increased autophagosome counts in LM-treated AML-12 cells (Fig. 6F and G). Together, these data suggested that canagliflozin induces autophagy in the liver of HFD-induced obese mice and hepatocytes.

Canagliflozin protects against hepatosteatosis by inducing autophagy in hepatocytes

The autophagy inhibitor 3-MA and autophagy activator rapamycin were used to assess canagliflozin-induced effects on LM-treated AML-12 hepatocytes and to determine whether canagliflozin exerts the protective effects described above on hepatosteatosis by inducing autophagy. Rapamycin partially mimicked and 3-MA partially abolished the effects of canagliflozin on increasing CPT1a, ACOX1, and ACADM protein levels and decreasing SREBP-1c and FASN protein expression in LM-treated AML-12 hepatocytes (Fig. 7A).

In addition, both 3-MA and rapamycin were also used to investigate whether autophagy is also involved in the canagliflozin-mediated anti-inflammatory effect on LM-treated

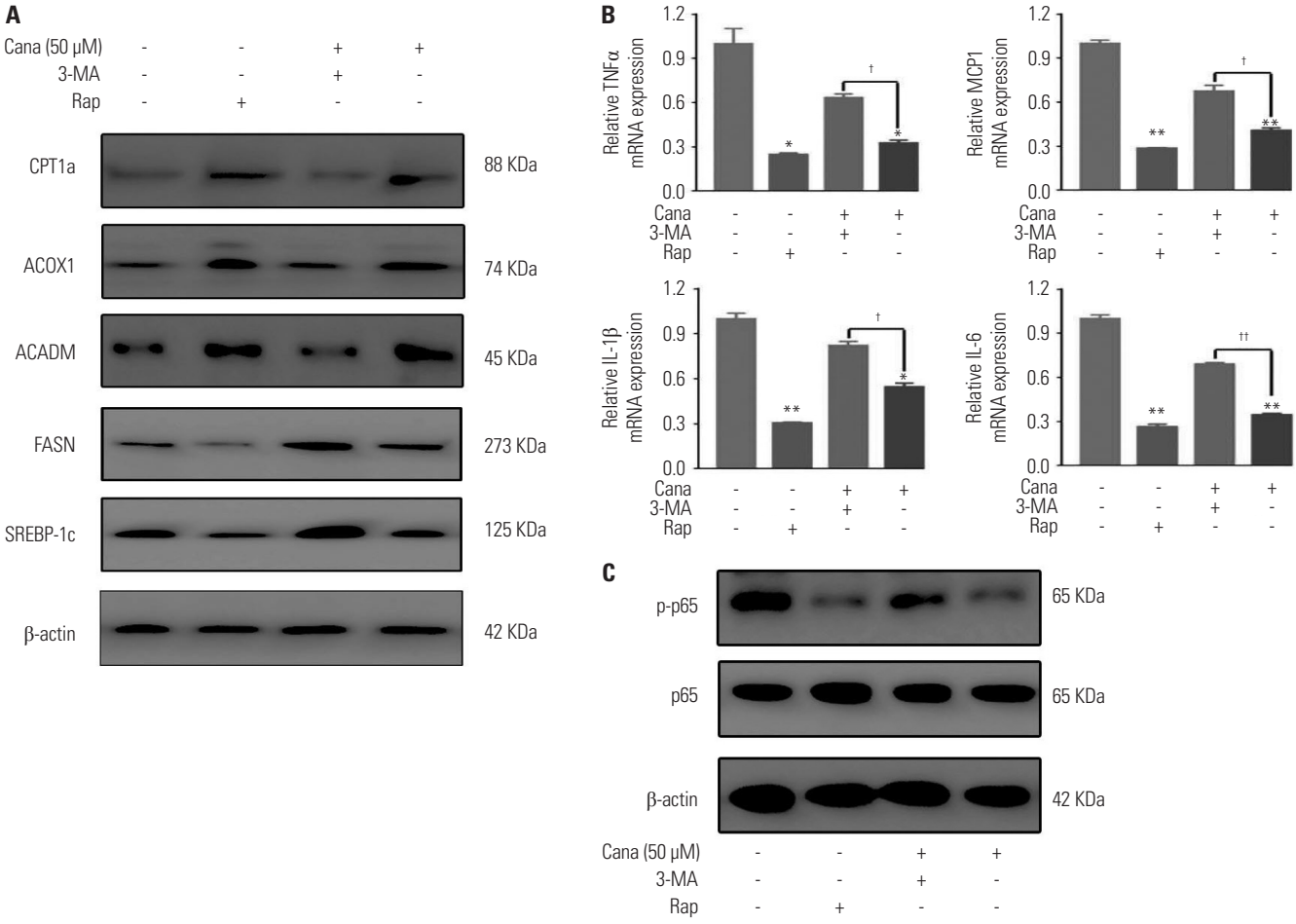

Fig. 7. Canagliflozin protects against hepatosteatosis by inducing autophagy in hepatocytes. AML-12 hepatocytes were treated with canagliflozin (50 μM) alone or in combination with either 3-MA or rapamycin for 24 h. (A) The expression of CPT1a, ACOX1, ACADM, FASN, and SREBP-1c proteins in the hepatocytes was measured using Western blots. (B) The expression of TNFα, MCP1, IL-1β, and IL-6 mRNA in the hepatocytes was detected using RT-PCR. (C) The expression of p-p65 and p65 proteins in the hepatocytes was measured using Western blots. Data are presented as means±SEM (n=3). *p<0.05 and **p<0.01 compared with the control group; $^{\dagger}p$<0.05 and $^{\dagger\dagger}p$<0.01 compared with the Cana+3-MA group.

AML-12 hepatocytes. Rapamycin partially mimicked and 3-MA partially abolished canagliflozin-induced downregulation of TNFα, MCP1, IL-1β, and IL-6 mRNA levels in LM-treated AML-12 hepatocytes (Fig. 7B). NF-κB is one of the most important mediators of inflammatory gene expression, like TNFα, MCP1, IL-1β, and IL-6, and contributes to inflammation-driven organ injury.[18] Based on this, we investigated the possibility that canagliflozin may downregulate the expression of inflammatory genes in hepatocytes via NF-κB signaling. Hepatocytes exposed to canagliflozin showed decreased levels of p-p65, and the addition of 3-MA could abrogate the effect of canagliflozin (Fig. 7C). Thus, we deemed that canagliflozin might protect against hepatosteatosis by inducing autophagy in hepatocytes (Fig. 8).

DISCUSSION

The present study provides novel insights into the mechanism underlying the protective effects of canagliflozin on NAFLD. Canagliflozin administration ameliorated NAFLD by promoting lipolysis and suppressing lipogenesis and inflammation in the livers of mice with HFD-induced obesity and in hepatocytes. These beneficial effects of canagliflozin appeared to be attributable to its ability to induce autophagy. To the best of our knowledge, no empirical research has been conducted on whether canagliflozin alleviates NAFLD through autophagy in the liver and hepatocytes.

NAFLD has gradually become the most common cause of chronic liver disease. This disease is often accompanied by metabolic syndromes, such as obesity, T2DM, and hyperlipidemia, which may seriously affect the physical and mental health of people.[19] As shown in our study, canagliflozin effectively decreased the body weight of HFD-fed mice independent of food intake. Moreover, canagliflozin treatment also decreased fat mass and the fat mass to body weight ratio and increased the lean mass to body weight ratio, consistent with a previous study by Liang, et al.[20] Obesity is closely correlated with insulin resistance and dyslipidemia. As a novel antihyperglycemic drug, our study also showed that canagliflozin improved glucose intolerance and insulin resistance, as evidenced by the results of the GTT and ITT. HFD-fed mice had higher plasma insulin concentrations and HOMA-IR, while canagliflozin significantly decreased the levels of these important insulin resistance markers. Next, we explored the effects of canagliflozin on lipid metabolism. Compared with the ND-fed mice, serum TG and TC levels were increased in the HFD-fed mice, while canagliflozin treatment significantly reduced TG and TC levels. The results were consistent with a previous study documenting the beneficial effects of canagliflozin on hyperlipidemia.[21] However, they contradicted another study that reported canagliflozin administration did not affect serum TG and TC levels.[22] The difference may be due to the use of different animal models, different treatment courses, and different concentrations of canagliflozin in the diet. Based on these results, canagliflozin might exert potential effects on alleviating obesity-induced glucose and lipid metabolism disturbances.

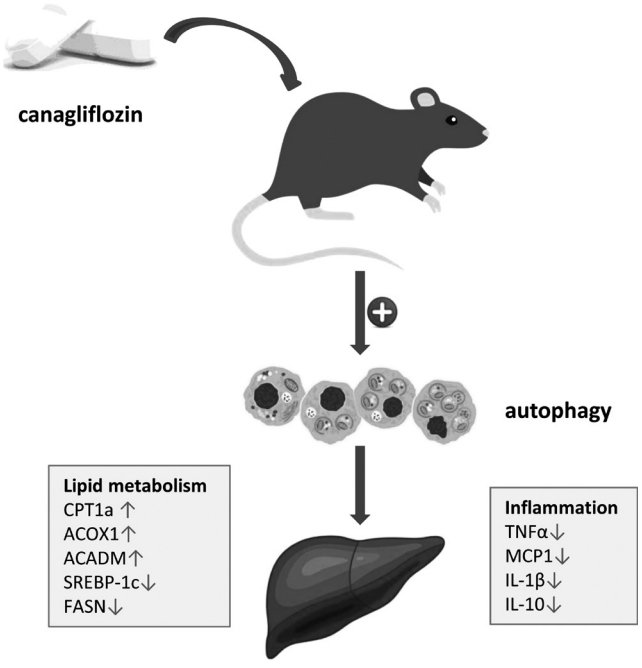

Fig. 8. Schematic diagram of the effect of canagliflozin on nonalcoholic fatty liver disease.

The liver plays an important role in lipid homeostasis. An imbalance in lipid metabolism leading to excessive accumulation of TG in hepatocytes is a hallmark of NAFLD. Therefore, we sought to evaluate whether canagliflozin might reduce the TG contents in the liver to ameliorate NAFLD. Canagliflozin inhibited lipid droplet formation in the liver as determined by Bodipy fluorescence staining in our study. H&E staining also showed that canagliflozin reduced areas of macrovesicular steatosis and hepatocellular ballooning, indicating that canagliflozin alleviates HFD-induced NAFLD. ALT and AST are conventional markers of liver injury in subjects with NAFLD.[23] In our study, canagliflozin administration decreased serum ALT levels. Moreover, serum AST levels showed a decreasing trend after canagliflozin treatment. Furthermore, canagliflozin also reduced TG content in the liver. Therefore, canagliflozin reduced hepatic TG accumulation to inhibit the development of NAFLD and ameliorate liver injury.

The liver is involved in lipolysis and lipogenesis. An abnormality in either process might contribute to excess TG accumulation in the liver. We focused on examining the expression of proteins related to lipolysis and lipogenesis to investigate the potential mechanisms of canagliflozin in NAFLD. CPT1a is a rate-limiting enzyme that transports long-chain fatty acids into mitochondria for β oxidation.[24] ACOX1 is the first and rate-limiting enzyme required for peroxisomal β-oxidation of long-chain fatty acids.[25] ACADM has also been shown to regulate fatty

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acid oxidation.[26] As observed in the present study, canagliflozin upregulated the expression of CPT1a, ACOX1, and ACADM proteins in hepatocytes. In addition to an increase in lipolysis, another alternative for reducing hepatic TG accumulation is to decrease lipogenesis. As an important transcriptional regulator of lipogenesis, SREBP-1c has been reported to activate many enzymes involved in fatty acid formation, including FASN.[27,28] In our study, SREBP-1c and FASN protein levels were increased by LM, while canagliflozin significantly reduced the levels of these proteins. In vivo, HFD decreased levels of the CPT1a, ACOX1, and ACADM proteins and increased SREBP-1c and FASN protein levels, while these changes induced by the HFD were reversed by canagliflozin. Obesity induces hepatic inflammation, which is characterized by increased production of inflammatory cytokines.[29] In our study, canagliflozin decreased the expression of the TNFα, MCP1, IL-1β, and IL-6 mRNA in the liver and hepatocytes. Based on these data, we deemed that canagliflozin might promote lipolysis and suppress lipogenesis and inflammation both in vivo and in vitro and that the beneficial effects of canagliflozin might partially contribute to ameliorating NAFLD. However, its underlying mechanism remained unclear.

We thus sought to elucidate the mechanisms underlying the beneficial effects of canagliflozin on NAFLD. Originally, SGLT2 was reported to be expressed in the renal proximal tubules.[30] In our study, we found the SGLT2 protein was not expressed in the liver and AML-12 hepatocytes, suggesting that the effect of canagliflozin on NAFLD is likely independent of SGLT2. Autophagy, a highly adaptive catabolic process that involves the degradation of excess or harmful organelles and proteins in the lysosomes, has been implicated in the development of metabolic disorders, such as NAFLD.[31,32] A growing body of research has shown that autophagy is closely related to hepatic steatosis, wherein the suppression of autophagy increases TG content in the liver by decreasing TG degradation.[33,34] A previous study showed significantly reduced levels of autophagy in the liver of individuals with NAFLD.[35] As lipid droplets are also substrates for autophagy, abnormal hepatic autophagy due to HFD-induced lipotoxicity is involved in the pathogenesis of NAFLD. Thus, the upregulation of autophagy might protect against hepatic steatosis. According to a study by Hsiao, et al.,[36] pharmacological promotion of autophagy by pioglitazone attenuates NAFLD by enhancing cytosolic lipolysis and β-oxidation in the liver. Sun, et al.[37] found that berberine inhibits lipid storage and promotes lipid utilization in the liver by inducing autophagy, thus protecting against hepatic steatosis. Therefore, we investigated whether canagliflozin altered autophagy in the liver. LC3 II/I has been used as a reliable marker of autophagy, and its levels are closely associated with the number of autophagosomes.[38] Atg7 has also been established as a good marker of autophagy.[39] The levels of both LC3 II/I and Atg7 in the liver were decreased in HFD-fed mice in our study, whereas the administration of canagliflozin increased the protein levels of these autophagy markers. Our in vitro data also revealed that canagliflozin upregulated LC3 II/I and Atg7 levels in hepatocytes, compared with LM-treated hepatocytes. Canagliflozin also increased autophagy flux in hepatocytes. As further validation of our results, pretreatment with an inhibitor of autophagy, 3-MA, prevented canagliflozin-induced upregulation of CPT1a, ACOX1, and ACADM and downregulation of SREBP-1c and FASN protein levels. Similarly, canagliflozin-induced reductions in hepatic production of inflammatory cytokines were also reversed by 3-MA. Finally, NF-κB signaling plays a key role in the inflammatory response during the development of NAFLD.[40] Our study found that canagliflozin might downregulate the NF-κB signaling in hepatocytes, leading to reduced levels of inflammatory genes, including TNFα, MCP1, IL-1β, and IL-6. These results provided strong evidence that canagliflozin regulates lipid metabolism and inhibits inflammation at least in part by modulating autophagy.

The present study has some limitations. First, an autophagy inhibitor was used to treat hepatocytes, while we did not use autophagy knockout mice or feed the mice autophagy inhibitor to further investigate underlying mechanisms in vivo. Such knockout study is needed to further demonstrate whether the effects of canagliflozin on improving NAFLD may be associated with its promotion of autophagy. Second, we only used one dose of canagliflozin, and this dose of canagliflozin in mice is higher than that used in humans. Third, although canagliflozin ameliorated HFD-induced NAFLD in mice, the pathophysiology of NAFLD in humans is slightly different from that in mice. Hence, clinical studies in humans are needed to confirm the protective effects of canagliflozin on NAFLD.

In conclusion, the present study indicated for the first time that canagliflozin improves NAFLD by regulating lipid metabolism and inhibiting inflammation, which may be associated with its induction of autophagy. These results suggest that canagliflozin may worth exploring as be a therapeutic option for NAFLD.

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AUTHOR CONTRIBUTIONS

Conceptualization: Dan Wei and Zhipeng Xu. Data curation: Wenxin Hu, Ting Xu, Dan Wei, Zhipeng Xu, and Jianning Wang. Formal analysis: Dan Wei, Zhipeng Xu, and Jianning Wang. Funding acquisition: Dan Wei and Zhipeng Xu. Investigation: Wenxin Hu, Zhipeng Xu, Bin Wang, and Dan Wei. Methodology: Wenxin Hu, Ting Xu, Jianning Wang, and Dan Wei. Project administration: all authors. Resources: Dan Wei and Zhipeng Xu. Software: Bin Wang. Supervision: Dan Wei.

Validation: Dan Wei and Zhipeng Xu. Visualization: Bin Wang. Writing—original draft: Wenxin Hu and Zhipeng Xu. Writing—review & editing: Wenxin Hu, Jianning Wang, and Dan Wei. Approval of final manuscript: all authors.

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