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Folic acid promotes autophagy to relieve metabolism-associated fatty liver disease by regulating NRF2

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ABSTRACT

Metabolism-associated fatty liver disease (MAFLD) is a liver disease characterized by hepatic steatosis and excessive accumulation of lipids, with a high global incidence, especially in populations with obesity, diabetes and metabolic syndrome (MetS). As an important B vitamin, folate (FA) is stored mainly in the liver where it regulates oxidative stress, chronic inflammation and lipid metabolism. However, its regulatory role and mechanism of action in MAFLD are still poorly understood. Therefore, this study was conducted to investigate the regulatory effect of FA on MAFLD. The MAFLD rat model was induced by a high-fat diet (HFD), and HepG2 cells were treated with 0.3 mM palmitic acid (PA) for 24 h to establish a cell model. The expression of relevant genes and proteins was detected by RT-qPCR and Western blotting. Injury to HepG2 cells and rat liver tissues was evaluated *via* hematoxylin and eosin staining, Oil red O staining, ELISA and CCK-8 assay. FA treatment inhibited body weight gain in rats and reduced the levels of liver injury indicators (aspartate and alanine aminotransferase, and Alkaline phosphatase), blood lipids (total cholesterol, triglycerides and free fatty acids) and inflammatory cytokines (TNF-α, IL-6, and IL-1β), reducing lipid accumulation and pathological damage in the liver and ultimately alleviating the progression of MAFLD. Moreover, FA treatment promoted the expression of the autophagy-related protein LC3 II/I, inhibited the expression of p62, and increased the formation of autophagosomes, thereby alleviating PA-induced damage to HepG2 cells. Furthermore, NRF2 expression is downregulated in MAFLD and can be upregulated by FA treatment. Further examination revealed that knocking down NRF2 could partially attenuate the inhibitory effect of FA on PA-induced HepG2 cell injury. In conclusion, FA activates autophagy by promoting the expression of NRF2, thereby alleviating the development of MAFLD.

Key words: metabolism-associated fatty liver disease; folic acid; autophagy; NRF2, lipid accumulation.

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Introduction

Metabolism-associated fatty liver disease (MAFLD), a chronic progressive disease that is characterized by steatosis and excessive accumulation of lipids in liver cells, affects approximately 30% of the global population.[1] As a multifactorial disease, MAFLD is closely associated with insulin resistance, oxidative stress, lipid metabolism, lifestyle and environmental factors.[2] In the process of lipid metabolism, autophagy simultaneously regulates the biosynthesis and degradation of LDs; this process is referred to as "lipophagy".[3] Evidence suggests that autophagy dysfunction or dysregulation is associated with MAFLD.[4] Improving the level of autophagy in the liver can reduce lipid deposition, thereby reducing the degree of steatosis.[5,6] Therefore, increasing autophagy activity through pharmaceutical means may be an effective strategy for improving MAFLD.

As an important B vitamin, folic acid (FA) is involved in one-carbon (1C) metabolic reactions, and these reactions play key roles in various physiological processes of the body, including nucleic acid and protein synthesis, amino acid homeostasis, redox defense, methylation modification and immune response.[7,8] FA deficiency is closely associated with various systemic diseases, such as cancer, cardiovascular diseases, and mental diseases.[9-11] In addition, as the main metabolic and storage site of FA, liver FA also affects oxidative stress, chronic inflammation and lipid metabolism in the liver, which are all pathogenic mechanisms of fatty liver.[12,13] Previous studies have shown that serum FA levels are correlated with the lipid profile, underscoring the importance of FA in liver lipid metabolism.[14] Notably, FA can also regulate the development of autophagy in liver cells.[15] Therefore, in this study, we investigated whether FA affects the development of MAFLD by regulating autophagy.

Nuclear factor erythrocyte 2-related factor 2 (NRF2) is a master regulator that maintains the cellular redox balance, and its expression is closely related to lipid homeostasis.[16] Therefore, NRF2 functions in regulating the progression of MAFLD. For example, honokiol can inhibit the development of MAFLD by activating the NRF2 signaling pathway.[17] In addition, NRF2 activation can promote mitochondrial autophagy by promoting the expression of key genes associated with autophagy and mitochondrial autophagy.[18] Genes such as p62/SQSTM1 and LC3 play central roles in the process of autophagy, and these genes are directly affected by NRF2.[18] However, no studies have reported that FA affects autophagy in MAFLD through the regulation of NRF2.

In this study, we conducted experiments at the cellular and animal levels to explore the role and mechanism of FA in the progression of MAFLD, providing new ideas and methods for the treatment of this disease.

Materials and Methods

Construction of the MAFLD rat model

Twenty 6-week-old specific pathogen-free (SPF) male SD rats (body weight: 180-200 g) were purchased from Beijing HFK Biotechnology Co., Ltd. (Beijing, China). After adaptive feeding for 1 week, the rats were randomly divided into a control group, a high-fat diet (HFD) group, an HFD+FA (15 mg/kg) group, and an HFD+FA (30 mg/kg) group, with 5 rats in each group. In accordance with previous methods,[19] the control group was fed standard feed, and the remaining three groups were fed high-fat feed (containing 88% standard feed, 10% lard and 2% cholesterol) for 8 weeks. Starting from the 9th week, the rats in the HFD+FA (15 mg/kg) group and the HFD+FA (30 mg/kg) group were orally gavaged with FA (Y0001979; Sigma-Aldrich, St. Louis, MO, USA) at dosages of 15 mg/kg/d and 30 mg/kg/d, respectively, and the rats in the control group and the HFD group were gavaged with normal saline at the same dosage. After the last drug treatment, all the rats were fasted for 12 h. The rats were weighed, and blood was collected. After the rats were anesthetized with 3% pentobarbital sodium (40 mg/kg; intraperitoneal injection; Sigma-Aldrich), they were euthanized by cervical dislocation. Afterward, the liver was isolated to detect changes in relevant indicators.

Cell culture and transfection

HepG2 human liver cancer cells were obtained from Wuhan Pricella Biotechnolog Co., Ltd. (Wuhan, China), and were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 100 U/mL streptavidin/penicillin (Sigma-Aldrich). The culture medium (Gibco) was routinely cultured in an incubator at 37 °C with 5% CO_2. After the cells were cultured, they were treated with 0.3 mM palmitic acid (PA; P0500; Sigma-Aldrich) for 24 h to induce the cell model.[20] The cells were treated with different concentrations of FA (0, 5, 10, 20, 40, or 80 μg/mL) for 24 h to determine the optimal therapeutic concentration of FA. To explore the role of autophagy in the effects of injury on HepG2 cells, HepG2 cells were treated with 50 μM chloroquine (CQ; HY-17589A; MedChemExpress, Monmouth Junction, NJ, USA) for 24 h. For cell transfection, HepG2 cells were seeded in 6-well plates. When the cell density reached 60%, lentiviruses in which NRF2 was knocked down (sh-NRF2#1, sh-NRF2#2) or the control (sh-NC) were added. The cells were infected with the vector (GenePharma, Shanghai, China) at 1×10^8 TU/mL (MOI = 100) for 14 h. Then, the medium was changed to complete medium. After 72 h of transfection, the cells were treated with 4 μg/mL puromycin (MedChemExpress) to obtain stably transfected cell lines, and the transduction effect was verified *via* Western blotting.

RT-qPCR

Total RNA from rat liver tissue and HepG2 cells was extracted *via* TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA), and the RNA was reverse transcribed to synthesize first-strand cDNA *via* a HiScript II First-Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). After that, fluorescence quantitative PCR was performed *via* 2 × SYBR Green PCR Master Mix (Solarbio, Beijing, China), and the experimental results were calculated *via* the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers used in this study are listed in Table 1.

Western blot analysis

Total protein was isolated from rat liver tissue and HepG2 cells using RIPA lysis buffer (Beyotime, Shanghai, China) containing a protease phosphatase inhibitor (Beyotime), and its concentration was determined *via* a Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of proteins were separated by SDS-PAGE and then transferred to PVDF membranes. After the membrane was blocked with 5% skim milk in PBS for 1 h at room temperature, it was incubated with primary antibodies against NRF2 (1:1000; PA5-27882; Thermo Fisher Scientific), LC3B (1:1000; ab192890; Abcam, Cambridge, UK), p62 (1:1000; ab109012; Abcam), and β-actin (1:1000; ab8227; Abcam) at 4°C overnight and then incubated with secondary antibodies (1:3000; ab6721; Abcam) for 2 h at room temperature. Detection was performed *via* a superenhanced chemiluminescence (ECL) detection kit (Thermo Fisher Scientific), and the results were analyzed *via* ImageJ software.

Immunofluorescence

HepG2 cells were fixed in 4% paraformaldehyde solution for 1 h, permeabilized in 0.5% Triton X-100 (Thermo Fisher Scientific) for 20 min, and then washed with PBS. The cells were blocked with bovine serum albumin (BSA; Thermo Fisher Scientific) for 1 h. Then, the samples were incubated with primary antibodies against NRF2 (1:200; PA5-27882; Thermo Fisher Scientific), LC3B (1:300; ab192890; Abcam), and p62 (1:200; ab109012; Abcam). The cells were incubated at 4°C overnight. After the cells were washed with PBS, they were incubated with secondary antibody (1:200; ab150081; Abcam) at room temperature for 2 h. The cells were then stained with DAPI (Beyotime) and washed with PBS after being incubated for 15 min. As a negative control, the primary antibody was replaced with PBS, and the fluorescently labeled secondary antibody was added directly to the samples, resulting in a negative reaction. Finally, the cells were examined *via* fluorescence microscopy (Eclipse 80i; Nikon, Tokyo, Japan) with a 100× objective. Three microscopic fields of view were taken for each sample. The positive areas (fluorescence intensity) were calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Monodansylcadaverine staining

Monodansylcadaverine (MDC) is a fluorescent dye that is usually used to detect specific markers of autophagosome formation. HepG2 cells were cultured in 24-well plates and treated according to the corresponding experimental groups. After treatment, 250 µL of MDC staining solution (Beyotime) was added to each well, and the samples were stained at room temperature for 30 min in the dark. The staining results were observed and photographed with a fluorescence microscope (Eclipse 80i; Nikon) with a 100× objective. Three microscopic fields of view were taken for each sample. The positive areas (fluorescence intensity) were calculated using ImageJ software.

CCK-8

A cell counting kit 8 (Beyotim) was used to measure cell viability. Briefly, HepG2 cells were seeded in 96-well plates at a density of 2,000 cells/well and incubated at 37°C overnight. After different treatments were performed, CCK-8 reagent (10 µL) was added to the 96-well plates. After incubation for 1 h, the absorbance of the HepG2 cells at 450 nm was measured *via* a microplate reader (Thermo Fisher Scientific).

Oil red O staining

Cells: HepG2 cells were inoculated into 12-well plates coated with cell slices. After the cells attached, they were grouped and administered the corresponding experimental treatments. The samples were subsequently washed with PBS, fixed in 4% paraformaldehyde for 10 min, washed with double-distilled water, and ready to use.

Tissues: fixed liver tissue was embedded in optimal cutting temperature (OCT) compound, and the thickness was set to 10 µm for cryosections.

The cell and tissue samples were quickly infiltrated with isopropanol, stained with Oil Red O staining solution (Beyotime), stained in the dark for 15 min, washed with double-distilled water, mounted with glycerol, observed and photographed under a microscope (Eclipse 80i; Nikon) with 20× or 80× objectives. After staining, the lipid droplets were stained orange–red to bright red. Three microscopic fields of view were taken for each sample.

Detection of lipid and liver function injury indicators

Triglyceride (TG, A110-1-1), total cholesterol (TC, A111-1-1), free fatty acid (FFA, A042-2-1), aspartate aminotransferase (AST, C010-2-1), alanine aminotransferase (ALT, C009-2-1) and alkaline phosphatase (ALP, A059-2) kits were purchased from Nanjing Jiancheng Bioengineering Institute. HepG2 cells were collected *via* centrifugation according to the instruction manual. The cells were disrupted, and the cell homogenate was collected for testing. Rat blood samples were collected, and serum samples were separated for testing. Each working solution was subsequently added according to the instruction manual to determine the contents of total cholesterol (TC), triglycerides (TG) and free fatty acids (FFA)in the cells and serum and the serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels.

Table 1. Primer sequences.

Target	Sequence (F: Forward primer; R: Reversed primer) (5′-3′)
SREBP1c (Hum)	F: TCTCTTAGAGCGAGCACTG R: TCCGAGAATTCCTTGTCCC
SREBP1c (Rat)	F: CTACCGTTCCTCTATCAATGAC R: GCAGATTTATTCAGCTTTGCC
SCD (Hum)	F: TCTAGCTCCTATACCACCACCA R: TCGTCTCCAACTTATCTCCTCC
SCD (Rat)	F: GAGTACCGCTGGCACATCAA R: GGAACTCAGAAGCCCAGAACT
PPARγ (Hum)	F: TACTGTCGGTTTCAGAAATGCC R: GTCAGCGGACTCTGGATTCAG
PPARγ (Rat)	F: GAGCACTTCACAAGAAATTACC R: CAAAGGAATGGGAGTGGTC
FABP1 (Hum)	F: GTGTCGGAAATCGTGCAGAAT R: GACTTTCTCCCCTGTCATTGTC
FABP1 (Rat)	F: GTGATCCACAATGAGTTCACC R: CTTAACCACTGCCTTGACC
β-actin (Hum)	F: CATGTACGTTGCTATCCAGGC R: CTCCTTAATGTCACGCACGAT
β-actin (Rat)	F: GGTCAGGTCATCACTATCGG R: GGATTCCATACCCAGGAAGG

Detection of inflammatory factors by ELISA

IL-6 (SEKR-0005 or SEKH-0013; Solarbio), IL-1β (SEKR-0002 or SEKH-0002; Solarbio) and TNF-α (SEKR-0009 or SEKH-0047; Solarbio) levels were determined in rat serum and HepG2 cell medium.

Hematoxylin and eosin staining

Rat liver tissue was collected, fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 5 μm thick sections. The tissue sections were then dewaxed in xylene for 5 min, rehydrated in gradient alcohol, and soaked in ddH_2O for 2 min. The samples were then stained with hematoxylin (Solarbio) for 5 min, washed twice with tap water, and stained with eosin for 2 min. After dehydration, the samples were cleared and sealed with neutral resin (Sigma-Aldrich), and the stained liver tissue was observed under a microscope (Eclipse 80i; Nikon) with a 40× objective.

Immunohistochemistry

Rat liver tissue was embedded, sectioned, and dewaxed as described above. The sections were placed in citrate buffer (pH 6.0) and boiled for 2 min, after which the activity of endogenous peroxidase was blocked for 10 min with 3% H_2O_2. After being washed with PBS, the sections were incubated with a primary antibody against NRF2 (1:200, PA5-27882; Thermo Fisher Scientific) at 4°C overnight and then incubated with a secondary antibody (1:200, ab150081; Abcam) at room temperature for 1 h. After the sections were washed, they were developed with a diaminobenzidine (DAB) kit (Solarbio) and observed and photographed *via* a light microscope (CKX53; Olympus, Tokyo, Japan) with an 80× objective. Three microscopic fields of view were taken for each sample. The immunolabeling results were evaluated by measuring the area of the positive signals using ImageJ software.

Statistical analysis

All the experimental data in this study are expressed as the means ± standard deviations (means ±SD). The data were analyzed and graphed *via* GraphPad Prism 8 (La Jolla, CA, USA). Comparisons between two groups were performed *via* a t test, and comparisons between multiple groups were performed *via* one-way analysis of variance (ANOVA) followed by Fisher's LSD test. A p-value <0.05 indicated that the difference was statistically significant.

Results

Folic acid inhibits HFD-induced liver injury in MAFLD rats

We first detected the effect of FA on liver injury in MAFLD rats. The results of the body weight measurements revealed that HFD consumption led to a significant increase in the body weight of the rats, and the body weight of the rats was significantly reduced after FA treatment (Figure 1A). Compared with those in the control group, the serum TC, TG and FFA levels in the HFD group were significantly greater, and FA treatment partially reversed the effects of the HFD, reducing serum TC, TG and FFA levels (Figure 1 B-D). Moreover, the serum levels of AST, ALT, and ALP in the rats in the HFD group significantly increased, and these levels significantly decreased after FA treatment (Figure 1 E-G). Pathology of the livers of the HFD-fed rats revealed that, compared with those in the control group, the volume of hepatocytes was greater, severe steatosis and inflammatory infiltration were more common, and lipid accumulation was significantly greater in the HFD-fed rats. FA treatment somewhat inhibited the increase in hepatocyte volume, improved steatosis and inflammatory infiltration, and reduced lipid accumulation (Figure 1 H-I). RT-qPCR detection of the expression of lipid metabolism-related genes revealed that, compared with those in the control group, the mRNA levels of SREBP1c and SCD in the livers of the HFD group were significantly greater, and the levels of PPARγ and FABP1 were significantly lower. FA treatment inhibited the expression of SREBP1c and SCD mRNAs and promoted the expression of PPARγ and FABP1 mRNAs (Figure 1 J-M). Finally, the levels of inflammatory factors in the rat serum were detected. The levels of TNF-α, IL-6, and IL-1β in the serum of HFD-fed rats significantly increased, and FA treatment decreased the levels of TNF-α, IL-6, and IL-1β in the serum (Figure 1 N-P). These results suggest that FA treatment can improve HFD-induced liver injury in MAFLD rats and that high-dose FA treatment has a better effect.

Folic acid regulates PA-induced autophagy in HepG2 cells

As mentioned above, autophagy dysfunction or dysregulation is related to MAFLD.[4] Therefore, we detected PA-induced changes in autophagy in HepG2 cells and the effects of FA on autophagy. First, we treated HepG2 cells with different concentrations of FA and reported that 5, 10, and 20 μg/mL FA did not affect the viability of HepG2 cells, whereas 40 and 80 μg/mL FA inhibited the viability of HepG2 cells. Therefore, 20 μg/mL FA was selected for future studies (Figure 2A). The results of Western blot and immunofluorescence analyses revealed that compared with the control group, PA treatment significantly inhibited the expression of the autophagy-related proteins LC3 II/I and promoted p62 expression. After FA treatment, LC3 II/I expression was upregulated, and p62 expression was downregulated (Figure 2 B-D). Moreover, the results of MDC staining revealed that PA treatment significantly inhibited the formation of autophagosomes, and FA treatment partially reversed the effect of PA, promoting the formation of autophagosomes (Figure 2E). These results suggest that FA can promote PA-induced autophagy in HepG2 cells.

Folic acid relieves PA-induced damage to HepG2 cells by promoting autophagy

Next, we detected whether FA relieves PA-induced damage to HepG2 cells by promoting autophagy. The results of the CCK-8 assay revealed that PA treatment significantly reduced the viability of HepG2 cells. Meanwhile, FA treatment improved cell viability, and further addition of the autophagy inhibitor CQ weakened the therapeutic effect of FA and reduced cell viability (Figure 3A). CQ inhibits autophagy by preventing the fusion of autophagosomes with lysosomes, resulting in increased levels of LC3II. By inhibiting the degradation of autophagosomes, CQ promotes the formation of autophagosomes. Western blot analysis revealed that compared with the PA+FA treatment, the addition of CQ significantly promoted the accumulation of LC3 II and the expression of p62 (Figure 3B). MDC staining revealed that compared with the PA+FA treatment, the addition of CQ significantly promoted autophagosome formation (Figure 3C). Compared with the control group, PA treatment significantly promoted lipid accumulation in HepG2 cells, and lipid accumulation was reduced after FA intervention. With the further addition of CQ, lipid accumulation was partially increased (Figure 3D). Compared with those in the control group, the TC, TG, and FFA levels in the PA group were significantly greater, and the TC, TG, and FFA levels were significantly lower after FA intervention. Further addition of CQ increased the TC, TG, and FFA levels (Figure 3 E-G). The detection of lipid metabolism-related genes revealed that compared with the control group, the PA group presented significantly increased SREBP1c and SCD mRNA levels and significantly

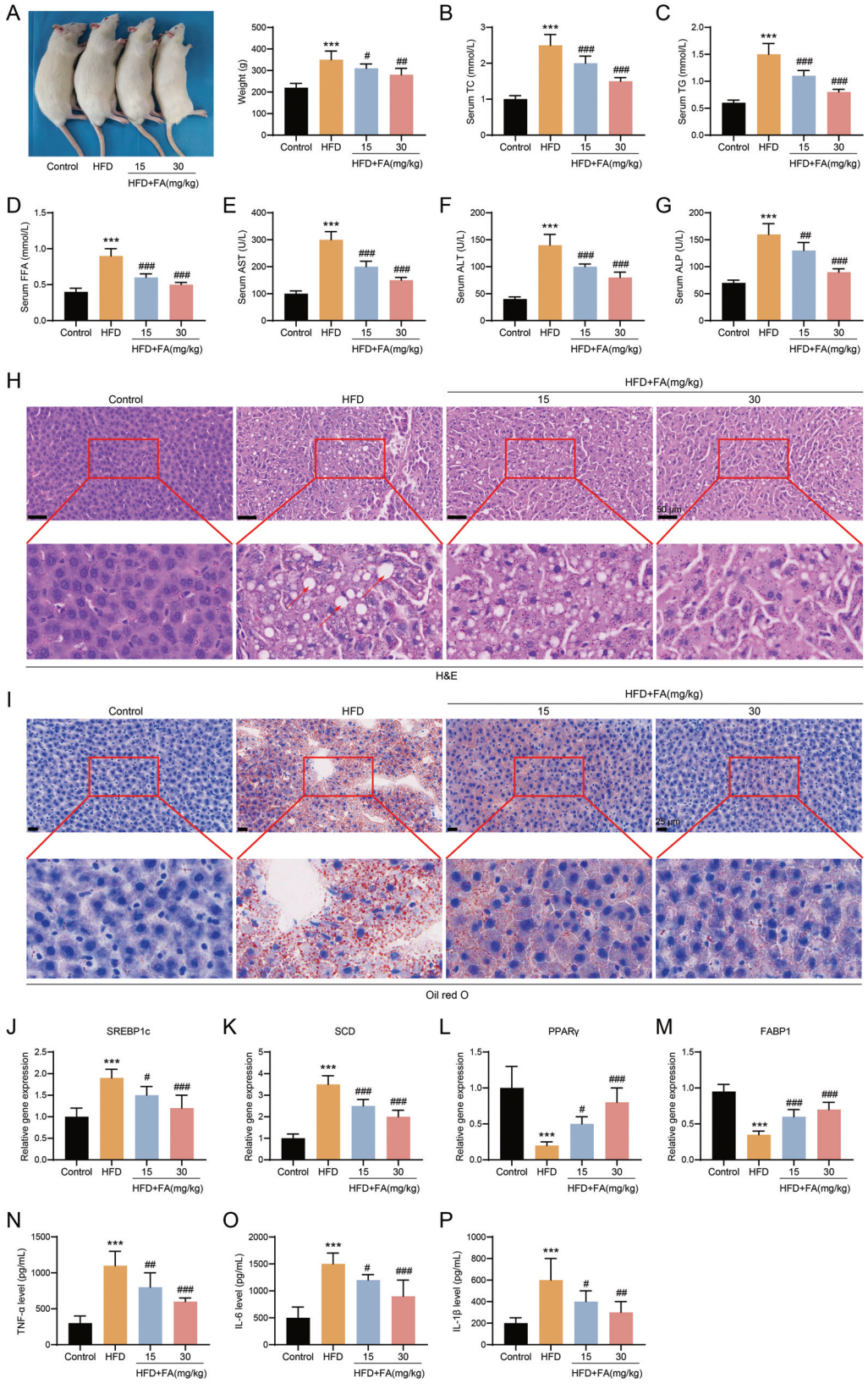

Figure 1. Folic acid inhibits HFD-induced liver injury in MAFLD rats. **A**) Rat weight; **B-D**) Rat serum TC, TG, and FFA levels detected by a kit. **E-G**) Rat serum liver function injury indicators AST, ALT, and ALP levels detected by a kit. **H**) Pathological changes in liver tissue observed with hematoxylin and eosin staining; scale bar: 50 μm. **I**) Lipid accumulation level in liver tissue detected by Oil Red O staining; scale bar: 25 μm. **J-M**) mRNA expression of the lipid metabolism-related genes SREBP1c, SCD, PPARγ, and FABP1 detected by RT-qPCR. **N-P**) Serum inflammatory cytokine TNF-α, IL-6 and IL-1β levels detected by ELISA. n=5 rats/group. ***p<0.001 *vs* control; $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 *vs* HFD.

reduced PPARγ and FABP1 mRNA levels. FA treatment inhibited the expression of SREBP1c and SCD mRNAs and promoted the expression of PPARγ and FABP1 mRNA. The expression of these genes was partially reversed after the addition of CQ (Figure 3 H-K). Finally, the results of inflammatory cytokine detection revealed that PA treatment significantly promoted the expression of TNF-α, IL-6, and IL-1β; however, after FA treatment, the levels of TNF-α, IL-6, and IL-1β decreased. Adding CQ led to increased levels of TNF-α, IL-6, and IL-1β (Figure 3 L-N). These results indicate that FA relieves PA-induced damage to HepG2 cells by promoting autophagy.

NRF2 expression is low in MAFLD and is regulated by folic acid

In addition, studies have shown that NRF2 not only regulates MAFLD development[17] but also affects the expression of autophagy-related genes.[18] Therefore, we detected NRF2 expression and found that NRF2 expression was downregulated in the liver tissue of HFD-fed rats and in PA-induced HepG2 cells, whereas FA intervention promoted NRF2 expression (Figure 4 A-D). These results indicate that NRF2 expression is low in MAFLD and that FA can promote the expression of NRF2.

Folic acid alleviates PA-induced HepG2 cell injury through the NRF2 autophagy pathway

To determine whether FA affects PA-induced autophagy and damage in HepG2 cells through the regulation of NRF2, we knocked down NRF2 in HepG2 cells. Compared with those in the sh-NC group, the levels of NRF2 in the sh-NRF2#1 and sh-NRF2#2 groups were significantly lower, and the knockdown effect in the sh-NRF2#2 group was greater (Figure 5A). Therefore, sh-NRF2#2 was selected for future experiments. The results of the

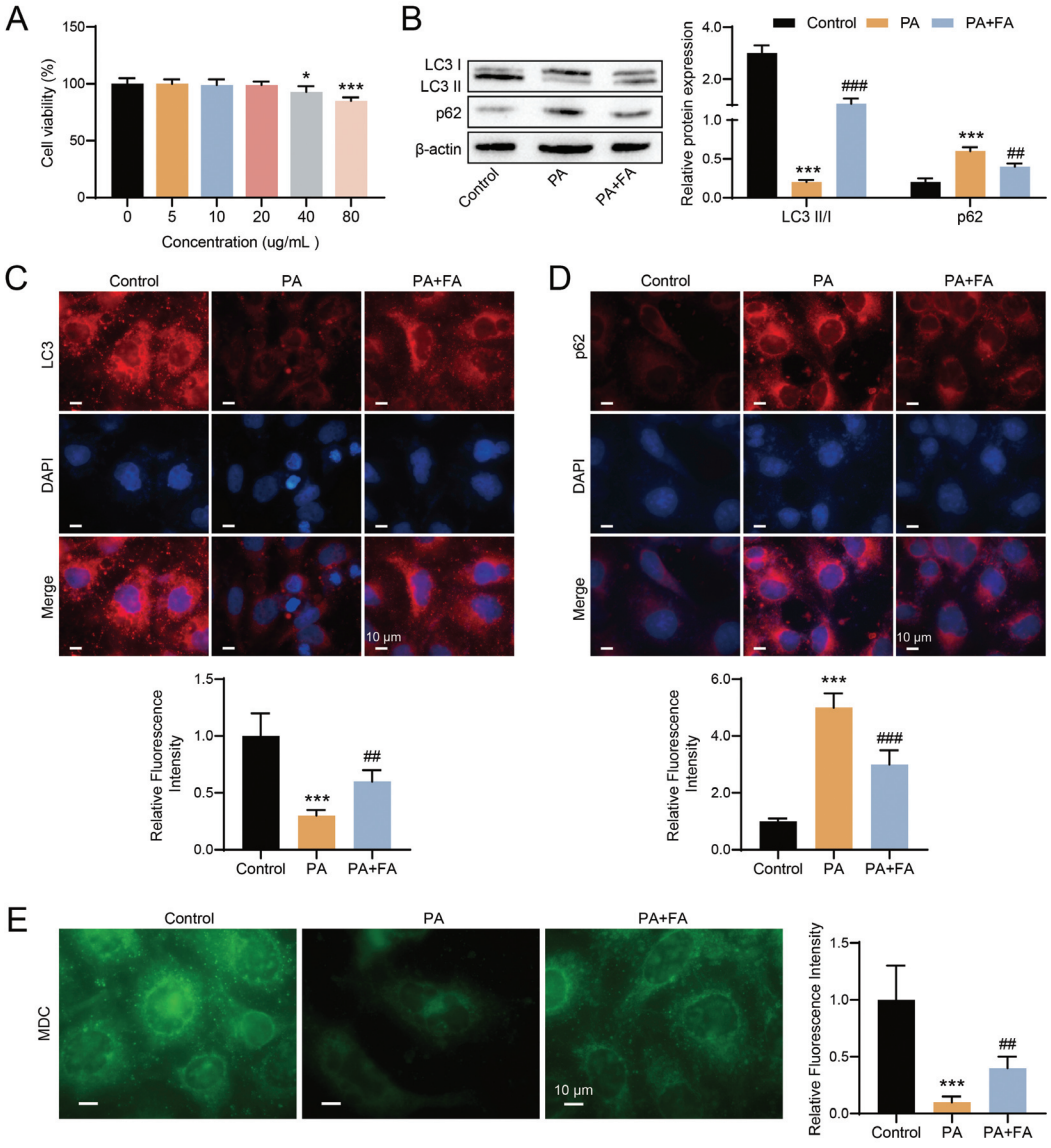

Figure 2. Folate regulates PA-induced autophagy in HepG2 cells. **A**) HepG2 cell viability determined by a CCK-8 assay. **B**) Western blot detection of the expression of the autophagy-related proteins LC3 II/I and p62. **C,D**) The expression of the autophagy-related proteins LC3 and p62 detected by immunofluorescence; scale bar: 10 μm. E) MDC staining to detect the formation of autophagosomes in HepG2 cells; scale bar: 10 μm. n=3. *p<0.05, ***p<0.001 *vs* 0 μg/mL or control; $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 *vs* PA.

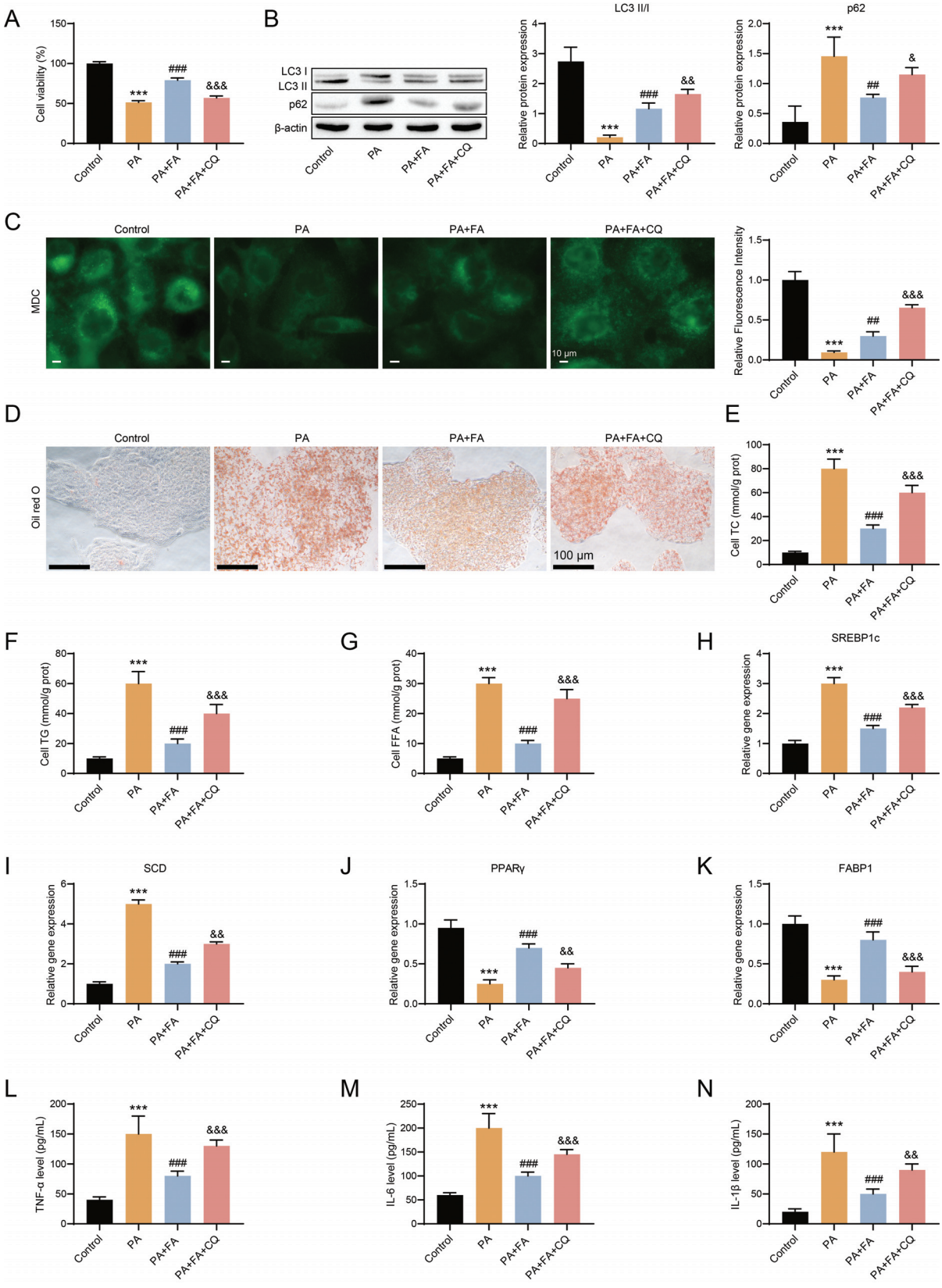

Figure 3. Folic acid relieves PA-induced damage to HepG2 cells by promoting autophagy. **A**) Cell viability determined by a CCK-8 assay. **B**) Western blot detection of the expression of the autophagy-related proteins LC3 II/I and p62. **C**) MDC staining for the formation of autophagosomes in HepG2 cells; scale bar: 10 μm. **D**) Oil Red O staining; scale bar: 100 μm. **E-G**) TC, TG, and FFA levels in HepG2 cells detected *via* kits. H-K) RT-qPCR detection of the mRNA expression of the lipid metabolism-related genes SREBP1c, SCD, PPARγ, and FABP1. **L-N**) The levels of the inflammatory cytokines TNF-α, IL-6 and IL-1β detected *via* ELISA. n=3. ***p<0.001 *vs* control; $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 *vs* PA; $^{\&}p$<0.05, &&p<0.01, $^{\&\&\&}p$<0.001 *vs* PA+FA.

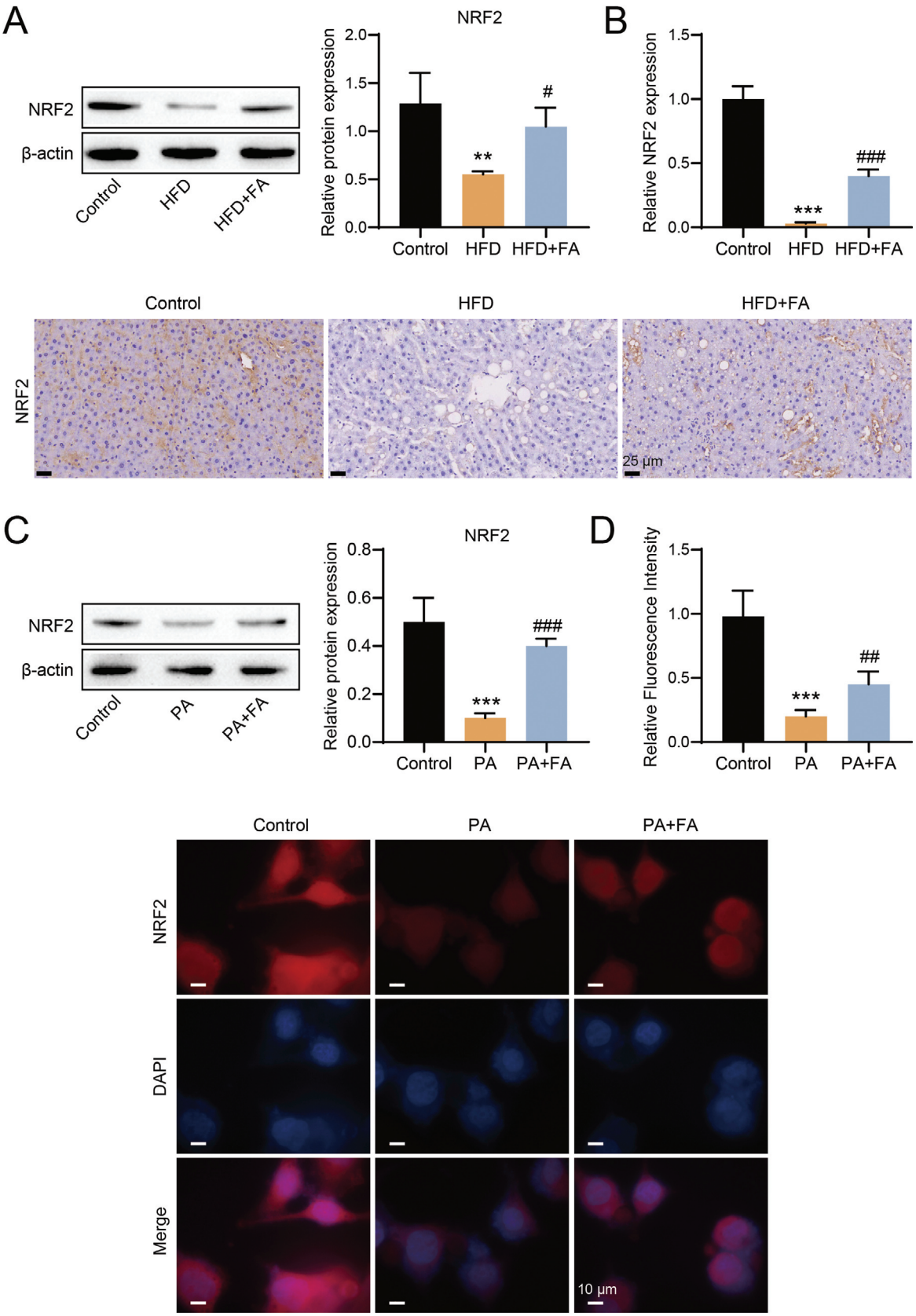

Figure 4. NRF2 expression is low in MAFLD and is regulated by folic acid. **A**) NRF2 expression in rat liver tissue detected by Western blotting. **B**) NRF2 expression in rat liver tissue detected by immunohistochemistry; scale bar: 25 μm. **C**) NRF2 expression in HepG2 cells detected by Western blotting. **D**) Immunofluorescence detection of NRF2 expression in HepG2 cells; scale bar: 10 μm. **A,B**) n=5 rats/group; **C,D**) n=3. **$p<0.01$, ***$p<0.001$ *vs* control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs* HFD or PA.

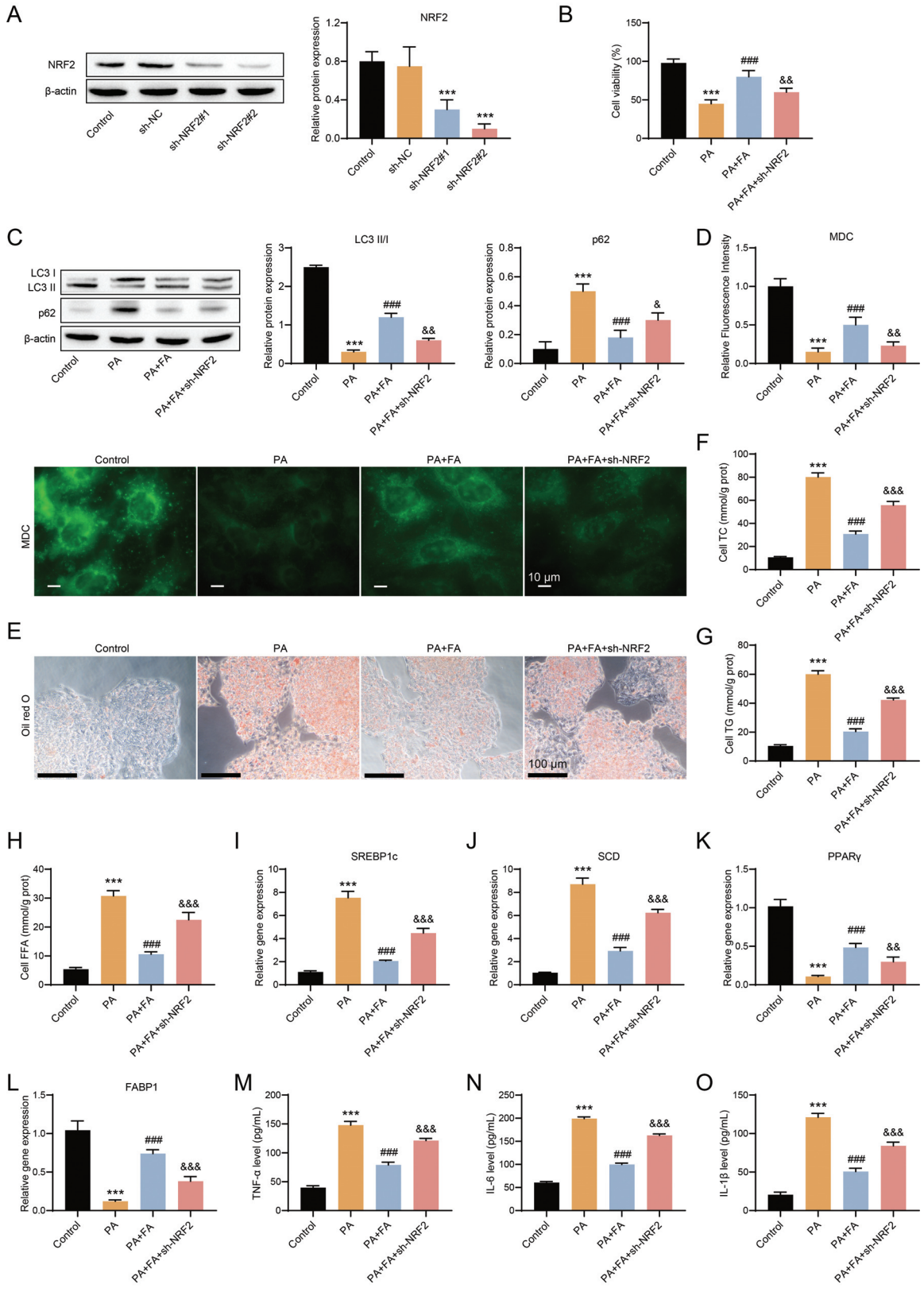

Figure 5. Folic acid attenuated PA-induced HepG2 cell damage through the NRF2 autophagy pathway. **A**) sh-NRF2 transduction efficiency was detected by Western blotting. **B**) Lipid accumulation in HepG2 cells was detected by a CCK-8 assay. **C**) Western blot detection of the expression of the autophagy-related proteins LC3 II/I and p62. **D**) Autophagosome formation in HepG2 cells was detected by MDC staining, scale bar: 10 μm. **E**) Lipid accumulation in HepG2 cells was detected by Oil Red O staining, scale bar: 100 μm. **F-H**) TC, TG, and FFA levels in HepG2 cells were detected with kits. **I-L**) RT–qPCR detection of the lipid metabolism-related genes SREBP1c, SCD, PPARγ, and FABP1 mRNAs. **M-O**) The levels of the inflammatory cytokines TNF-α, IL-6 and IL-1β were detected *via* ELISA. n=3. ***p<0.001 *vs* sh-NC or control; $^{###}p$<0.001 *vs* PA; $^{&}p$<0.05, $^{&&}p$<0.01, $^{&&&}p$<0.001 *vs* PA+FA.

CCK-8 assay revealed that, compared with PA+FA, NRF2 knockdown significantly reduced the viability of HepG2 cells (Figure 5B). The results of the Western blot analysis revealed that, compared with the PA+FA group, NRF2 knockdown significantly inhibited the expression of LC3 II/I and increased the expression of p62 (Figure 5C). MDC staining revealed that, compared with the PA+FA group, NRF2 knockdown significantly inhibited autophagosome formation (Figure 5D). Oil red O staining revealed that NRF2 knockdown promoted lipid accumulation in HepG2 cells (Figure 5E). Compared with those in the PA+FA group, NRF2 knockdown significantly increased the TC, TG, and FFA levels (Figure 5 F-H). The detection of lipid metabolism-related genes revealed that NRF2 knockdown partially attenuated the therapeutic effect of FA, promoting the expression of SREBP1c and SCD mRNAs and inhibiting the expression of PPARγ and FABP1 mRNAs (Figure 5 I-L). Finally, the results of inflammatory cytokine detection revealed that the inhibitory effect of FA treatment on the levels of TNF-α, IL-6, and IL-1β was partly attenuated by knocking down NRF2, which upregulated the levels of TNF-α, IL-6, and IL-1β (Figure 5 M-O). These results indicate that FA activates autophagy through NRF2, thereby alleviating PA-induced HepG2 cell damage.

Discussion

MAFLD is a prevalent chronic disease worldwide that can lead to severe nonalcoholic steatohepatitis (NASH), liver cirrhosis and hepatocellular carcinoma (HCC).[21] Epidemiological surveys have shown that the incidence of MAFLD is increasing sharply, mainly due to the intake of a high-fructose diet and the increasing incidence of obesity and diabetes.[22] Currently, for MAFLD, the main treatments for dialysis include antidiabetic drugs, antilipidemia drugs, and antihypertensive drugs, but there is still a lack of direct treatment drugs approved by the FDA.[23] FA is an important B vitamin that is stored mainly in the liver. Previous studies have reported that low serum FA levels are an independent risk factor for MAFLD in a Chinese population; incorporating serum FA levels into the existing MAFLD predictive scoring system can significantly improve the predictive accuracy of MAFLD.[24] Reduced serum FA levels in patients with MAFLD were closely related to an increased risk of all-cause death. Therefore, avoiding low serum FA levels might reduce the risk of death in MAFLD patients.[25] Sid *et al*.[26] reported that FA supplementation could reduce hepatic lipid accumulation and the aggregation of inflammatory foci caused by HFD feeding, thereby improving MAFLD in mice. In this study, FA treatment inhibited body weight gain; reduced lipid accumulation in rat liver tissues; decreased serum TC, TG, FFA, AST, ALT, ALP, TNF-α, IL-6 and IL-1β levels in HFD-fed rats; and improved pathological injury to liver tissue. Our results and those of Sid *et al*.[26] showed that FA can improve the progression of MAFLD. However, our study used rats as the research object, which further confirmed the potential of FA in the treatment of MAFLD in different animals.

In the liver, two main pathways regulate the catabolism of lipid droplets: lipolysis and lipophagy. In addition to affecting the clearance of lipid droplets in liver cells, autophagy affects liver non-parenchymal cells, including hepatic stellate cells, hepatic macrophages and liver sinusoidal endothelial cells, thereby affecting MAFLD-related proinflammatory and fibrotic responses during progression.[27] Both genetic and diet-induced obesity mouse models exhibit defects in hepatic autophagy and chaperone-mediated autophagy (CMA), which lead to obesity-associated hepatic steatosis and insulin resistance.[28, 29] Interestingly, FA also regulates the development of autophagy in liver cells. Chi *et al*.[15] reported that FA deficiency in liver cells interfered with one-carbon metabolism and inhibited autophagy, resulting in lipid accumulation. Huang *et al*.[30] reported that FA can promote autophagy by promoting the expression of ATG12, thereby inhibiting lipid accumulation in liver cells both *in vivo* and *in vitro*. Similarly, FA supplementation can reduce blood homocysteine concentrations and restore autophagy through transmethylation, thereby attenuating the progression of NASH and reversing inflammation and fibrosis in mice.[31] Here, the addition of FA promoted the expression of LC3 II/I, inhibited p62 expression, and restored the number of autophagosomes in PA-induced HepG2 cells. Moreover, the inhibitory effect of FA on PA-induced lipid accumulation and inflammation in HepG2 cells could be partially reversed by the autophagy inhibitor CQ, indicating that FA can relieve PA-induced damage to HepG2 cells through the activation of autophagy. Although the results of our study are similar to those of previous studies, the differences in molecular mechanisms still need further exploration.

NRF2 is a redox-sensitive transcription factor that regulates the expression of a series of genes related to detoxification, antioxidant defense, cell protection, cell cycle regulation and autophagy.[32] More specifically, autophagy-related factors are also target genes of the NRF2/antioxidant response element ARE transcriptional pathway.[33] Currently, detailed reports on the role of NRF2/autophagy in various diseases continue to emerge. In spinal cystic echinococcosis, NRF2 activates autophagy by regulating oxidative stress to induce angiogenesis.[32] In granulosa cell injury, activation of the NRF2/autophagy pathway can reduce cisplatin-induced granulosa apoptosis.[34] In addition, NRF2 can also protect the liver from lipid toxicity, and NRF2 deficiency may lead to increased lipid accumulation in the liver.[35] Zhang *et al*.[36] reported that caffeic acid reduces lipid accumulation, lipotoxicity and oxidative damage in the liver through activating NRF2, thereby alleviating MAFLD. Similarly, berberine can reduce liver biochemical indicators, lipid levels, the expression of proinflammatory mediators and the fatty liver index in MAFLD mice through activating NRF2, ultimately improving MAFLD.[37] In the present study, the expression of NRF2 was downregulated in MAFLD, and FA treatment promoted NRF2 expression. Further tests revealed that the beneficial effects of FA treatment on PA-induced autophagy and injury in HepG2 cells could be partially reversed by knocking down NRF2. Our results showed that FA improved HepG2 cell injury by activating the NRF2/autophagy pathway. Furthermore, Liu *et al*.[38] reported that after treatment with 100 mg/kg tea tree oil (TTO), the expression levels of NRF2, Beclin1 and LC3 increased, activating autophagy and reducing hepatopancreatic lipid deposition. Treatment with 1000 mg/kg TTO increased the expression of NRF2, while the expression of Beclin1 and LC3 decreased, inhibiting autophagy and increasing hepatopancreatic lipid deposition. These findings indicate that whether the upregulation of NRF2 can promote autophagy may be related to the type of drug, dosage, duration, disease progression and location of disease occurrence. However, how different doses of FA mediate autophagy through NRF2 and play a role in the progression of MAFLD still requires further investigation.

Overall, our study revealed for the first time that FA activates autophagy by promoting the expression of NRF2, thereby reducing lipid accumulation and alleviating the development of MAFLD. However, our study has several limitations. First, the short-term effect of FA may be significant, but the safety and efficacy of its long-term use remain unclear. Moreover, long-term activation of autophagy may cause side effects, leading to cell damage. Second, this study did not explore in depth the mechanism of NRF2 in an MAFLD animal model. In the future, we need to monitor the effects of FA over a long period and conduct additional *in vivo* experiments to verify the mechanism of NRF2.

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