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Original Article

Dimethyl fumarate suppresses hepatocellular carcinoma progression via activating SOCS3/JAK1/STAT3 signaling pathway

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Abstract: Dimethyl fumarate (DMF) is generally used to treat psoriasis and multiple sclerosis. In the present study, we aimed to investigate the effects of DMF on hepatocellular carcinoma progression and its mechanism of action. *In vitro*, cell viability was examined using CCK-8 assay; cell cycle was analyzed by flow cytometry; angiogenesis was detected using tube formation assay; and autophagic flux assay results were examined using fluorescence microscopy. We also used western blotting to explore the potential mechanisms. *In vivo*, tumor xenograft experiment was performed with nude mice, and liver function, renal function, and routine blood counts were assessed using biochemical tests. Dimethyl fumarate inhibited tumor growth and angiogenesis in hepatocellular carcinoma, both *in vitro* and *in vivo*. Dimethyl fumarate decreased autophagy in hepatocellular carcinoma cells. Treatment with DMF activated SOCS3, which led to repression of JAK1 and STAT3 phosphorylation. DMF inhibited cell proliferation, angiogenesis, and autophagy via activation of the SOCS3/JAK1/STAT3 signaling pathway. This finding may provide a novel approach for the treatment of hepatocellular carcinoma.

Keywords: Dimethyl fumarate (DMF), SOCS3/JAK1/STAT3 pathway, cell proliferation, autophagy, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is one of most common malignancies worldwide [1]. Sorafenib is the standard treatment for patients with advanced HCC; however, the outcomes remain poor [2]. Several systemic therapeutic drugs against HCC have been tested in an attempt to overcome the poor anti-tumor effects and toxicity but satisfactory results have not been observed, for various reasons [3].

Dimethyl fumarate (DMF) is an inexpensive compound commonly used in industrial chemistry. Initially, it was used to treat psoriasis [4]. Recently, DMF was approved by the US Food and Drug Administration and the European Medicines Agency for the treatment of multiple sclerosis (MS) [5]. Long-term intake of DMF does not cause serious adverse effects [5-7]. Dimethyl fumarate exerts anti-inflammatory effects by inhibiting the expression of inflammatory cytokines such as NOS, IL-1β, and IL-6 in glial cells [8, 9]. Furthermore, DMF has also been found to exhibit anti-tumor effects. It not only inhibits melanoma growth and metastasis but also induces colon cancer apoptosis and necroptosis [10, 11]. However, the effects of DMF on HCC and its mechanism of action remain unknown.

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is constitutively activated in cancers and is involved in a variety of cellular functions such as differentiation, survival, proliferation as well as apoptosis [12-14]. STAT3 protein plays a key role in tumorigenesis, tumor cell proliferation, survival, and invasion [15]. STAT3 transcriptional activity is primarily activated by phosphorylation of a single tyrosine residue Tyr705. Tyrosine phosphorylation of STAT3 can be catalyzed by the

kinase JAK [16]. Once activated, STAT3 regulates the transcription of many target genes, such as the angiogenesis-related gene VEGF [17] and the autophagy-related genes BECN1 [16] and ATG5 [18]. Suppressors of cytokine signaling protein 3 (SOCS3) was reported to negatively regulate the JAK/STAT pathway [19] through attenuation of JAK activity. In addition, recent studies have demonstrated universal activation of the JAK/STAT pathway in human HCC [20], suggesting that JAK/STAT inhibitors could be used for treating HCC [21].

In the present study, we demonstrated that DMF suppressed hepatocellular carcinoma progression via the SOCS3/JAK1/STAT3 signaling pathway, which may provide a novel solution for the treatment of liver cancer.

Materials and methods

Cell culture

Two HCC cell lines (Huh-7 and HCC-LM3), a hepatoblastoma cell line (HepG2), and a human umbilical vein endothelial cell (HUVEC) line were used in the present study. The cells were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences, and were cultivated as described by the suppliers. Huh7, HCC-LM3 and HepG2 cells were cultured in modified Eagle's medium (MEM; Biological Industries, CA, USA) supplemented with 10% fetal bovine serum, and HUVECs were cultured in endothelial cell growth medium at 37°C and 5% CO_2.

Reagents

DMF (CAS: D106460) was purchased from Aladdin Industrial Corporation (Shanghai, China). Rapamycin (CAS: S1039) was purchased from Selleck Chemicals (Shanghai, China). DMSO (CAS: 67-68-5, D8418) was obtained from Sigma-Aldrich, Inc. (Darmstadt, Germany). Methyl cellulose (CAS: 9004-67-5) was obtained from Sangon (Shanghai, China).

Antibodies

STAT3 (#30835), phospho-STAT3 (Tyr705) (#9145), JAK1 (#50996), phospho-JAK1 (#74129), Atg5 (#9980), Atg7 (#8558), LC3B (#3868), Beclin1 (#3495), and phospho-VEGF receptor 2 (#3770) antibodies were purchased from Cell Signaling Technology (USA). VEGFA (Cat. No. 66828--Ig), CKD2 (Biotech Cat. No. 10122-1-AP), (Cat. No. 11026-1-AP), P21 (Cat. No. 10-355-1-AP), cyclin D1 (Cat. No. 60186-1-Ig), and Ki67 (Cat. No. 27309-1-AP) antibodies were obtained from Proteintech (Rosemont, IL, USA). Anti-CD31 antibody (#GB1306) was obtained from Servicebio (China). Bcl-XL antibody (#db-225) was obtained from Bio-Technology Co., Ltd. (Hangzhou, China). c-Myc (ab32072) and Anti-AKT1 (phospho S473) (ab81283) antibodies were obtained from Abcam (USA).

Immunohistochemical analysis

Tumor tissues from mice were sliced into 4 μm thick sections using a thin semi-automatic slicer. All sections were deparaffinized in xylene and rehydrated in a series of graded alcohol dilutions. Antigen retrieval was performed by heating in a microwave oven. The sections were then incubated with 3% H_2O_2 for 10 min and then incubated with 10% normal goat serum for 15 min at room temperature to block endogenous peroxidase as well as non-specific antigen binding. The samples were then incubated with primary antibodies. Tissue sections were immunostained overnight at 4°C with the following antibodies: anti-Ki67 antibody (1:1000 dilution; #27309-1-AP, Proteintech, Rosemont, IL, USA), anti-LC3B antibody (1:200 dilution; #3868, Cell Signaling Technology, USA) and anti-CD31 antibody (1:200 dilution; GB1306, Servicebio, China). After washing thrice with phosphate buffered saline (PBS), the sections were incubated with horseradish peroxidase-labeled secondary antibody (1:2000 dilution) for 30 min at room temperature. Finally, 3,3-diaminobenzidine was used to visualize the signal development. The sections were then counterstained with hematoxylin. Finally, the sections were photographed under an inverted microscope.

CCK-8 assay

Cell vitality was determined with the cell counting kit-8 (CCK-8) according to the manufacturer's protocol (DOJ INDO Laboratories, Kumamoto, Japan). In brief, cells were seeded into 96-well plates at a density of 5000 cells per well. 12 h later, cells were treated with different concentrations of DMF in a humidified incubator for 24, 48, or 72 h. After removing the supernatant by vacuum, 100 μL serum-free medium and 10 μL of CCK-8 were premixed and added to each well, followed by incubation for 1 h. Absorbance was measured at 450 nm using a microplate reader (BioTek, USA). Each assay was performed in triplicate.

Colony formation assay

Cells were seeded in 6-well plates at an equal density (1000 cells per well) and were treated with different concentrations of DMF. Fresh medium was replaced every 4 days. Cells were maintained in culture for another 10 days; the medium was removed and cells were fixed with methanol for 10 min and then stained with crystal violet. The total number of colonies in each dish were counted and recorded from three independent experiments.

Tube formation assay

Matrigel (40 μL) was uniformly coated on 96-well plates, and HUVECs were seeded at a density of 2×10^4 cells/well in Matrigel-coated 96-well plates containing 50, 100, or 200 μM DMF. After cells were cultured for 6-8 h, the capillary-like structures of HUVECs were photographed under an inverted microscope. The branch points of formed tubes, which represent the extent of angiogenesis *in vitro*, were scanned and quantified at 100 × magnification.

Flow cytometry analysis

Cells (1.5×10^5 per well) were seeded into 6-well plates and treated with different DMF concentrations for 48 h. Cells were harvested and fixed with pre-cooled 75% ethanol at -20°C for 24 h, then resuspended in 300 μL of DNA-Prep stain (Beckman Coulter, USA), and incubated for 30 min at room temperature in the dark. Suspended cells were analyzed by flow cytometry (BD FACSCalibur, USA).

Autophagic flux assay

Huh7 cells (2×10^4) were seeded in 6-well plates and incubated overnight. mCherry-GFP-LC3 adenovirus (HANBIO, Shanghai, China) was transfected with 5 μg/mL polybrene. The transfection mixture was replaced with 10% fetal bovine serum containing MEM overnight and cultured for an additional 24 h. Cells were then treated with rapamycin or rapamycin in combination with DMF for 24 h. Green (GFP) and red (mCherry) fluorescence was observed under a fluorescence microscope.

Western blot assay

After the cells were treated with different concentrations of DMF, cells were harvested and lysed in RIPA buffer, and supernatants were collected and stored at -20°C after centrifugation at 12000 × *g* for 15 min. Protein concentrations were determined by Bradford assays (Bio-Rad, USA). After denaturation, proteins were separated by gel electrophoresis using 10% or 12% SDS-polyacrylamide gels and then transferred to a PVDF membrane for 1 h followed by blocking in 5% non-fat milk. Membranes were washed once with TBST (0.1% Tween 20) and then incubated overnight at 4°C with the relevant antibodies (1:1000 dilution). The next day, membranes were washed three times with TBST for 10 min each time and then incubated with secondary antibody (goat anti-rabbit IgG, 1:2000 dilution) for 1 h at room temperature. After a washing procedure as in the previous step, ECL liquid was added, and samples were placed in a dark room to allow the reaction to proceed. GAPDH (1:1000 dilution) was used as a positive control.

Tumor xenograft experiments

All experimental protocols were approved by the animal experimental ethics committee of First Affiliated Hospital of Zhejiang University School of Medicine. The procedures were carried out in accordance with the approved guidelines. HCC-LM3 cells (3×10^6) were resuspended in 50 μL phosphate buffered saline and injected subcutaneously into the flanks of immunodeficient mice. Tumor formation was monitored by palpation, and when tumors were detected, mice were randomized into control or DMF treatment groups. Mice were injected daily with vehicle (0.8% methylcellulose) or DMF (30 mg/kg suspended in 0.8% methylcellulose). Tumor size was measured daily using electronic calipers and calculated using the following equation: Volume (mm^3) = width^2 (mm^2) × length (mm)/2. Tumor growth was monitored until the total tumor burden reached humane endpoint criteria. All tumors were harvested for immunostaining and western blotting assays.

Biochemical tests

Each blood sample (1 mL) was divided into two Eppendorf tubes containing heparin, and one of the tubes was centrifuged at 3000 × *g* for 5 min to obtain serum. Typical biochemical markers of hepatocyte lysis, such as circulating alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase

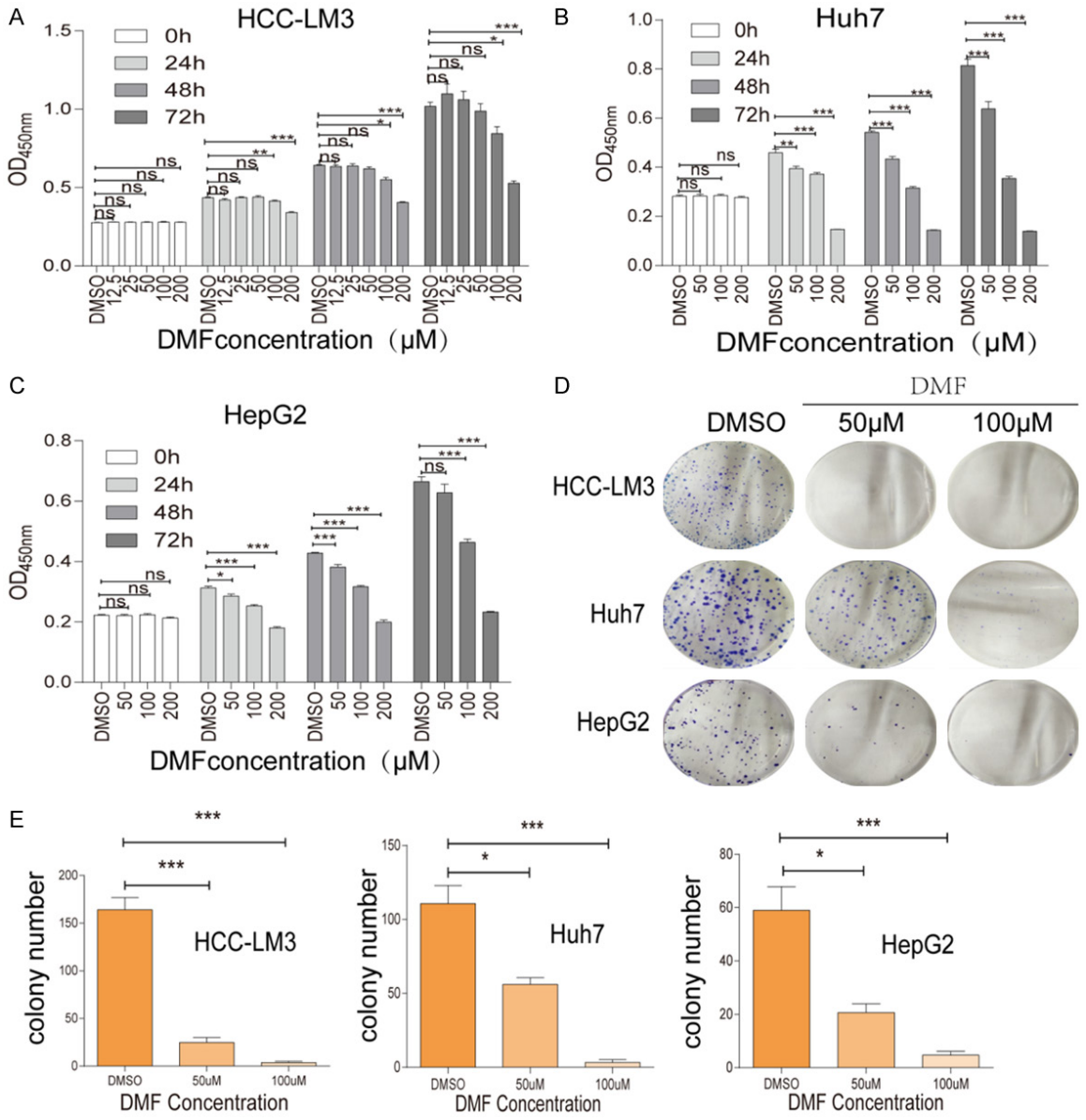

Figure 1. DMF suppressed cell proliferation of HCC. A-C. Cell Counting Kit-8 assay was used to detect the proliferation of HCC-LM3, Huh7 and HepG2 cells after treated with DMF. D and E. Colony formation assay evaluated the growth of HCC-LM3, Huh7 and HepG2 cells treating with 50 μmol and 100 μmol DMF. Values are presented as the mean ± SD, every experiment was repeated three times. *P < 0.05, **P < 0.01 and ***P < 0.001.

(ALP), and those of renal function, such as creatinine (CR), blood urea nitrogen (BUN), and uric acid (UA), were analyzed. The other blood sample tube was used to detect the red blood cell, white blood cell, and platelet counts.

Statistical analysis

SPSS 18.0 software was used for statistical analysis. Quantitative data were expressed as mean ± SD. Means were compared using Student's t-test. *P* values < 0.05 were considered statistically significant.

Results

Dimethyl fumarate inhibits cell proliferation in HCC

Previous studies have shown that DMF is capable of inhibiting melanoma and colon cancer cell growth [10, 11]. In the present study, we analyzed whether DMF treatment also exhibits cytotoxicity against HCC cells. The CCK-8 assay was performed to investigate the effect of DMF on proliferation in HCC cells (HCC-LM3, Huh7, and HepG2). As shown in **Figure 1A-C**, cell via-

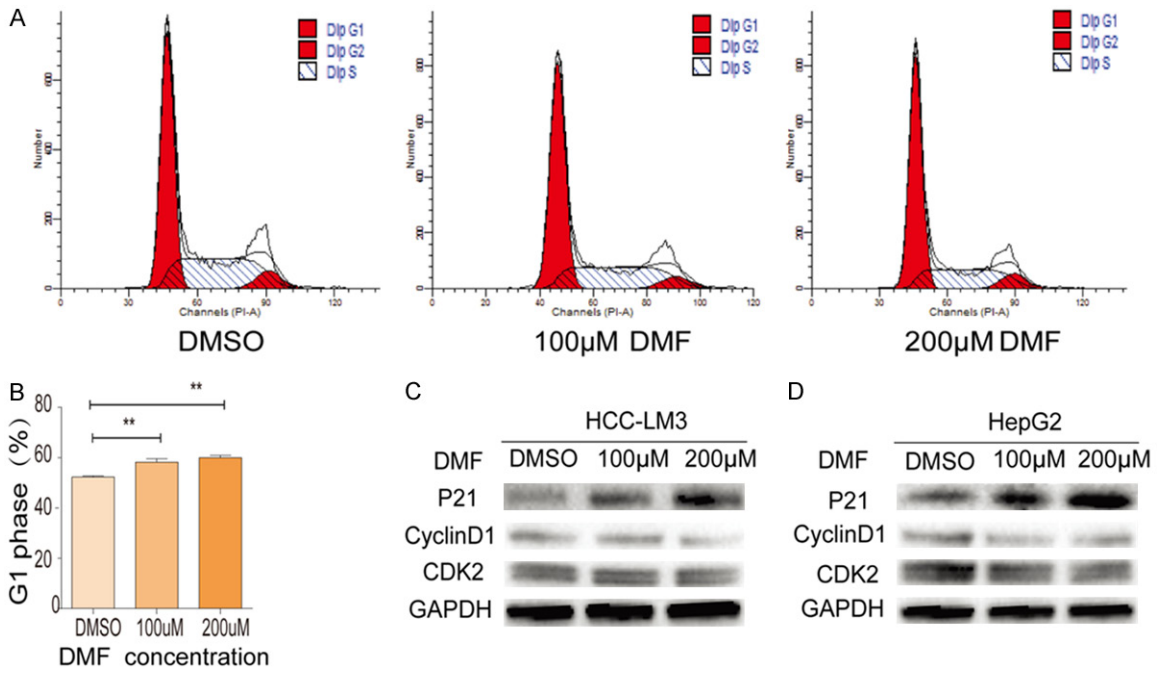

Figure 2. DMF arrested HCC cell cycle at G1 phase. A and B. Cell cycle arrest was examined by Flow cytometry for the treatment of HCC cells with DMF. Values are presented as the mean ± SDs, every experiment was repeated three times. **P < 0.01 compared with DMSO group. C and D. HCC-LM3 cells and HepG2 cells were treated by DMF. Cell cycle associated protein p21, CDK2 and cylinD1 were examined by western blot.

bility of HCC-LM3, Huh7, and HepG2 was significantly inhibited by different concentrations of DMF in a dose-dependent manner ($P < 0.05$ compared with the DMSO group). Results of the colony formation assay also showed that DMF significantly suppressed the growth of HCC-LM3, Huh7, and HepG2 cells in a dose dependent manner ($P < 0.05$ compared with the DMSO group) (**Figure 1D** and **1E**).

Dimethyl fumarate arrests cell cycle at G1 phase

Based on the results that DMF alters HCC cell proliferation, we assumed that DMF treatment may affect the cell cycle. Therefore, flow cytometry was performed. We found a significant increase in G1 phase arrest in the DMF group, accompanied by a significant decrease in the S phase (**Figure 2A** and **2B**) ($P < 0.05$).

To elucidate the molecular mechanism underlying the changes in cell cycle, we performed western blot and found that after DMF treatment, cell cycle-associated protein p21 was upregulated and CDK2 and cyclin D1 were downregulated in HCC-LM3 and HepG2 cells (**Figure 2C** and **2D**).

Dimethyl fumarate inhibits angiogenesis of HUVECs

To analyze whether DMF exhibits distinct anti-angiogenic effects in addition to its known anti-tumor function, we used tube formation assays to investigate the effect of DMF on the ability of HUVECs to form capillary-like structures. Dimethyl fumarate inhibited angiogenesis in HUVECs in a dose-dependent manner, as determined by the tube formation assay (**Figure 3A** and **3B**).

To elucidate the molecular mechanism underlying inhibition of angiogenesis by DMF, western blot assay was used to analyze the expression of p-VEGFR2 and VEGFA in HUVECs after DMF treatment. After DMF treatment, p-VEGFR2 and VEGFA were found to be downregulated in HUVECs. Thus, the anti-angiogenic effects of DMF corresponded with suppression of p-VEGFR2 and VEGFA protein expression (**Figure 3C**).

Dimethyl fumarate activates SOCS3 and inhibits JAK1/STAT3 signaling pathway

In addition to affecting proliferation, DMF also affects apoptosis of HCC cells. Our results

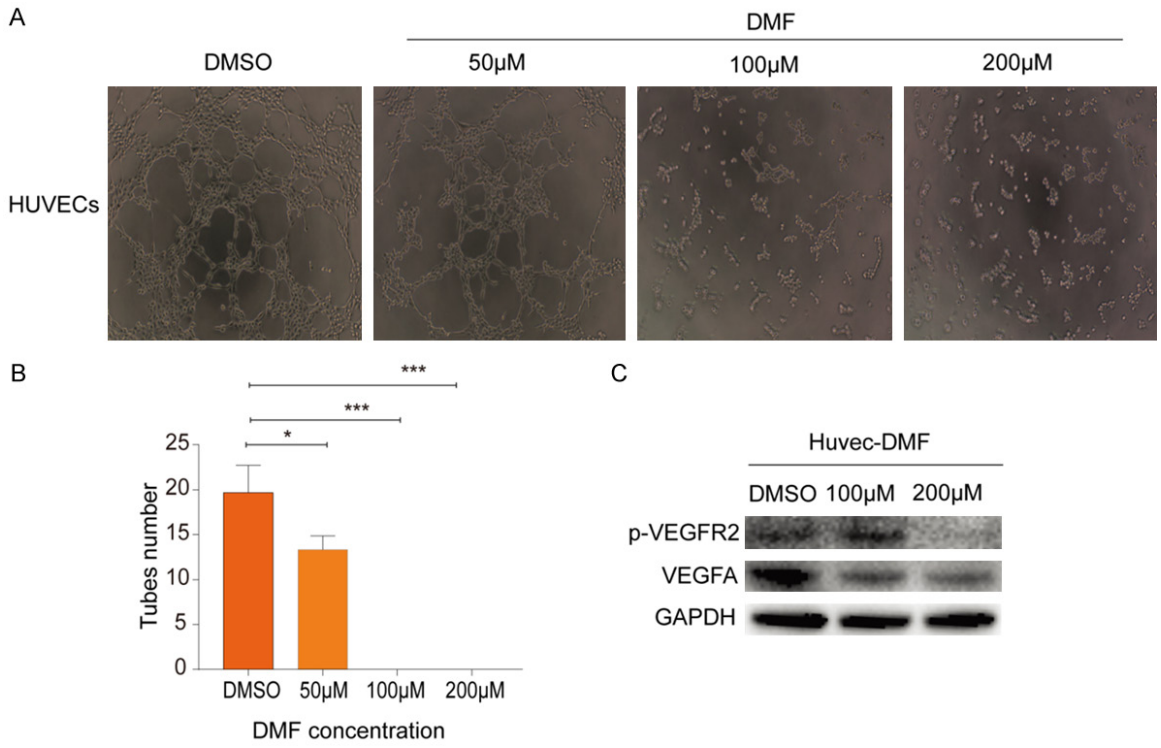

Figure 3. DMF inhibited tube formation of HUVECs. A. DMF inhibited HUVECs tubulogenesis *in vitro*. B. The number of tubes treated with different concentrations of DMF. Values are presented as the mean ± SD, every experiment was repeated three times. *P < 0.05 and ***P < 0.001. C. Expression of angiogenesis related proteins p-VEGFR2 and VEGFA in HUVECs after treating with DMF were analyzed by western blot.

(Figure S1A-C) were consistent with those reported by a previous study [11]. PI3-K/Akt1 pathway is one of the pathways affected by DMF in systemic sclerosis fibrosis [22]. We found that DMF treatment decreased p-AKT and c-MYC levels in HCC cells (**Figure 4A**). To further investigate the mechanism of DMF in HCC cells, we analyzed the proteins that affected by DMF. DAVID bioinformatics database was used to perform Gene Ontology analysis to analyze the pathway regulated by DMF [23]. Gene Ontology analysis suggested that the JAK/STAT signaling pathway was associated with DMF treatment in HCC cells (**Figure 4B**).

Activation of STAT3 signaling increases tumor cell proliferation, survival, and invasion [12, 13]. Moreover, studies have also demonstrated the importance of the JAK/STAT pathway in HCC development. The JAK/STAT pathway is modulated by negative regulators such as SOCS proteins [24]. SOCS proteins can bind both cytokine receptors and JAK and are recruited to the tyrosine-phosphorylated receptor, facilitating inhibition of JAK [24, 25]. SOCS3, one of SOCS family members, is also likely involved in the suppression of tumor growth, vascular invasion, and metastasis of HCC [26, 27].

To demonstrate the effects of DMF on SOCS3/JAK1/STAT3 signaling pathway, HCC-LM3 and HepG2 cells were treated with 100 μM and 200 μM DMF for 48 h. Cells were harvested for western blot analysis with SOCS3/JAK1/STAT3 signaling pathway antibodies. Western blot analysis showed that in HCC-LM3 and HepG2 cells, after DMF treatment, SOCS3 protein expression was upregulated (**Figure 4C** and **4D**), and phosphorylation of JAK1 (**Figure 4C** and **4D**) and STAT3 (**Figure 4A** and **4B**) was severely inhibited. Therefore, the results indicated that DMF activated SOCS3 protein expression and inhibited the JAK1/STAT3 signaling pathway with SOCS3 negative feedback, thus efficiently suppressing proliferation and angiogenesis in HCC. These results suggested that SOCS3/JAK1/STAT3 pathway was a potential mechanism of action in DMF-treated HCC cells.

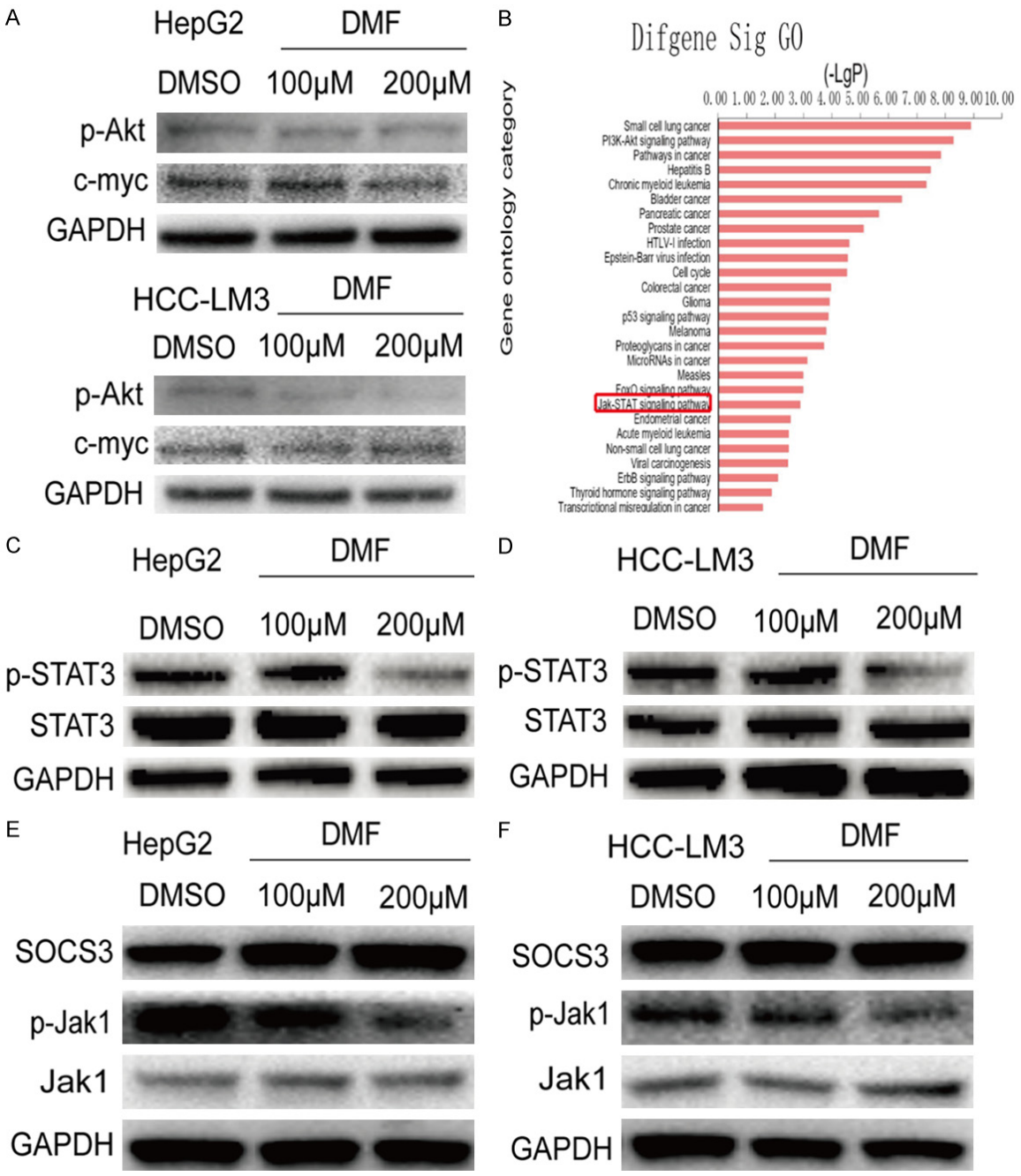

Figure 4. DMF activated the expression of SOCS3 and inhibited JAK1/STAT3 signaling. A. p-AKT and c-MYC were examined in HCC-LM3 and HepG2 cells after treating with DMF. B. Gene Ontology analysis of the signaling pathway regulated by DMF. Jak-STAT signaling pathway was found associated with DMF treatment in HCC cells. C and D. HCC-LM3 and HepG2 cells were treated with DMF for 48 hours. p-STAT3 and STAT3 were analyzed by western blot. E and F. HCC-LM3 and HepG2 cells were treated with DMF for 48 hours. SOCS3, p-Jak1 and Jak1 were evaluated by western blot.

Dimethyl fumarate decreases autophagy in HCC cells

STAT3 plays a crucial role in regulating autophagy. Inhibition of the STAT3 signaling pathway would decrease autophagy [18]. In the present study, we have demonstrated that DMF inhibited the STAT3 signaling pathway. Therefore, to explore the effect of DMF towards autophagy, we analyzed cells treated with different concentrations of DMF. The changes in autophagy markers Atg5, Atg7, and LC3B were all detected by western blot assays in HCC-LM3 and HepG2 cells. We found that Atg5, Atg7, and LC3B pro-

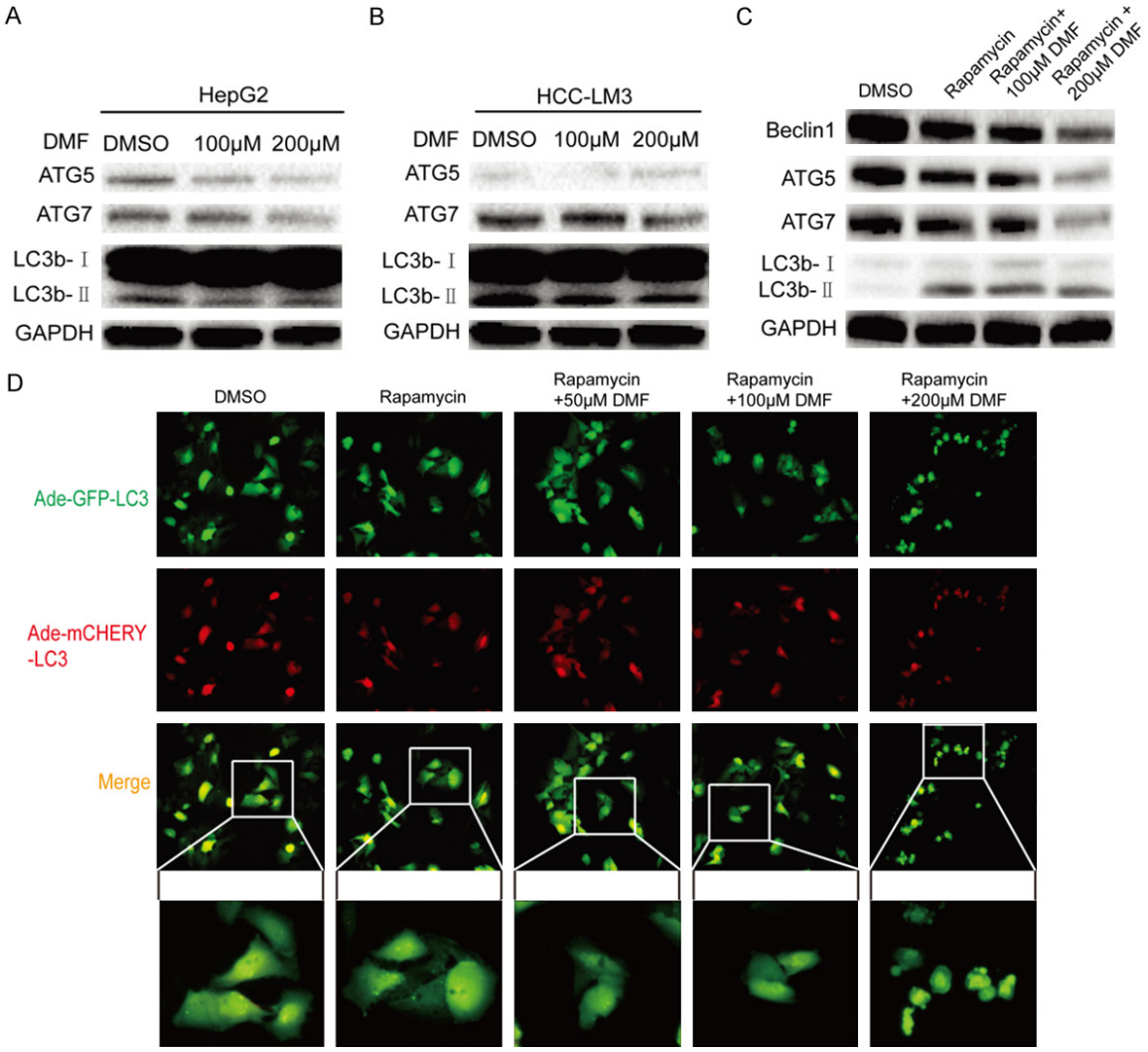

Figure 5. DMF decreased autophagy level in HCC cells. A and B. HepG2 and HCC-LM3 cells were treated with 100 μM and 200 μM DMF for 24 hours. Autophagy associated proteins Atg5, Atg7 and LC3b were examined by western blot. C. Huh7 cells were treated with Rapamycin or Rapamycin combined with DMF. Autophagy markers, LC3b, ATG5, ATG7 and Beclin1 were detected by western blot assay. D. Representative fluorescence images of autophagosomes and autolysosomes in Huh7 cells treated with Rapamycin or Rapamycin combined with DMF by autophagic flux assay (magnification, × 200).

tein levels significantly decreased (**Figure 5A** and **5B**). In order to verify the inhibitory effects of DMF on autophagy, Huh7 cells were treated with autophagy inducer rapamycin or rapamycin combined with DMF. Autophagy was activated after treatment with rapamycin, whereas autophagy was inhibited after treatment with rapamycin combined with DMF (**Figure 5C**). To detect changes in autophagic flux induced by DMF, we transduced mCherry-GFP-LC3 adenovirus into Huh7 cells, and then treated the cells with rapamycin or rapamycin combined with DMF. Cells were observed under a fluorescence microscope. Autophagy was found to be activated in the rapamycin-treated group; in contrast, the level of autophagy decreased in the group treated with rapamycin combined with DMF (**Figure 5D**).

Dimethyl fumarate suppresses tumorigenicity in vivo

Anticancer property of DMF was further assessed in mice models. HCC-LM3 cells (3×10^6) were subcutaneously injected into 6-week-old male nude mice; tumor formation was monitored by palpation. When tumors could be detected, mice were randomized into either the control or the DMF treatment groups. The DMF treatment group showed significantly lower tumor size and body weight than the control group (**Figure 6A-C**).

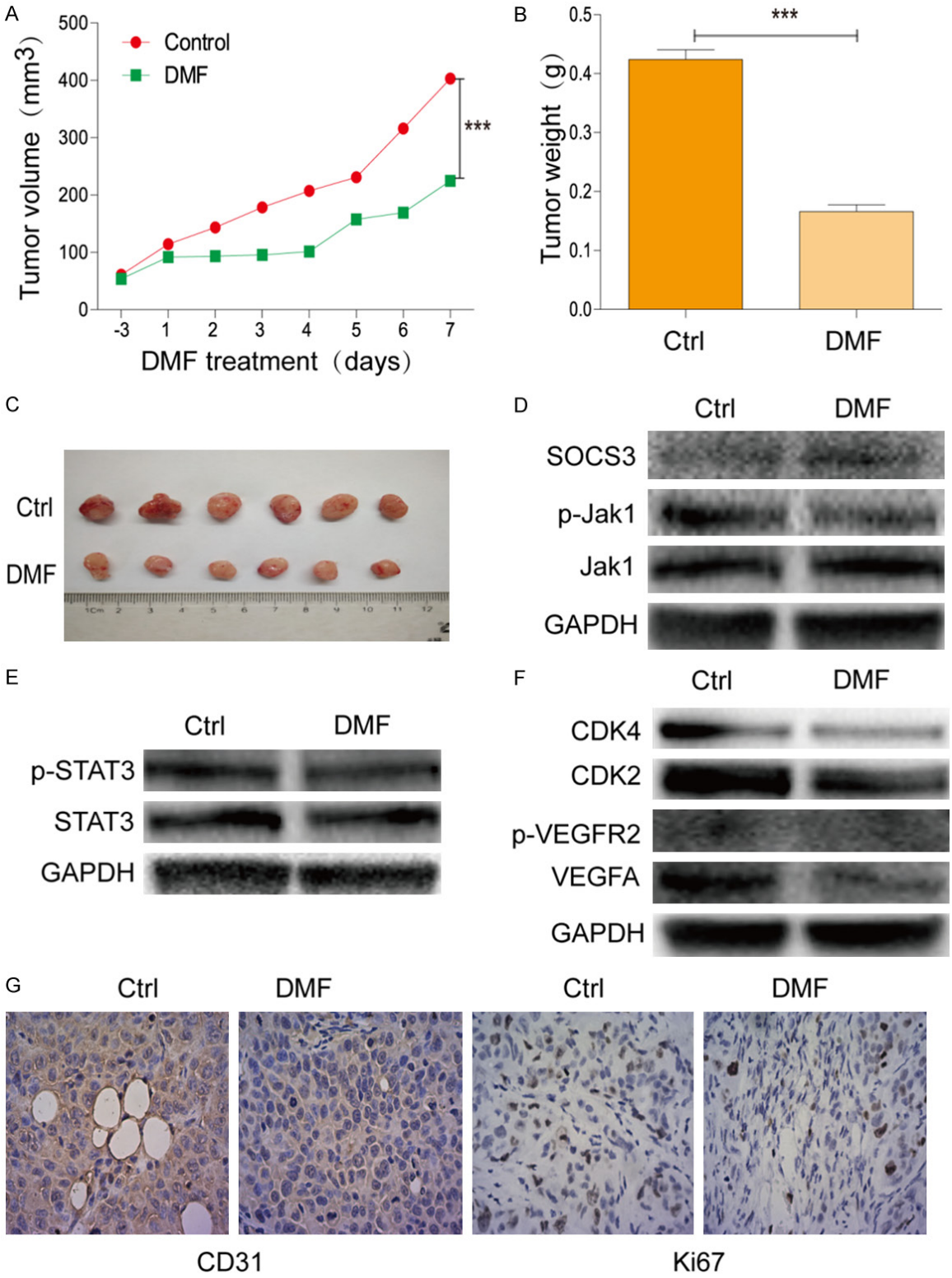

Figure 6. DMF suppressed the tumorigenicity of HCC cell lines in vivo. A and B. DMF inhibited the growth and weight of HCC tumors in nude mice. C. Photographic images of xenograft tumors. D and E. Western blot assay were used to examine the expression of SOCS3, STAT3, p-STAT3, Jak1 and p-Jak1 in xenograft tumor tissue. F. Cell cycle-associated proteins CDK2, CDK4 as well as angiogenesis related proteins VEGF and p-VEGFR2 were detected by western blot in xenograft tumor tissue. G. Tumor sections for Ki67 and CD31 protein expression were analyzed by immunohistochemistry (magnification, × 200).

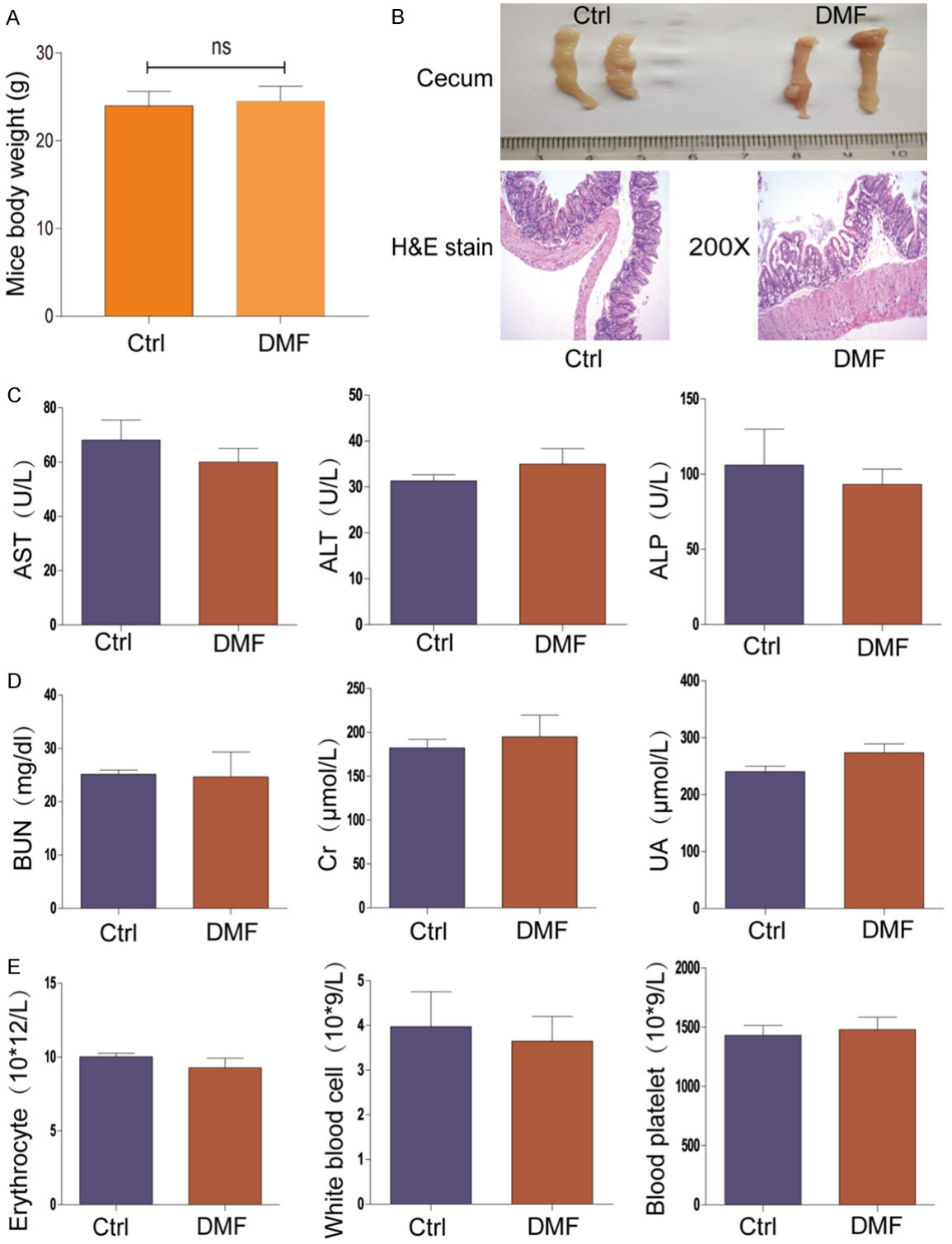

Figure 7. DMF showed non-toxic effects on mice. A. Mice body weight of xenograft nude mice was measured. Values are presented as the mean ± SD, P > 0.05 was seen as no significant difference. B. Cecums were observed from macroscopic observation and H&E stain. C. Liver function related alkaline phosphatase (ALP), aspartate aminotransferase (AST) and aminotransferase (ALT) were examined. Values are presented as the mean ± SD. D. BUN, Cr and UA levels of kidney function index were measured. Values are presented as the mean ± SD. E. Erythrocytes, white blood cells and blood platelets levels of blood routine index were evaluated. Values are presented as the mean ± SD.

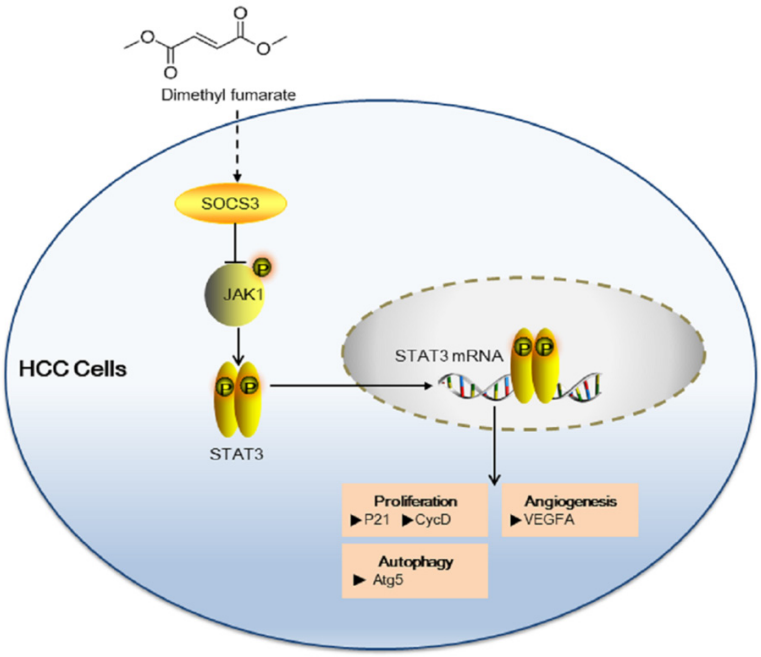

Figure 8. A scheme for DMF induced SOCS3/JAK1/STAT3 signaling pathway in HCC cells.

To verify whether the mechanism of HCC inhibition by DMF *in vivo* is consistent with that observed *in vitro*, tumor tissues from the control and DMF treatment groups were harvested to carry out western blot analysis. We found that DMF activated SOCS3 protein and, sequentially abolished the phosphorylation of JAK1 and STAT3 (**Figure 6D** and **6E**). With the suppression of JAK1/STAT3 signaling, cell cycle-associated proteins CDK2 and CDK4 were downregulated (**Figure 6F**). Moreover, the proteins involved in angiogenesis, VEGFA and p-VEGFR2, were also effectively inhibited (**Figure 6F**). These results demonstrated that molecular mechanisms of HCC inhibition by DMF *in vivo* and *in vitro* are consistent.

Immunohistochemistry showed that Ki67 expression in the DMF treatment group was lower than that in the control group (**Figure 6G**). This suggests that DMF effectively inhibited proliferation of HCC cells *in vivo*. Moreover, CD31 expression significantly decreased in the DMF group (**Figure 6G**), which indicates that DMF inhibited angiogenesis of HCC *in vivo*.

Dimethyl fumarate shows good biocompatibility in vivo

To verify the toxicity of DMF on mice, body weight was measured. No significant difference in body weight was observed between the DMF-treated group and the DMSO-treated group (**Figure 7A**).

Dimethyl fumarate, as an oral drug, was reported to have corrosive effects on human intestine. In order to examine whether DMF has corrosive effects on the intestine, we obtained mouse cecum from both the groups. In comparison with the control group, no significant corrosion was observed from macroscopic observations and H&E staining in the DMF-treated group (**Figure 7B**).

Liver function, renal function as well as routine blood counts of mice were examined with standard biochemical markers that indicate hepatic and renal cytolysis. Activity of ALP and AST in the DMF group was slightly lower than that in the control group (**Figure 7C**). However, ALT activity in the DMF group was higher than that in the control group (**Figure 7C**). Additionally, CR, BUN, and UA levels were monitored to examine renal function. However, no significant changes in renal function were found between the DMF group and the control group (**Figure 7D**). Routine blood counts in the DMF group also showed negligible changes (**Figure 7E**). These results suggest that treatment with DMF leads to low hepatic and renal cytolysis.

Discussion

In the present study, we demonstrated that SOCS3 was upregulated after DMF treatment, and then the phosphorylation of JAK1 and STAT3 decreased with SOCS3 overexpression. Consequently, inhibition of the JAK1/STAT3 pathway efficiently downregulated proliferation-associated factor Ki67 in xenograft tumor treating with DMF. Moreover, cell cycle-associated factors CDK2, CDK2, and cyclin D1 were downregulated. Protein levels of autophagy markers LC3B, ATG5, and ATG7 also decreased. Angiogenesis was also suppressed as suggested by the inhibition of VEGFA and p-VEGFR2 expression. The mechanism underlying induction of the SOCS3/JAK1/STAT3 signaling path-

way by DMF in HCC cells is presented in **Figure 8**.

Hepatocellular carcinoma has a high mortality rate in developing countries, especially in China. Although many treatment options are currently available for HCC-such as surgery, local chemotherapy, and liver transplantation-the treatment is still limited and the prognosis of HCC remains poor [28]. Therefore, it is necessary to find a novel treatment strategy for HCC. Dimethyl fumarate is a methyl ester of fumaric acid, a commonly used industrial compound that is inexpensive and easy to obtain. Studies have reported that DMF has anti-tumor effects on certain cancers [10, 11]. Our results suggest that DMF reduces the growth of HCC cells both *in vivo* and *in vitro*, and shows a relatively moderate toxicity for mice. Dimethyl fumarate also plays a role in inhibiting tumor migration, which may be due to tumor cell apoptosis induced by DMF [10].

The anti-tumor mechanism of DMF treatment is still rare. We explored a deeper mechanism for HCC treatment with DMF. SOCS3 expression plays a role in the negative feedback loop to attenuate the JAK1/STAT3 signaling pathway, inhibiting JAK1 activity and phosphorylation of STAT3 downstream [19]. Excessive activation of STAT3 is ubiquitous in many malignancies. However, STAT3 signaling also regulates tumor cell proliferation, migration, invasion, autophagy, and tumor angiogenesis [12, 13]. Therefore, inhibition of STAT3 is a viable therapeutic target for the treatment of HCC.

Autophagy is a process that maintains cellular homeostasis in response to a range of extracellular stress stimuli. STAT3 signaling pathway is involved in various aspects of the autophagy process. Different subcellular localizations of STAT3 have recently been reported to regulate autophagy in various manners [18]. Our data showed that DMF significantly inhibited the autophagy pathway as indicated by the decreased levels of the autophagic markers LC3B, Atg5, and Atg7. Moreover, DMF also inhibits autophagy caused by the autophagy inducer rapamycin. Dimethyl fumarate can also inhibit the formation of autophagic flux. In conclusion, these data indicated that DMF suppressed autophagy via inhibition of STAT3 signaling.

Tumor angiogenesis plays a vital role in HCC tumor invasion, metastasis, and progression [29]. VEGFA is an endothelial cell-specific growth factor and a key regulator of angiogenesis in tumor progression. These functions are primarily based on the interactions of VEGF with three structurally related tyrosine kinase receptors: VEGFR-1, VEGFR-2, and VEGFR-3. Extensive studies have been conducted to inhibit VEGF/VEGFR2 signaling to prevent angiogenesis [30]. Markus's study provides evidence for suppression of VEGFR2 expression in human endothelial cells by DMF treatment [31]. This finding was also supported by our data. Accordingly, controlling tumor angiogenesis remains a crucial target for the successful treatment of HCC.

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Disclosure of conflict of interest

None.

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References

[1] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87-108.
[2] Keane FK, Hong TS and Zhu AX. Evolving systemic therapy in hepatocellular carcinoma: current management and opportunities for integration with radiotherapy. Semin Radiat Oncol 2018; 28: 332-341.
[3] Kudo M. Systemic therapy for hepatocellular carcinoma: 2017 update. Oncology 2017; 93 Suppl 1: 135-146.
[4] Sheremata W, Brown AD and Rammohan KW. Dimethyl fumarate for treating relapsing multiple sclerosis. Expert Opin Drug Saf 2015; 14: 161-170.

[5] Burness CB and Deeks ED. Dimethyl fumarate: a review of its use in patients with relapsing-remitting multiple sclerosis. CNS Drugs 2014; 28: 373-387.
[6] Hoefnagel J, Thio H, Willemze R and Bouwes Bavinck JN. Long-term safety aspects of systemic therapy with fumaric acid esters in severe psoriasis. Br J Dermatol 2003; 149: 363-9.
[7] Atwan A, Ingram JR, Abbott R, Kelson MJ, Pickles T, Bauer A and Piguet V. Oral fumaric acid esters for psoriasis: abridged Cochrane systematic review including GRADE assessments. Br J Dermatol 2016; 175: 873-881.
[8] Wierinckx A, Breve J, Mercier D, Schultzberg M, Drukarch B and Van Dam AM. Detoxication enzyme inducers modify cytokine production in rat mixed glial cells. J Neuroimmunol 2005; 166: 132-143.
[9] Wilms H, Sievers J, Rickert U, Rostami-Yazdi M, Mrowietz U and Lucius R. Dimethylfumarate inhibits microglial and astrocytic inflammation by suppressing the synthesis of nitric oxide, IL-1β, TNF-α and IL-6 in an in-vitro model of brain inflammation. J Neuroinflammation 2010; 7: 30.
[10] Loewe R, Valero T, Kremling S, Pratscher B, Kunstfeld R, Pehamberger H and Petzelbauer P. Dimethylfumarate impairs melanoma growth and metastasis. Cancer Res 2006; 66: 11888-11896.
[11] Xie X, Zhao Y, Ma CY, Xu XM, Zhang YQ, Wang CG, Jin J, Shen X, Gao JL, Li N, Sun ZJ and Dong DL. Dimethyl fumarate induces necroptosis in colon cancer cells through GSH depletion/ROS increase/MAPKs activation pathway. Br J Pharmacol 2015; 172: 3929-3943.
[12] Bromberg JF. Activation of STAT proteins and growth control. Bioessays 2001; 23: 161-169.
[13] Kisseleva T, Bhattacharya S, Braunstein J and Schindler C. Signaling through the JAK/STAT pathway, recent advances and future challenges. Gene 2002; 285: 1-24.
[14] Yu H and Jove R. The STATs of cancer--new molecular targets come of age. Nat Rev Cancer 2004; 4: 97-105.
[15] Yu H, Pardoll D and Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer 2009; 9: 798-809.
[16] You L, Wang Z, Li H, Shou J, Jing Z, Xie J, Sui X, Pan H and Han W. The role of STAT3 in autophagy. Autophagy 2015; 11: 729-739.
[17] Ji Y, Wang Z, Li Z, Li K, Le X and Zhang T. Angiotensin II induces angiogenic factors production partly via AT1/JAK2/STAT3/SOCS3 signaling pathway in MHCC97H cells. Cell Physiol Biochem 2012; 29: 863-74.
[18] Han YF, Zhao YB, Li J, Li L, Li YG, Li SP and Li ZD. Stat3-Atg5 signal axis inducing autophagy to alleviate hepatic ischemia-reperfusion injury. J Cell Biochem 2018; 119: 3440-3450.
[19] Yasukawa H, Sasaki A and Yoshimura A. Negative regulation of cytokine signaling pathways. Annu Rev Immunol 2000; 18: 143-64.
[20] Calvisi DF, Ladu S, Gorden A, Farina M, Conner EA, Lee JS, Factor VM and Thorgeirsson SS. Ubiquitous activation of Ras and Jak/Stat pathways in human HCC. Gastroenterology 2006; 130: 1117-1128.
[21] Giacomin A, Sergio A, Vanin V, Gazzola A, Cazzagon N and Farinati F. Molecular targeted therapy in hepatocellular carcinoma: present achievements and future challenges. Dig Dis 2012; 30: 284-288.
[22] Toyama T, Looney AP, Baker BM, Stawski L, Haines P, Simms R, Szymaniak AD, Varelas X and Trojanowska M. Therapeutic targeting of TAZ and YAP by dimethyl fumarate in systemic sclerosis fibrosis. J Invest Dermatol 2018; 138: 78-88.
[23] Huang DW, Sherman BT and Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009; 4: 44-57.
[24] Riehle KJ, Campbell JS, McMahan RS, Johnson MM, Beyer RP, Bammler TK and Fausto N. Regulation of liver regeneration and hepatocarcinogenesis by suppressor of cytokine signaling 3. J Exp Med 2008; 205: 91-103.
[25] Elliott J. SOCS3 in liver regeneration and hepatocarcinoma. Mol Interv 2008; 8: 19-21, 12.
[26] Croker BA, Kiu H and Nicholson SE. SOCS regulation of the JAK/STAT signalling pathway. Semin Cell Dev Biol 2008; 19: 414-422.
[27] Fujimoto M and Naka T. Regulation of cytokine signaling by SOCS family molecules. Trends Immunol 2003; 24: 659-66.
[28] Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015; 136: E359-386.
[29] Fernandez M, Semela D, Bruix J, Colle I, Pinzani M and Bosch J. Angiogenesis in liver disease. J Hepatol 2009; 50: 604-620.
[30] Ellis LM and Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. Nat Rev Cancer 2008; 8: 579-591.
[31] Meissner M, Doll M, Hrgovic I, Reichenbach G, König V, Hailemariam-Jahn T, Gille J and Kaufmann R. Suppression of VEGFR2 expression in human endothelial cells by dimethylfumarate treatment: evidence for anti-angiogenic action. J Invest Dermatol 2011; 131: 1356-64.

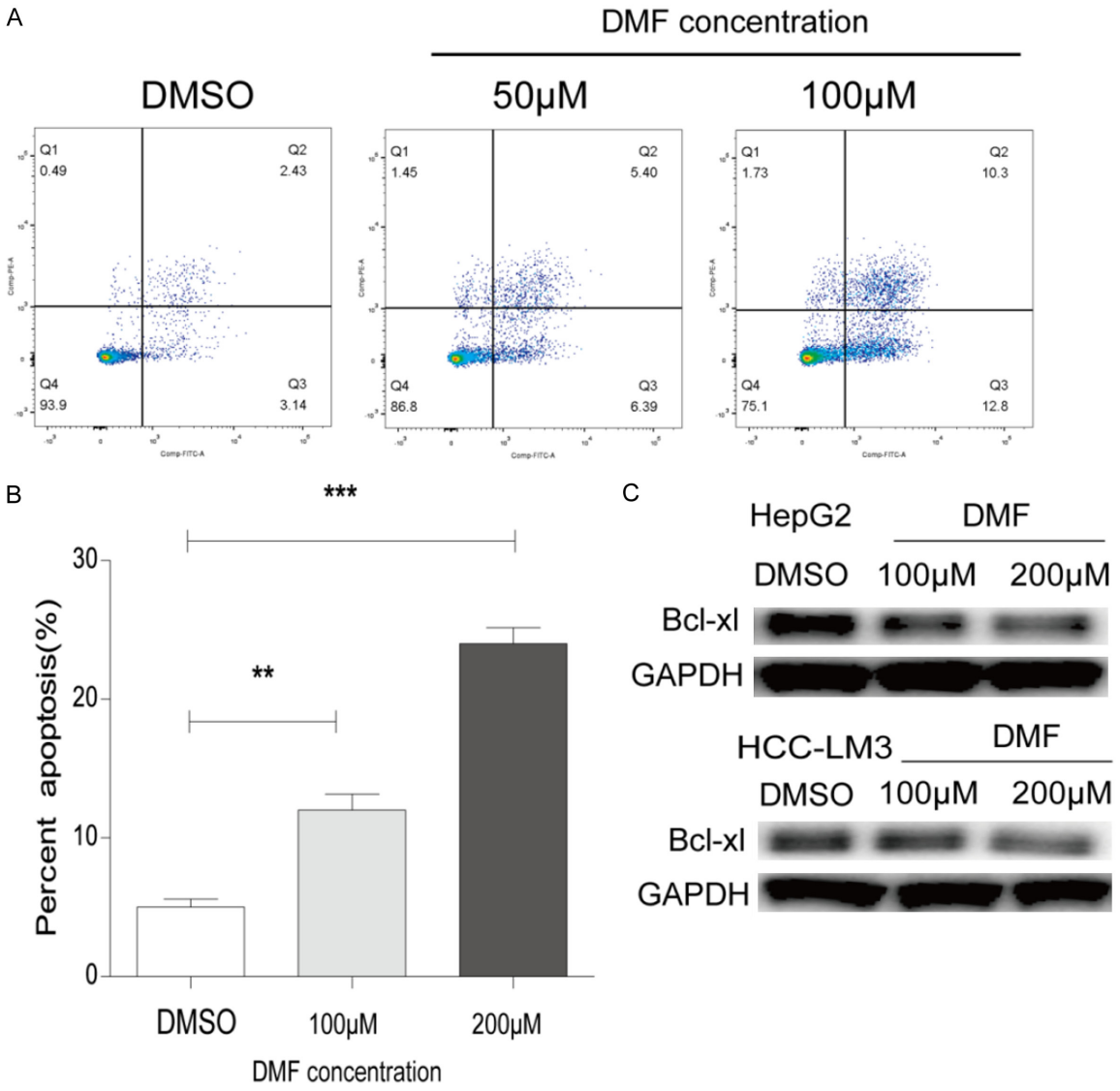

Figure S1. DMF promotes the apoptosis of HCC cells. A. The apoptosis level of Huh7 cells were increased with DMF treatment. B. Percentage of apopotic HCC cells, Data is expressed as mean ± SD, $^{**}P < 0.01$, $^{***}P < 0.001$. C. DMF decreased the expression of Bcl-xl in HCC-LM3 and HepG2 cells with treatment of DMF.