GDF11 inhibits the malignant progression of hepatocellular carcinoma via regulation of the mTORC1-autophagy axis

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Abstract. Hepatocellular carcinoma (HCC) is a common malignant tumor, which is associated with a poor prognosis and high mortality rate. It is well known that growth differentiation factor 11 (GDF11) acts as a tumor suppressor in various types of cancer, including HCC. The present study aimed to determine the tumor-suppressive properties of GDF11 in HCC and to assess the intrinsic mechanisms. In the present study, the human hepatoma cell line Huh-7 was transfected with the GDF11 overexpression plasmid (Oe-GDF11) for gain-of-function experiments to investigate the effects of GDF11 on the biological behaviors of HCC cells, including proliferation, colony formation, apoptosis, cell cycle arrest, migration, invasion, epithelial-mesenchymal transition (EMT) and angiogenesis. The proliferation, colony formation, apoptosis, cell cycle, migration, invasion and angiogenesis of HCC cells were assessed by CCK-8, EdU staining, colony formation, flow cytometry, wound healing, Transwell and tube formation assays, respectively. Apoptosis-, cell cycle-, EMT-related key factors were also determined by western blot assay. Furthermore, Oe-GDF11-transfected Huh-7 cells were treated with the mammalian target of rapamycin (mTOR) activator MHY1485 for rescue experiments to explore whether GDF11 could exert antitumor effects against HCC via mediating the mTOR complex 1 (mTORC1)-autophagy axis. In the present study, GDF11 was verified to be lowly expressed in HCC cells. Overexpression of GDF11 inhibited the proliferation, colony formation, migration, invasion, EMT and angiogenesis of HCC cells, and facilitated the apoptosis and cell cycle arrest of HCC cells. Additionally, it was verified that overexpression of GDF11 inactivated the mTORC1 signaling pathway to enhance autophagy in HCC cells. Treatment with the mTOR activator MHY1485 partially reversed the tumor-suppressive effects of GDF11 overexpression on HCC. In conclusion, GDF11 may exert tumor-suppressive properties in HCC cells through inactivating the mTORC1 signaling pathway to strengthen autophagy.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignant tumor and the third leading cause of cancer-related mortality worldwide (1,2). Due to lack of clear symptoms in the early stages of HCC, the majority of patients with HCC have advanced stage cancer upon diagnosis, with a 5-year survival rate of <20% (3-5). Even after curative resection, the prognosis of patients with HCC remains unsatisfactory owing to the biological characteristics of HCC cells, including metastasis and recurrence (6). Therefore, identifying the molecular basis underlying HCC and exploring potential molecular targets for HCC therapy are of great urgency.

Growth differentiation factor 11 (GDF11), also known as bone morphogenetic protein (BMP)11, is a member of the transforming growth factor-β superfamily and BMP subfamily (7). Evidence has indicated that GDF11 serves as a critical modulator in tumor progression. GDF11 acts as a tumor suppressor in triple-negative breast cancer by preserving epithelial cell-cell adhesion and inhibiting cell invasion (8). Furthermore, colorectal cancer-associated human intestinal lymphatic endothelial cells have been reported to promote tumor cell proliferation via the soluble matrisome component GDF11 (9). Exosome-transmitted microRNA-3124-5p promotes cholangiocarcinoma development by targeting GDF11 (10). GDF11 overexpression in pancreatic cancer cells suppresses the proliferation, migration and invasion abilities *in vitro* (11). In addition, it has been reported that GDF11 suppresses adipogenesis and improves the metabolic functioning of mature adipocytes via the WNT/β-catenin and ALK5/SMAD2/3 pathways (12). GDF11 is also involved in metabolic reprogramming and lipid metabolism dysregulation in HCC cells through ALK5-dependent signaling (13). Notably, GDF11 has been verified to be lowly expressed in liver cancer

tissues and cell lines compared with that in normal liver tissues and cells (14), and GDF11 exerts tumor-suppressive properties in HCC cells by restricting proliferation, clonogenicity, spheroid formation and cellular function (15). However, the intrinsic mechanisms underlying the antitumor effects of GDF11 in HCC have not yet been fully elucidated.

GDF11 has been suggested to serve as a mammalian target of rapamycin (mTOR) inhibitory factor in HCC cells (16). mTOR, a serine/threonine protein kinase, exists as two structurally and functionally different complexes, known as mTOR complex (mTORC)1 and mTORC2 (17,18). mTORC1 can modulate cell proliferation and metabolism and can suppress autophagy (19). Autophagy is critical for maintaining cellular homeostasis and survival. By contrast, inhibition of mTORC1 activates autophagy, which is required for the clearance of dysfunctional cellular components (20).

In the present study, the biological role of GDF11 in the malignant behavior of HCC cells was assessed. Moreover, whether GDF11 could exert antitumor effects against HCC via mediating the mTORC1-autophagy axis was discussed.

Materials and methods

Cell culture. The human normal hepatocyte cell line HHL-5 and the human hepatoma cell line Huh-7 were purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. The human hepatoma cell lines SNU-449 and Hep3B, and the immortalized hybrid human umbilical vein endothelial cell (HUVEC)/EAhy926 cell line were purchased from the American Type Culture Collection. All cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a 5% CO_2 incubator.

Cell transfection. The GDF11 overexpression plasmid (Oe-GDF11), which was established by inserting the GDF11 gene into the pcDNA3.1 vector, and the empty vector [Oe-negative control (NC)] were designed by Shanghai GenePharma Co., Ltd. Cell transfection with either Oe-GDF11 or Oe-NC was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, Oe-GDF11 or Oe-NC (4 μg) and Lipofectamine 2000 (10 μl) were added to Opti-MEM (250 μl; Gibco; Thermo Fisher Scientific, Inc.) and incubated for 10 min at room temperature. Subsequently, diluted vectors were mixed with diluted Lipofectamine 2000 and then incubated for 15 min at room temperature. Huh-7 cells were re-plated in serum-free DMEM, and the transfection mixtures were separately added to the cells when the cell confluence reached 85% for 4 h of incubation at 37˚C. After another 48 h of incubation in complete DMEM at 37˚C, the cells were collected for subsequent experiments.

Cell treatment. Huh-7 hepatoma cells were divided into the control group (cells cultured under normal conditions), the Oe-NC group (cells transfected with Oe-NC), the Oe-GDF11 group (cells transfected with Oe-GDF11) and the Oe-GDF11 + MHY1485 group [cells transfected with Oe-GDF11 and treated with 10 μM mTOR activator MHY1485 (MedChemExpress) for 4 h at 37˚C].

Western blotting. Total protein from HHL-5, SNU-449, Hep3B and Huh-7 cells was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology) containing protease inhibitors, and the protein concentration was quantified using the BCA method. Protein samples (30 μg/lane) were separated by 5-10% SDS-PAGE and transferred to PVDF membranes. After blocking in 5% BSA (Thermo Fisher Scientific, Inc.) for 2 h at 37˚C, the membranes were incubated on a shaker with primary antibodies against GDF11 (1:5,000; cat. no. ab234647), phosphorylated (p)-mTOR (1:1,000; cat. no. ab109268), mTOR (1:10,000; cat. no. ab134903), p-p70 S6K T389 (1:1,000; cat. no. ab2571), p70 S6K (1:1,000; cat. no. ab308113), p-S6 (1:5,000; cat. no. ab215214), S6 (1:1,000; cat. no. ab127980), LC3-II/I (1:2,000; cat. no. ab192890), Beclin-1 (1:2,000; cat. no. ab207612), p62 (1:10,000; cat. no. ab109012), Bcl-2 (1:2,000; cat. no. ab182858), Bax (1:10,000; cat. no. ab32503), cleaved caspase-3 (1:500; cat. no. ab32042), caspase-3 (1:5,000; cat. no. ab32351), CDK4 (1:10,000; cat. no. ab108357), CDK6 (1:50,000; cat. no. ab124821), cyclin D1 (1:200 dilution; cat. no. ab16663), E-cadherin (1:25; cat. no. ab227639), N-cadherin (1:5,000; cat. no. ab76011), Snail (1:1,000; cat. no. ab216347), Vimentin (1:5,000; cat. no. ab92547), GAPDH (1:2,500; cat. no. ab9485) and β-actin (1:5,000; cat. no. ab8227) (all from Abcam) overnight at 4˚C. Subsequently, the membranes were incubated with HRP-conjugated secondary antibodies (1:20,000; cat. no. ab6721; Abcam) for 1 h at room temperature. GAPDH served as the internal controls. Protein bands were visualized using an ECL detection kit (Beyotime Institute of Biotechnology) and were semi-quantified using ImageJ (version 1.42; National Institutes of Health).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from HHL-5, SNU-449, Hep3B and Huh-7 cells was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and was then reverse-transcribed into cDNA using the Prime Script RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol. qPCR was carried out using the SYBR Green PCR Kit (Takara Bio, Inc.) on an ABI Detection System (PerkinElmer, Inc.). The qPCR thermocycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 64˚C for 30 sec. GAPDH served as the internal control. The following primers were employed: GDF11, 5'-CCACCACCGAGACCGTCATT-3' (forward) and 5'-GAGGGCTGCCATCTGTCTGT-3' (reverse); GAPDH, 5'-GCACCGTCAAGGCTGAGAAC-3' (forward) and 5'-ATGGTGGTGAAGACGCCAGT-3' (reverse). The mRNA expression levels of GDF11 were calculated using the $2^{-\Delta\Delta Cq}$ method (21).

Immunofluorescence (IF) staining. After fixation in 4% paraformaldehyde for 30 min at room temperature, Huh-7 cells were permeabilized with 0.1% Triton X-100 for 15 min and then blocked with 5% BSA (Beyotime Institute of Biotechnology) for 2 h at room temperature. Subsequently, the cells were incubated with an anti-LC3 primary antibody (1:250; cat. no. ab225382, Abcam) overnight at 4˚C and were then incubated with a FITC-conjugated secondary antibody (1:1,000; cat. no. ab150077, Abcam) for 1 h at room temperature. DAPI was used to counterstain the nuclei for 5 min in the

dark at room temperature. Fluorescence images were captured under a fluorescence microscope (magnification, x200).

Cell viability assay. Cell viability was investigated using a Cell Counting Kit-8 (CCK-8) assay (Beyotime Institute of Biotechnology). Huh-7 cells (5,000 cells/well) grown in a 96-well plate were cultured for 24, 48 and 72 h. Subsequently, 10 μl CCK-8 reagent was added to each well for an additional 2 h of incubation. The optical density value was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

EdU proliferation assay. Cell proliferative ability was investigated using a commercial EdU cell proliferation kit (Beyotime Institute of Biotechnology). Huh-7 cells were incubated with EdU reaction cocktail for 2 h at 37˚C to complete EdU labeling. The cells were then fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.5% Triton X-100 for 20 min at room temperature. DAPI was applied to counterstain the nuclei for 5 min in the dark at room temperature. Fluorescence images were captured under a fluorescence microscope (magnification, x100).

Colony formation assay. Cell colony-forming ability was investigated using a colony formation assay. Huh-7 cells (600 cells/well) grown in a 6-well plate were continuously cultured in 5% CO_2 at 37˚C for 14 days. Cell colonies were fixed in 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 15 min at room temperature. Images of visible colonies (≥50 cells) were captured under a light microscope and colonies were counted using ImageJ (version 1.42).

Flow cytometry. For cell apoptosis analysis, Huh-7 cells were trypsinized, collected by centrifugation at 1,000 x g for 5 min at room temperature, resuspended in 300 μl binding buffer at a concentration of $1x10^6$ cells/ml, and doubly stained with 5 μl Annexin V-FITC and PI (Beyotime Institute of Biotechnology) in the dark for 15 min at room temperature. The cells were then subjected to BD FACSCanto™ flow cytometry (FACSCalibur; BD Biosciences) and analyzed using FlowJo™ software (version 10.8.1; FlowJo LLC) to assess cell apoptosis. For cell cycle analysis, Huh-7 cells ($5x10^5$) were trypsinized, collected by centrifugation at 1,000 x g for 5 min at room temperature, resuspended in PBS and then fixed in 70% ice-cold ethanol at 4˚C for 4 h. Thereafter, the cells were stained with 1 ml PI/RNase dye (50 μg/ml) for 30 min at room temperature in the dark and were subjected to BD FACSCanto™ flow cytometry (FACSCalibur; BD Biosciences) and analyzed using FlowJo™ software (version 10.8.1; FlowJo LLC) to assess cell cycle distribution.

Wound healing assay. Cell migration was investigated using a wound healing assay. Huh-7 cells were grown in a 6-well plate until 95% confluence. The cell monolayer was scraped with a 200-μl sterile pipette tip to create a wound, followed by a 24-h incubation in serum-free DMEM at 37˚C. Images of the wounds were captured at 0 and 24 h under a light microscope (magnification, x100).

Transwell assay. Cell invasion ability was investigated using a Transwell invasion assay. Huh-7 cells suspended in fresh serum-free DMEM at a density of $2x10^4$ cells were seeded into the upper chamber of Transwell plates (8-μm pore size; Costar; Corning, Inc.) precoated with Matrigel at 37˚C for 30 min. DMEM supplemented with 10% FBS was added to the lower chamber to serve as a chemoattractant. After a 24-h incubation at 37˚C, non-migrated cells in the upper chamber were removed with cotton swabs and cells that had invaded into the lower chamber were fixed with 4% paraformaldehyde for 30 min at room temperature, stained with 0.5% crystal violet for 10 min at room temperature and captured under a light microscope (magnification, x200).

Tube formation. The conditioned media (CM) of normal Huh-7 cells, Huh-7 cells transfected with Oe-NC, Huh-7 cells transfected with Oe-GDF11, and Huh-7 cells transfected with Oe-GDF11 and treated with MHY1485 were collected after ~24 h incubation at 37˚C. HUVECs ($2x10^4$ cells/well) seeded in 96-well plates precoated with Matrigel were cultured in the collected CM at 37˚C for 24 h. Images of tube formation were captured under a light microscope (magnification, x100).

Statistical analysis. Data analysis was performed using GraphPad Prism (version 9.1; GraphPad Software; Dotmatics). Experimental data from three independent repeats are presented as the mean ± standard deviation. Comparisons among multiple groups were performed using one-way analysis of variance followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

GDF11 strengthens autophagy in HCC cells by suppressing the mTORC1 signaling pathway. Differences in the expression levels of GDF11 in HHL-5 human normal hepatocytes and the human hepatoma cell lines SNU-449, Hep3B and Huh-7 were investigated by RT-qPCR and western blotting. In comparison with HHL-5 cells, GDF11 mRNA and protein expression levels were markedly downregulated in HCC cells, particularly in Huh-7 cells (Fig. 1A). Thus, Huh-7 cells were selected for subsequent research. Huh-7 cells were transfected with Oe-GDF11 or Oe-NC for functional experiments and transfection with Oe-GDF11 significantly upregulated GDF11 expression compared with the Oe-NC group (Fig. 1B). In addition, IF staining revealed that GDF11 overexpression increased LC3 accumulation compared with that in the Oe-NC group (Fig. 1C). Furthermore, the expression levels of genes in the mTORC1-autophagy axis were analyzed. GDF11 overexpression decreased the expression levels of p-mTOR, p-p70 S6K T389, p-S6 and p62, and increased the expression levels of LC3-II/LC3-I and Beclin-1 compared with those in the Oe-NC group (Fig. 1D). These findings indicated that GDF11 overexpression suppressed the mTORC1 signaling pathway and enhanced autophagy in Huh-7 cells. Moreover, the enhancement of LC3 accumulation caused by GDF11 overexpression was partially reversed by treatment with mTOR activator MHY1485 (Fig. 2A). Decreased p-mTOR, p-p70 S6K T389, p-S6 and p62 protein

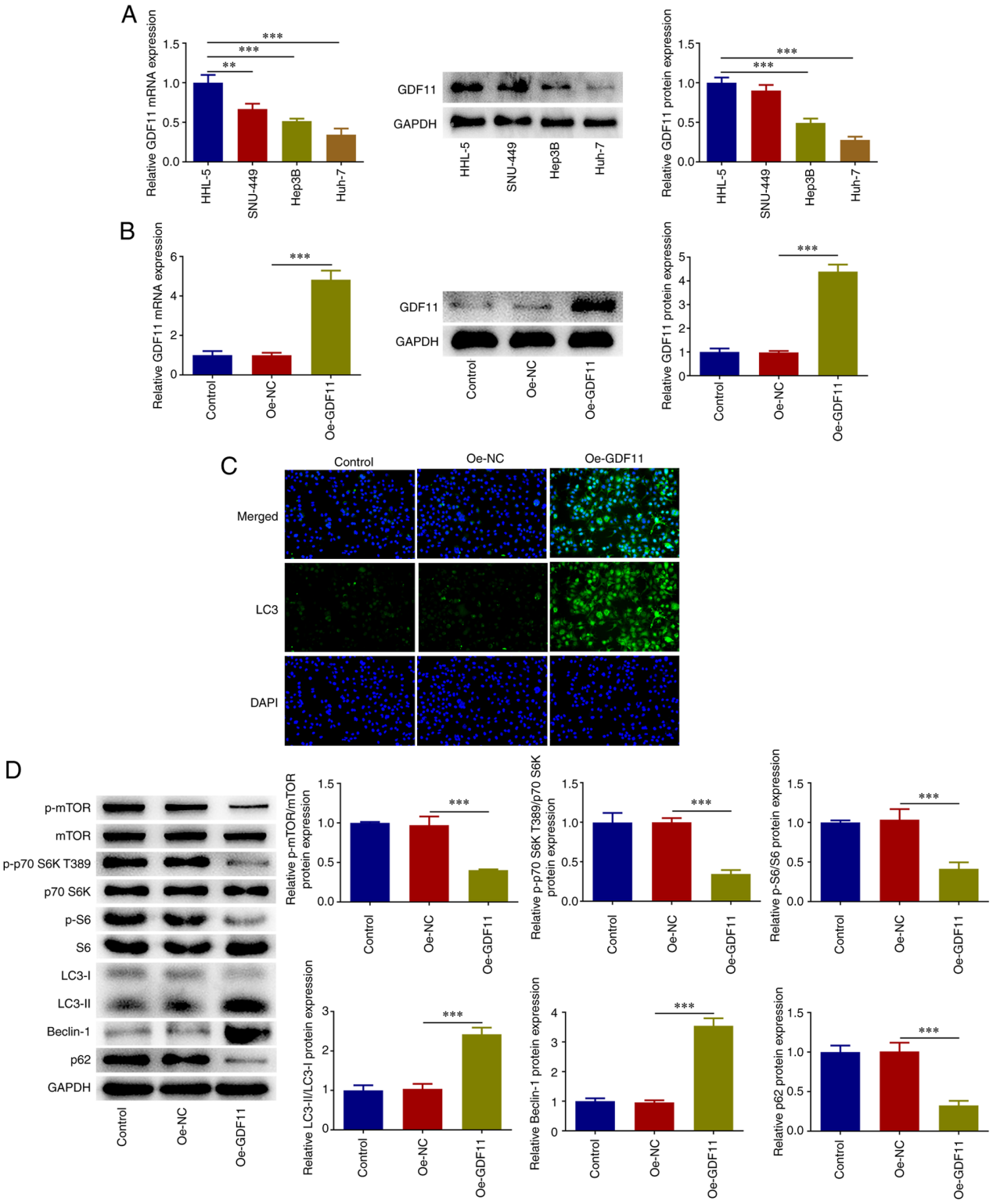


Figure 1. GDF11 suppresses the mTORC1 signaling pathway and strengthens autophagy in hepatocellular carcinoma cells. (A) Differences in GDF11 expression in HHL-5 human normal hepatocytes and the human hepatoma cell lines SNU-449, Hep3B and Huh-7 were detected by RT-qPCR and western blotting. (B) Huh-7 cells were transfected with either Oe-GDF11 or Oe-NC, and transfection efficiency was validated by RT-qPCR and western blotting. (C) Huh-7 cells were transfected with either Oe-GDF11 or Oe-NC. LC3 expression was determined by immunofluorescence staining. (D) Huh-7 cells were transfected with either Oe-GDF11 or Oe-NC. p-mTOR, mTOR, p-p70 S6K T389, p70 S6K, p-S6, S6, LC3-I, LC3-II, Beclin-1 and p62 protein expression levels were detected by western blotting. **P<0.01, ***P<0.001. GDF11, growth differentiation factor 11; mTORC1, mammalian target of rapamycin complex 1; RT-qPCR, reverse transcription-quantitative PCR; Oe, overexpression plasmid; NC, negative control; p, phosphorylated.

expression levels, as well as increased LC3-II/LC3-I and Beclin-1 protein expression levels induced by GDF11 overexpression were partially reversed by MHY1485 treatment (Fig. 2B). These results indicated that GDF11 could strengthen autophagy in HCC cells by suppressing the mTORC1 signaling pathway.

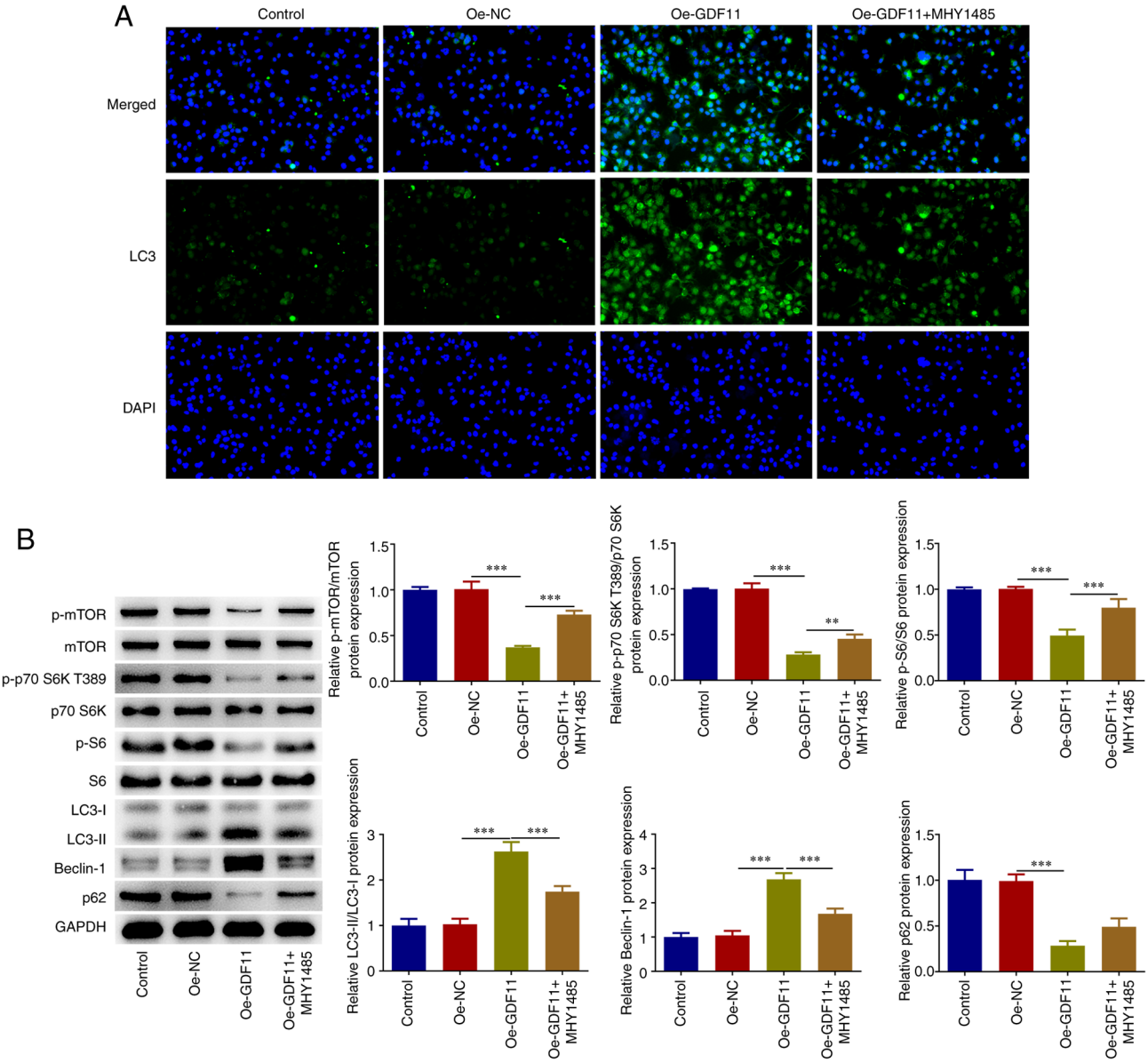


Figure 2. GDF11 strengthens autophagy in hepatocellular carcinoma cells by suppressing the mTORC1 signaling pathway. Huh-7 cells were transfected with either Oe-GDF11 or Oe-NC. Oe-GDF11-transfected Huh-7 cells were treated with the mTOR activator MHY1485. (A) LC3 expression was determined by immunofluorescence staining. (B) p-mTOR, mTOR, p-p70 S6K T389, p70 S6K, p-S6, S6, LC3-I, LC3-II, Beclin-1 and p62 protein expression levels were detected by western blotting. **P<0.01, ***P<0.001. GDF11, growth differentiation factor 11; mTORC1, mammalian target of rapamycin complex 1; Oe, overexpression plasmid; NC, negative control; p, phosphorylated.

GDF11 inhibits the proliferation and colony-forming ability of HCC cells by suppressing the mTORC1 signaling pathway. The results of the CCK-8 assay indicated that GDF11 overexpression suppressed proliferation of Huh-7 cells compared with that in the Oe-NC group, whereas MHY1485 treatment partially restored the impaired cell proliferative capacity (Fig. 3A). As determined by EdU staining, GDF11 overexpression suppressed cell proliferation compared with in the Oe-NC group, as indicated by the reduced number of EdU^+ Huh-7 cells, and this was partially reversed by MHY1485 treatment (Fig. 3B). Furthermore, the colony formation assay revealed that GDF11 overexpression suppressed the colony formation of Huh-7 cells compared with that in the Oe-NC group, which was partially reversed by MHY1485 treatment (Fig. 3C). These findings suggested that GDF11 could inhibit the proliferation and colony-forming ability of HCC cells by suppressing the mTORC1 signaling pathway.

GDF11 facilitates HCC cell apoptosis by suppressing the mTORC1 signaling pathway. Flow cytometry was used for the cell apoptosis analysis. Apoptotic rate was calculated as the sum of the early apoptosis rate and the late apoptosis rate. It was revealed that GDF11 overexpression elevated the apoptotic rate of Huh-7 cells compared with that in the Oe-NC group, which was partially reversed by MHY1485 treatment (Fig. 4A). Additionally, GDF11 overexpression reduced Bcl-2 protein expression levels, and elevated Bax and cleaved caspase-3 protein expression levels compared with the Oe-NC group. By contrast, MHY1485 treatment partially reversed the regulatory effects of GDF11 overexpression on apoptosis-associated

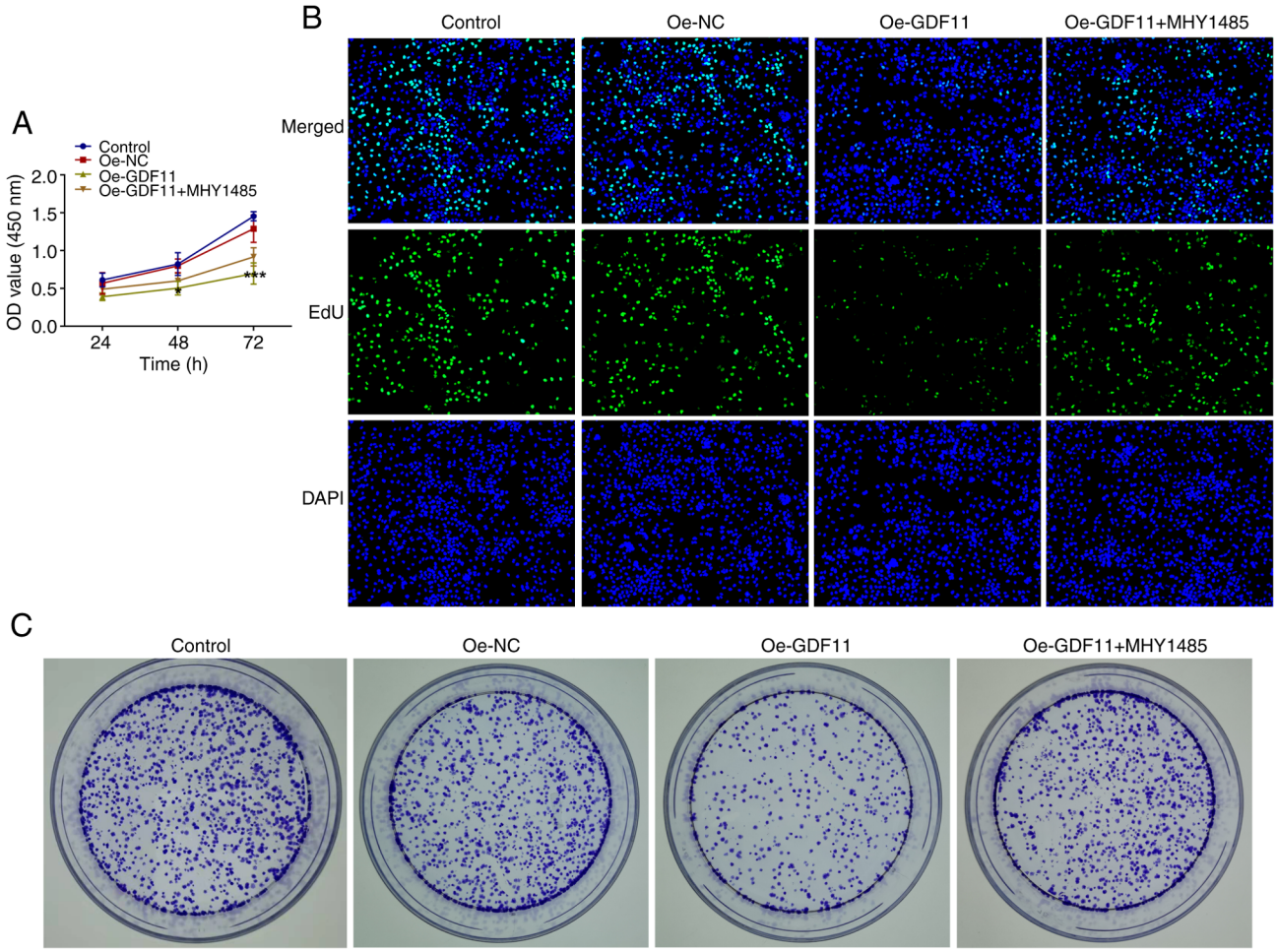


Figure 3. GDF11 inhibits the proliferation and colony-forming ability of hepatocellular carcinoma cells by suppressing the mTORC1 signaling pathway. Huh-7 cells were transfected with either Oe-GDF11 or Oe-NC. Oe-GDF11-transfected Huh-7 cells were treated with the mTOR activator MHY1485. (A) Cell viability was investigated using a Cell Counting Kit-8 assay. (B) Cell proliferative ability was investigated using EdU staining. (C) Cell colony-forming ability was investigated using a colony formation assay. *P<0.05, ***P<0.001, vs. Oe-NC group. GDF11, growth differentiation factor 11; mTORC1, mammalian target of rapamycin complex 1; Oe, overexpression plasmid; NC, negative control.

proteins (Fig. 4B). These results suggested that GDF11 may facilitate HCC cell apoptosis by suppressing the mTORC1 signaling pathway.

GDF11 induces HCC cell cycle arrest by suppressing the mTORC1 signaling pathway. Flow cytometry was used for cell cycle distribution analysis and it was revealed that GDF11 overexpression elevated the proportion of cells at the G_1 stage and reduced the proportion of cells at the S stage compared with the Oe-NC group. Furthermore, the results showed a decrease in the proportion of cells at the G_1 stage and an increase in the proportion of cells at the S stage after MHY1485 treatment in comparison with the Ov-GDF11 group (Fig. 5A). GDF11 induced Huh-7 cell cycle arrest, which was partially reversed by MHY1485 treatment. In addition, GDF11 overexpression reduced CDK4, CDK6 and cyclin D1 protein expression levels compared with those in the Oe-NC group, whereas MHY1485 treatment partially reversed the regulatory effects of GDF11 overexpression on cell cycle-associated proteins (Fig. 5B). Thus, GDF11 may induce HCC cell cycle arrest by suppressing mTORC1 signaling pathway.

GDF11 inhibits HCC migration, invasion, EMT and angiogenesis by suppressing mTORC1 signaling pathway. As determined by wound healing and Transwell assays, GDF11 overexpression suppressed the migration and invasion of Huh-7 cells compared with that in the Oe-NC group, which was partially reversed by MHY1485 treatment (Fig. 6A and B). Tumors derive their metastatic capacity through epithelial-mesenchymal transition (EMT) (22). EMT-associated biomarkers were thus detected. GDF11 overexpression elevated E-cadherin protein expression levels, and reduced N-cadherin, Snail and Vimentin protein expression levels compared with those in the Oe-NC group, whereas MHY1485 treatment partially reversed the regulatory effects of GDF11 overexpression on EMT-associated proteins (Fig. 6C). These findings indicated that GDF11 suppressed EMT in Huh-7 cells, which was partially reversed by MHY1485 treatment. Angiogenesis is responsible for nutritional provision of tumor metastasis (23). The results of the tube formation assay revealed that GDF11 overexpression suppressed the *in vitro* angiogenesis of HUVECs compared with the Oe-NC group, whereas MHY1485 treatment partially reversed the suppressive effect of GDF11 overexpression on

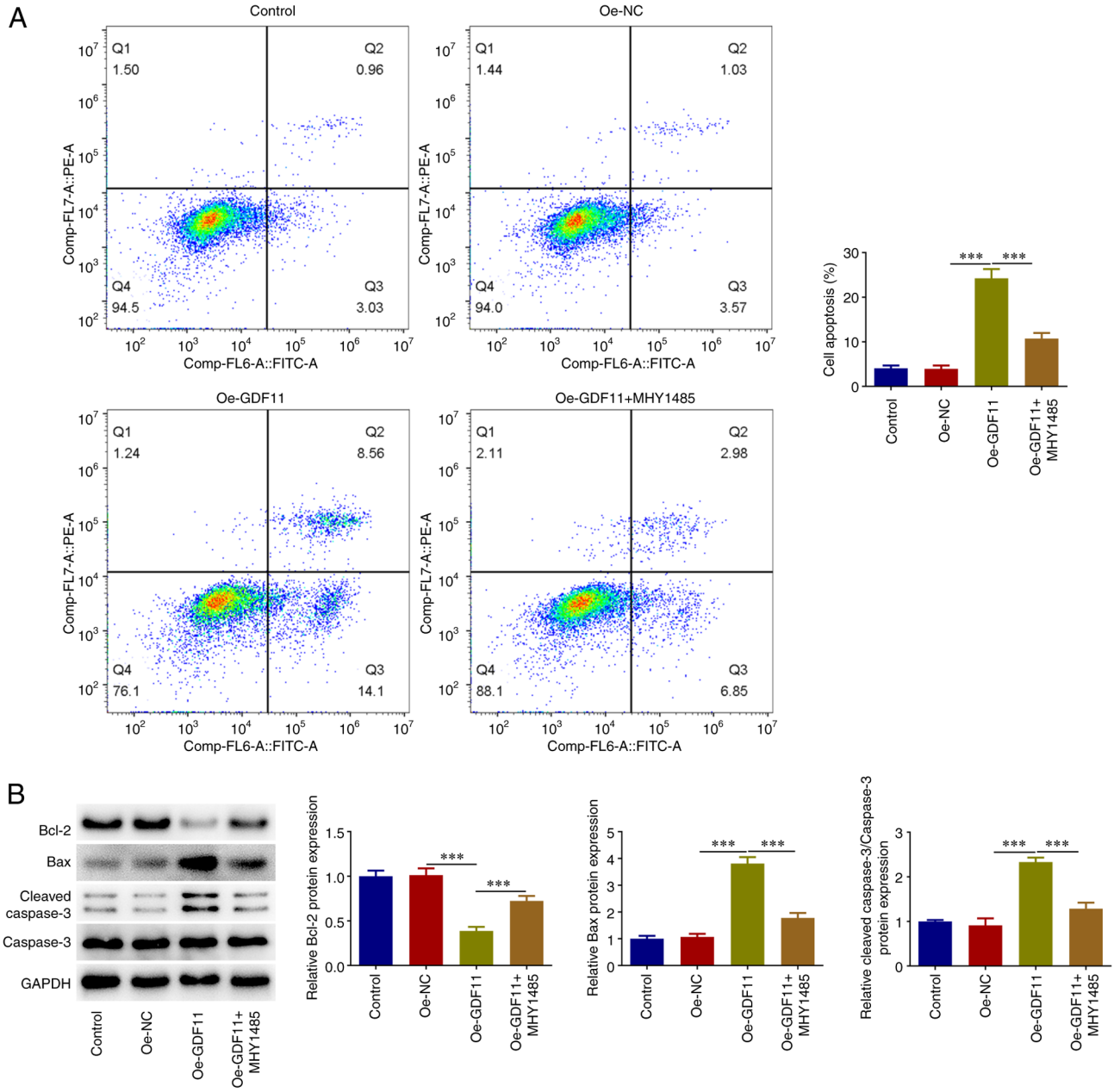


Figure 4. GDF11 facilitates hepatocellular carcinoma cell apoptosis by suppressing the mTORC1 signaling pathway. Huh-7 cells were transfected with either Oe-GDF11 or Oe-NC. Oe-GDF11-transfected Huh-7 cells were treated with the mTOR activator MHY1485. (A) Cell apoptosis was investigated using flow cytometry. (B) Bcl-2, Bax, cleaved caspase-3 and caspase-3 protein expression levels were detected by western blotting. ***P<0.001. GDF11, growth differentiation factor 11; mTORC1, mammalian target of rapamycin complex 1; Oe, overexpression plasmid; NC, negative control.

angiogenic ability (Fig. 6D). Thus, GDF11 may inhibit HCC metastasis by suppressing the mTORC1 signaling pathway.

Discussion

As a major contributor to global cancer-related mortality, HCC poses a challenging threat to public health (24). The malignant proliferation, migration and invasion of hepatoma cells triggers the metastasis and recurrence of HCC, resulting in an unfavorable prognosis of HCC (25,26). Therefore, the present study investigated the functional role of GDF11 in HCC proliferation, colony-forming ability, apoptosis, cell cycle progression, migration, invasion, EMT and angiogenesis, aiming to provide novel perspectives on the biological mechanisms of HCC and to identify promising targets for HCC therapy.

In the present study, GDF11 was verified to be lowly expressed in HCC cells. Overexpression of GDF11 inhibited the proliferation, colony-forming ability, migration, invasion, EMT and angiogenesis of HCC cells, and facilitated the apoptosis and cell cycle arrest of HCC cells.

Dysregulated autophagy has been implicated in various types of cancer, including HCC (27,28). Luteolin can induce autophagy by increasing the number of autophagosomes and enhancing Beclin-1 expression, thereby promoting HCC cell apoptosis (29). Knockdown of the oncogene UBE2I can inhibit cellular proliferation, migration and invasion via the autophagy-related pathway in HCC (30).

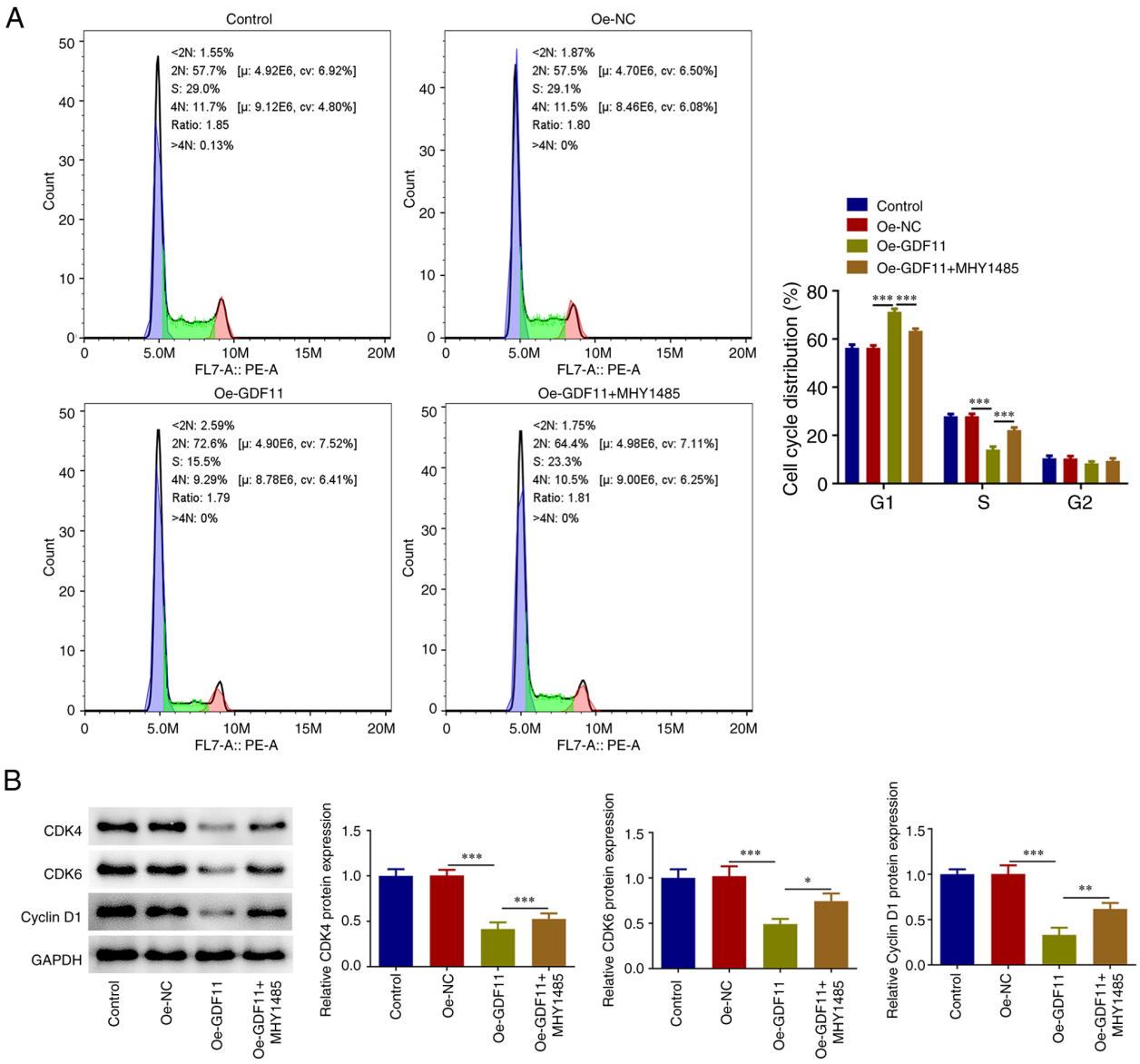


Figure 5. GDF11 induces hepatocellular carcinoma cell cycle arrest by suppressing the mTORC1 signaling pathway. Huh-7 cells were transfected with either Oe-GDF11 or Oe-NC. Oe-GDF11-transfected Huh-7 cells were treated with the mTOR activator MHY1485. (A) Cell cycle distributions were investigated using flow cytometry. (B) CDK4, CDK6 and cyclin D1 protein expression levels were detected by western blotting. *P<0.05, **P<0.01, ***P<0.001. GDF11, growth differentiation factor 11; mTORC1, mammalian target of rapamycin complex 1; Oe, overexpression plasmid; NC, negative control.

The E2F1/USP11 positive feedback loop can facilitate HCC cell proliferation and metastasis, and can promote tumor growth *in vivo*, by activating the ERK/mTOR pathway to inhibit autophagy (31). The novel mTOR inhibitor Torin-2 can induce autophagy by inactivating mTORC1 to suppress HCC cell proliferation and promote HCC cell apoptosis (32). In addition, DHX15 can inhibit autophagy through the mTORC1 pathway, thereby promoting HCC cell proliferation (33). In the present study, it was verified that overexpression of GDF11 inactivated the mTORC1 signaling pathway to enhance autophagy in HCC cells. MHY1485 is a potent cell-permeable mTOR activator, which can induce the activation of mTOR via two possible mechanisms: i) MHY1485 may bind a different site from an ATP-binding site of mTOR; and ii) MHY1485 could indirectly activate mTOR through elevation of p-mTOR at ser2448 (34). Treatment with the mTOR activator MHY1485 partially reversed the tumor-suppressive effects of GDF11 overexpression on HCC in the current study.

Literature reports that LAIR-1 can inhibit HCC cell proliferation and invasion via suppressing the PI3K-AKT-mTOR pathway (35). Furthermore, it has been verified that GDF11 can regulate the PI3K-AKT pathway in HCC cells (13). The results of the current study indicated that GDF11 could inhibit proliferation and colony formation, facilitate apoptosis, induce cell cycle arrest, and restrain migration and invasion of HCC cells by suppressing the mTORC1 signaling pathway. Therefore, AKT could be involved in the mTOR-dependent mechanism of GDF11 action in HCC. The aforementioned prospective molecular mechanisms require further investigation in future studies.

In conclusion, GDF11 may exert tumor-suppressive effects on HCC cells through inactivating the mTORC1 signaling pathway to strengthen autophagy. These findings are beneficial

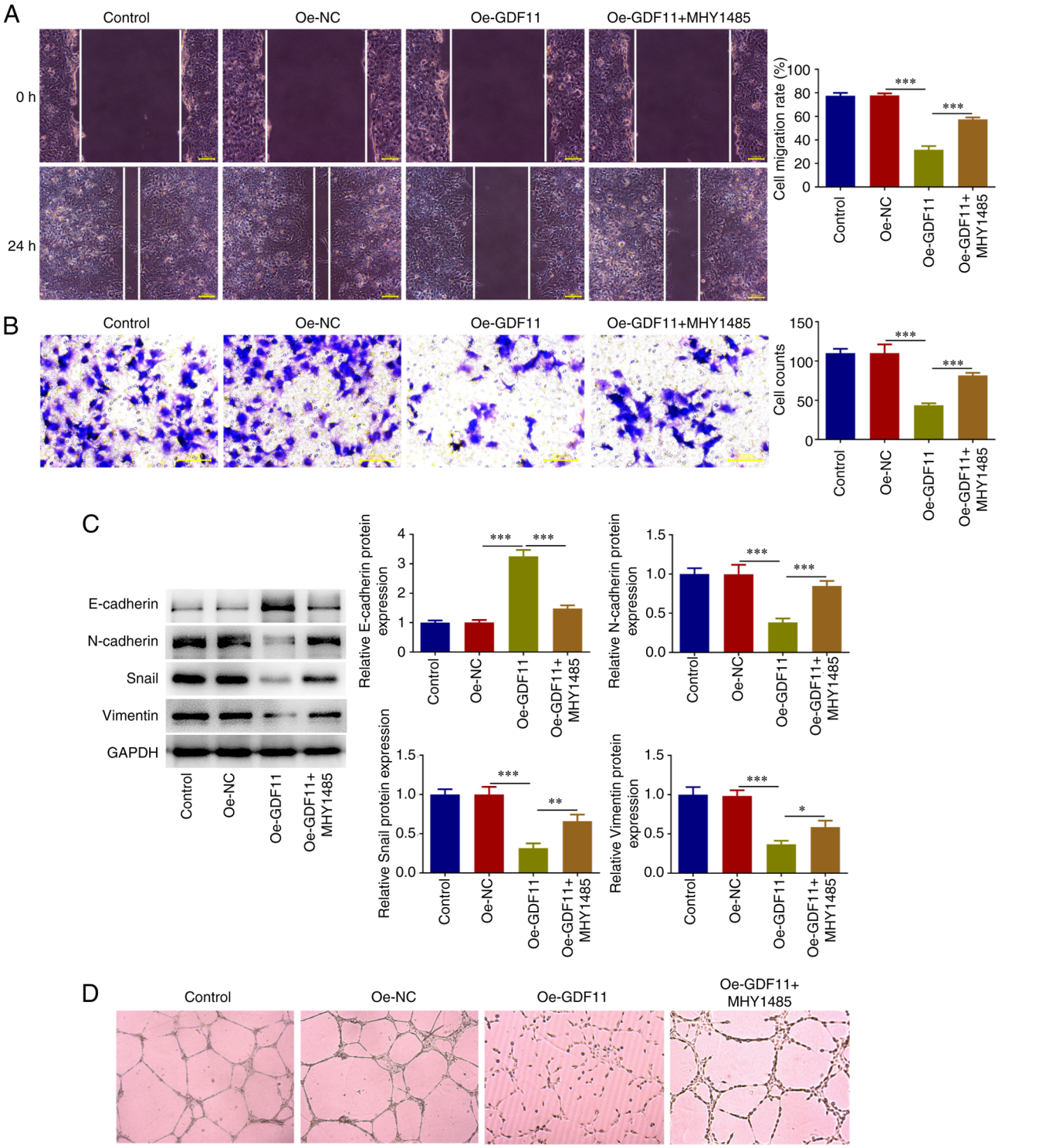


Figure 6. GDF11 inhibits hepatocellular carcinoma migration, invasion, EMT and angiogenesis by suppressing the mTORC1 signaling pathway. Huh-7 cells were transfected with either Oe-GDF11 or Oe-NC. Oe-GDF11-transfected Huh-7 cells were treated with the mTOR activator MHY1485. (A) Cell migration was investigated using a wound healing assay. (B) Cell invasion was investigated using a Transwell invasion assay. (C) E-cadherin, N-cadherin, Snail and Vimentin protein expression levels were detected by western blotting. (D) HUVECs were incubated with the conditioned media of Huh-7 cells at 37˚C for 24 h. *In vitro* angiogenesis of HUVECs was investigated using a tube formation assay. *P<0.05, **P<0.01, ***P<0.001. GDF11, growth differentiation factor 11; mTORC1, mammalian target of rapamycin complex 1; Oe, overexpression plasmid; NC, negative control; HUVECs, human umbilical vein endothelial cells.

to the development of a promising approach for HCC therapy. Modulation of GDF11 serves as an attractive marker for HCC prediction, prevention and novel therapy. Notably, it has been verified that spermidine can inhibit high glucose-induced endoplasmic reticulum stress in HT22 cells via the upregulation of GDF11, and can prevent liver fibrosis and HCC by activating MAP1S-mediated autophagy (36,37). These previous findings indicated that spermidine may be developed as a suitable drug candidate for the induction of GDF11 in HCC treatment. In addition, the exploration of more specific GDF11 agonists may

be used to upregulate GDF11 in HCC therapy. Furthermore, *in vivo* animal experiments should be conducted in the future to further support the obtained conclusions and to assess the predictive values of GDF11 and the mTORC1-autophagy axis.

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Availability of data and materials

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

Authors' contributions

QW contributed to study conception, designed the research study, performed the experiments, collected the data, performed data analysis, and wrote and critically revised the manuscript. CF designed the research study, performed the experiments, collected the data, performed data analysis and wrote the manuscript. KL performed the experiments, collected data and performed data analysis. JT contributed to study conception, designed the research study, and wrote and critically revised the manuscript. All authors read and approved the final manuscript, and confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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