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Original Article

Original Article

Mitochondrial fission factor promotes cisplatin resistance in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the most common primary liver tumor and one of the leading causes of cancer-related death worldwide. Chemotherapeutic agents/regimens such as cisplatin (DDP) are frequently used for advanced HCC treatment. However, drug resistance remains a major hindrance and the underline mechanisms are not fully understood. In this study, we investigated the expression pattern and function of mitochondrial fission factor (Mff) in cisplatin-resistant HCC. We found that Mff is highly expressed in cisplatin-resistant HCC tissues and cell lines. Knockdown of Mff suppresses cell proliferation and promotes cell apoptosis of HCC/DDP cells. In addition, knockdown of Mff sensitizes Huh-7/DDP cells to cisplatin treatment, inhibits cell proliferation, migration and invasion, and enhances cell apoptosis. Confocal imaging showed that knockdown of Mff inhibits the mitochondrial fission and downregulates the expression of GTPase dynamin-related protein 1 (Drp1) in cisplatin-resistant Huh-7/DDP cells. Moreover, xenograft tumor model revealed that knockdown of Mff sensitizes Huh-7/DDP xenograft tumor to cisplatin treatment *in vivo*. In summary, our findings suggest that Mff regulates mitochondrial Drp1 expression and promotes cisplatin resistance in HCC, which provides a potential therapeutic target for the treatment of cisplatin-resistant HCC.

Key words hepatocellular carcinoma, mitochondrial fission factor, dynamin-related protein 1, cisplatin resistance

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver tumor and one of the leading causes of cancer-related death worldwide [1,2]. The primary treatment of HCC is surgical resection or liver transplantation, while multiple strategies including chemotherapy and radiotherapy are also employed to treat HCC [3]. However, HCC is considered as a chemoresistant tumor, and multiple chemotherapeutic drugs used in HCC treatment such as cisplatin and 5-fluorouracil showed limited therapeutic outcome [4]. Thus, it is of great interest to understand the chemoresistance in HCC treatment and develop novel efficient therapeutic strategies.

Cisplatin, *cis*-diamminedichloroplatinum (DDP), is a well-known chemotherapeutic drug used to treat various human tumors such as ovarian cancer, breast cancer, and brain cancer [5–8]. Cisplatin treatment may lead to DNA damage, inhibit cell proliferation and induce cell apoptosis [9]. Due to the chemoresistance character of HCC, cisplatin is not used as a first-line treatment due to its low sensitivity [10]. Combined HCC treatment with cisplatin and other chemotherapeutic drugs such as gemcitabine or capecitabine have been reported to show improved therapeutic efficacy [11,13].

Mitochondria are important cytoplasmic organelles that play critical roles in regulating cellular metabolism [14]. Dysregulated mitochondria are found in various malignant tumors and targeting mitochondria provides new therapeutic strategies [15]. Somatic mitochondrial DNA alterations, such as point mutation, insertion, deletion and copy number changes, have been demonstrated to be associated with HCC [16]. Recent studies also showed that mitochondrial dysfunction plays a critical role in the transition from non-alcoholic steatohepatitis to HCC [17]. Mitochondria are also involved in the drug-resistance of HCC, but the underlying mechanisms need further exploration [18]. Mitochondrial fission factor (Mff) is a mitochondrial receptor assisting the fission process of

mitochondria. Previous studies have shown that Mff plays a critical role in the survival of non-small cell lung cancer [19] and colorectal cancer [20]. Tak *et al.* [21] found that Mff is augmented in the cancerous liver tissues compared to the corresponding non-tumor tissues. A recent study by Wu *et al.* [22] revealed that the expression of Mff is positively correlated with the chemosensitivity in head and neck squamous cell carcinoma samples. However, the exact role of Mff in the chemoresistance of HCC remains unclear.

In this study, we found that Mff is highly expressed in cisplatin-resistant HCC tissues and cell lines. Knockdown of Mff sensitizes HCC/DDP to cisplatin treatment both *in vitro* and *in vivo*. We demonstrated that knockdown of Mff inhibits the mitochondrial fission and downregulates the expression of membrane Drp1 in cisplatin-resistant Huh-7/DDP cells. Our findings suggest that Mff/Drp1 in mitochondrial promotes cisplatin resistance in HCC, which provides a potential target for cisplatin-resistant HCC therapy.

Materials and Methods

HCC tumor samples

Hepatocellular carcinoma tissues were obtained from HCC patients with good or poor response to cisplatin treatment during May 2018 to February 2019 at Xijing Hospital. Tissues were snap-frozen in liquid nitrogen until further use. All patients signed the informed consents and the study was approved by the Ethics Committee of Xijing Hospital and conducted in accordance with the Helsinki Declaration.

Cell culture

Huh-7 and HepG2 cells were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Huh-7 and HepG2 cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin in a 5% CO_2 incubator at 37°C. Huh-7/DDP and HepG2/DDP cells were established by gradually exposing the normal Huh-7 or HepG2 cells to increasing concentrations of DDP. Briefly, cells were cultured for 2 weeks in culture medium containing 0.0375 μg/mL DDP (Solarbio, Beijing, China). The cells were passaged every 4–5 days or at a confluence level of 80%. After 2 weeks, the cells were seeded into a new flask and cultured in culture medium containing increased concentrations (1.5-fold of the previous concentration) of cisplatin. This procedure was repeated until the cells exhibited stable growth and proliferation in a culture medium with 0.6 μg/mL DDP. DDP-resistant cell lines were cultured with continuous treatment of DDP to maintain the chemoresistance prior to the experiments.

Transfection

ShRNA knockdown vectors targeting Mff (shMff) and negative control (NC) were obtained from Ribobio (Guangzhou, China), target sequences are as follows: shMff-1, 5′-TAGCTATTATA GACCTGTA-3′; shMff-2, 5′-ATGGAATATACTGAAGGCA-3′; shMff-3, 5′-CAAGGATTCCAAGAAGGAG-3′; NC, 5′-TCTTTAGGGGTGT GCGTAGG-3′. Huh-7/DDP or HepG2/DDP cells were transfected with shMff or NC using lipofectamine 3000 (Invitrogen, Waltham, USA) following the manufacturer's protocol.

CCK-8 assay

Cell viability was analyzed by CCK-8 assay using a detection kit (AccuRef Scientific, Xi'an, China). Briefly, cells with different treatments were seeded into 96-well plates at a density of 2000 cells/well. After culture for 48 h, 10 μL CCK-8 reagent was added to the cell culture medium and further incubated for 4 h. Absorbance of each well was measured at 450 nm using a microplate reader to determine the cell viability.

Colony formation assay

Cells with different treatment were seeded into 6-well plates at a density of 500 cells/well and cultured for 2 weeks. Cell colonies were fixed and stained with 0.5% crystal violet and counted under an Alphaphot YS2 microscope (Nikon, Shanghai, China).

Annexin V/propidium iodide (PI) staining

Huh-7/DDP cells with indicated treatment were collected and stained with Annexin V-FITC and PI (BD Bioscience, San Jose, USA). Samples were analyzed by flow cytometry and data were analyzed using Flowjo software (Flowjo, Treestar, USA).

Transwell assay

Cell invasion or migration was evaluated using Transwell chamber (8 μm; Corning Co., Corning, USA) with or without Matrigel coating. Briefly, cells were seeded into the upper chamber in serum-free medium, and complete medium with 20% FBS was added to the lower chamber. After culture for 48 h, the invaded cells or migrated cells were fixed and stained with 0.5% crystal violet and counted under the Nikon Alphaphot YS2 microscope.

Wound healing assay

Cell migration was evaluated by wound-healing assay. Briefly, cells with different treatments were seeded into 6-well plates and cultured to confluence. An artificial wound was created using a sterile 200-μL tip, and floating cells were washed away with saline buffer. Cells were cultured with serum-free medium for additional 48 h. The images of wounds were captured using an TS100 inverted phase contrast microscope (Nikon) rat 0 h and 48 h, and the cell migration ability was calculated.

Mitochondrial mass assay

Huh-7/DDP cells with indicated treatment were collected and stained with MitoTracker™ Deep Red FM (M22426; ThermoFisher, Waltham, USA). Samples were examined by confocal microscopy, and the fluorescence intensities were quantified by ImageJ software (Version 1.60) to calculate the mitochondrial mass as previously reported [23,24].

Immunofluorescence staining and confocal imaging

Huh-7/DDP cells with indicated treatment were stained with the MitoTracker™ Green FM (M7514; ThermoFisher) and then fixed with 4% paraformaldehyde, followed by permeabilization with 0.1% Triton X-100. Then, cells were stained with rabbit monoclonal antibody to Drp1 (ab184247; Abcam, Cambridge, UK) and goat anti-rabbit IgG H&L (Cy3®) preadsorbed secondary antibody (ab6939; Abcam). Cell nuclei were counter-stained with DAPI (D9542; Sigma-Aldrich, St Louis, USA). Sample images were recorded using a confocal microscope (Leica, Wetzlar, Germany). The mitochondrial fission was quantified under the confocal microscope from three independent experiments.

Mitochondrial localization

Mitochondrial/cytosol fractionation was prepared using a Mi-

tochondrial/cytosol fractionation kit (ab65320; Abcam) following the manufacturer's protocol and the localization of Drp1 in mitochondrial was analyzed by western blot analysis.

Xenograft tumor model

The animal experiments were approved by the Animal Care Committee of Xijing Hospital and performed in accordance with the Institutional Animal Care and Use Committee (IACUC) procedures. Male BALB/C nude mice (5–6 weeks) were obtained from Shanghai SLAC Animal Center (Shanghai, China). Huh-7/DDP cells stably transfected with shMff or NC were inoculated subcutaneously into the flanks of nude mice and treated with saline control or 5 mg/kg cisplatin every 4 days. The tumor growth was monitored and tumor size was measured every 4 days. At the 25th day after inoculation, mice were sacrificed and tumor weights were determined. Tumor volumes were calculated by length × width^2.

Immunohistochemical staining

Xenograft tumor tissues were fixed and embedded with paraffin. Tissue sections (6 μm) were incubated with rabbit polyclonal antibody to Ki-67 (ab15580; Abcam) or rabbit polyclonal antibody to cleaved-caspase 3 (ab2302; Abcam), and goat anti-rabbit IgG H&L (HRP) secondary antibody (ab6721; Abcam). The sections were further treated with 3,3′-diaminobenzidine chromogen (DAB) (D7051; Solarbio), and positively-stained cells were determined under the Nikon Alphaphot YS2 microscope.

Real Time quantitative PCR (RT-qPCR)

Total RNA was purified from cultured cells or HCC tissues using Trizol (Invitrogen) and reverse-transcribed into cDNA using the PrimeScript RT reagent kit (Takara, Dalian, China). Quantitative PCR was performed using PowerUp SYBR Green master mix (Applied Biosystems, Foster City, USA) with CFX96 real-time PCR machine (Bio-Rad, Hercules, USA). The expression of Mff relative to internal control GAPDH was calculated by the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers used in this study are listed as follows: Mff forward, 5′-GCTCTCAGCCAACCACCTC-3′ and Mff reverse, 5′-GGAGAAGGAAATGCTGCCCT-3′; GAPDH forward, 5′-GAATGGGCAGCCGTTAGGAA-3′ and GAPDH reverse, 5′-AAAAGCATCACCCGGAGGAG-3′.

Western blot analysis

Total protein was extracted using RIPA buffer (Beyotime, Beijing, China) and quantified using BCA kit (AccuRef Scientific). Equal amount of protein was separated by 12% SDS-PAGE and transferred on to a PVDF membrane (Millipore, Burlington, USA). The membranes were blocked with 5% non-fat milk, followed by incubation with primary antibodies and HRP-conjugated secondary antibody. The protein bands were visualized using an enhanced chemiluminescence kit (Pierce, Dallas, USA). β-Actin was used as an internal loading control. The primary antibodies used in the study are listed as follows: rabbit monoclonal antibody to Bcl-2 (ab32124; Abcam), rabbit monoclonal antibody to Bax (ab32503; Abcam), rabbit polyclonal antibody to cleaved-caspase 3 (ab2302; Abcam), rabbit monoclonal antibody to pro-caspase 3 (ab32499; Abcam), rabbit polyclonal antibody to cleaved-caspase 9 (ab2324; Abcam), rabbit polyclonal antibody to pro-caspase 9 (ab138412; Abcam), rabbit monoclonal antibody to cleaved-PARP (ab32064; Abcam), rabbit polyclonal antibody to PARP (ab74290; Abcam), rabbit monoclonal antibody to cytochrome c (ab133504; Abcam), rabbit monoclonal antibody to Hsp60 (ab190828; Abcam), rabbit polyclonal antibody to β-actin (ab8227; Abcam), rabbit monoclonal antibody to E-cadherin (ab40772; Abcam), rabbit monoclonal antibody to N-cadherin (ab76011; Abcam), rabbit monoclonal antibody to MMP-2 (ab92536; Abcam), rabbit monoclonal antibody to MMP-9 (ab76003; Abcam), rabbit monoclonal antibody to COX IV (ab202554; Abcam), rabbit monoclonal antibody to Mff (ab129075; Abcam), rabbit anti-CXCR4 polyclonal antibody (11073-2-AP; Proteintech, Wuhan, China), rabbit anti-CXCR7 polyclonal antibody (20423-1-AP; Proteintech), and rabbit monoclonal antibody to Phospho-MFF (Ser146) (49281; Cell Signaling Technology, Danvers, USA). The secondary antibody is HRP-conjugated goat anti-rabbit IgG H&L (ab6721; Abcam).

Statistical analysis

The statistical analysis was conducted using GraphPad Prism software (V6.07; GraphPad Software, San Diego, USA). Data were presented as the mean ± standard derivation. Comparison between groups was analyzed by Student's *t*-test, and comparisons among multiple groups were analyzed using one-way ANOVA analysis. $P < 0.05$ was considered as statistically significant.

Results

Mff is upregulated in cisplatin-resistant HCC

The expression of Mff was examined in HCC tissues with good or poor responses to cisplatin therapy. Our results showed that HCC tissues resistant to cisplatin therapy had significantly higher Mff mRNA level than HCC tissues with good responses to cisplatin therapy (Figure 1A). To further verify the expression of Mff in cisplatin-resistant HCC, HCC cell line Huh-7 and its cisplatin-resistant cell line Huh-7/DDP were treated with different concentrations of cisplatin. Huh-7/DDP with cisplatin-resistance exhibited remarkably higher cell viability than regular Huh-7 cells treated with cisplatin (Figure 1B). The cisplatin-resistance was also confirmed in HepG2/DDP cells (Figure 1C). Moreover, cisplatin-resistant Huh-7/DDP and HepG2/DDP cells had significantly higher mRNA levels of Mff than regular Huh-7 or HepG2 cells (Figure 1D). Moreover, our western blot analysis results showed that both phosphorylated and total Mff were elevated in DDP-resistant cells (Figure 1E). Thus, our findings suggested that Mff is upregulated in cisplatin-resistant HCC tissues and HCC cells.

Knockdown of Mff suppresses cell proliferation and promotes cell apoptosis of HCC/DDP cells

To explore the function of Mff in HCC, knockdown of Mff was performed using shRNA-targeting Mff and the knockdown efficiency was verified by western blot analysis (Figure 2A). Among the three shRNAs with different targets, two of them were found to significantly reduce the expression of Mff (Supplementary Figure S1A,B) and the proliferation of Huh-7/DDP or HepG2/DDP cells (Supplementary Figure S1C). Since shRNA-2 showed the most significant RNA silencing efficiency, we used it for the subsequent experiments. Functionally, we demonstrated that knockdown of Mff inhibits cell proliferation (Figure 2B) and colony formation of Huh-7/DDP and HepG2/DDP cells (Figure 2C). In contrast, knockdown of Mff significantly promotes the apoptosis of Huh-7/DDP and HepG2/DDP cells, as demonstrated by Annexin V/Propidium Iodide analysis (Figure 2D). These data indicated that Mff is

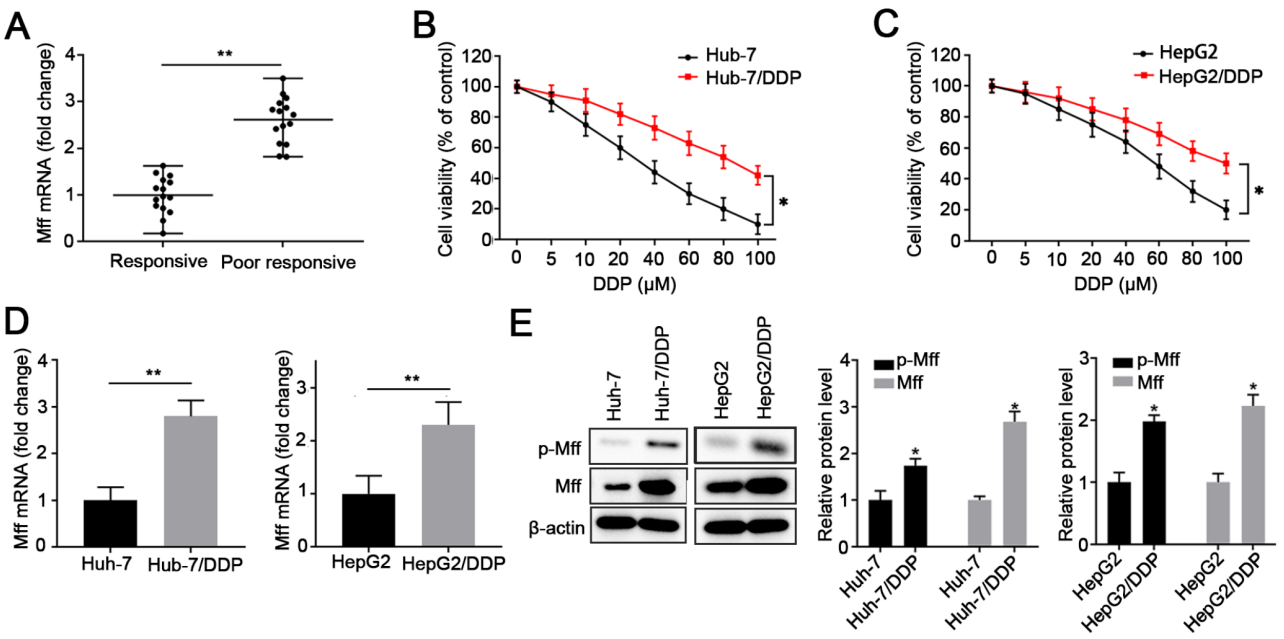

Figure 1. Mff is upregulated in cisplatin-resistant hepatocellular carcinoma (A) The mRNA expression of Mff in HCC tumor samples with good or poor responses to cisplatin therapy was analyzed by qPCR ($n = 15$ pairs). (B) Huh-7 and Huh-7/DDP cells were treated with different concentration of cisplatin (DDP) for 48 h. Cell viability was analyzed by CCK-8 assay. (C) HepG2 and HepG2/DDP cells were treated with different concentration of cisplatin (DDP) for 48 h. Cell viability was analyzed by CCK-8 assay. (D) The mRNA expression of Mff in Huh-7, Huh-7/DDP, HepG2, and HepG2/DDP cells was analyzed by qPCR. (E) The protein expression of Mff in Huh-7, Huh-7/DDP, HepG2, and HepG2/DDP cells was analyzed by western blot analysis. β-Actin was used as an internal control. Each experiment was repeated three times. $^*P < 0.05$, $^{**}P < 0.01$.

positively associated with the proliferation and survival of HCC/DDP cells.

Knockdown of Mff sensitizes cisplatin-resistant Huh-7/DDP cells to cisplatin treatment *in vitro*

We further evaluated the effect of Mff knockdown on cisplatin cytotoxicity. While cisplatin treatment or knockdown of Mff inhibited cell proliferation and colony formation, simultaneous cisplatin treatment of the Mff-downregulated Huh-7/DDP cells further suppressed their proliferation and colony formation capability (Figure 3A–C). Consistently, Huh-7/DDP cells with Mff knockdown and cisplatin treatment showed higher cell apoptosis rate compared with that in the negative control group, cisplatin treatment only group, or Mff knockdown only group (Figure 3D). Moreover, cell survival and apoptosis protein expressions were examined in Huh-7/DDP cells. Compared with the control group, cisplatin treatment or knockdown of Mff decreased the expression of Bcl-2 and enhanced the expression of Bax, while simultaneous cisplatin treatment and Mff knockdown had the lowest ratio of Bcl-2/Bax (Figure 3E). In addition, cisplatin treatment or Mff knockdown was found to enhance the expressions of cleaved-caspase 9, cleaved-caspase 3, cleaved-PARP expression, and cytosol cytochrome C expression, without changing the expressions of caspase 9, caspase 3, PARP, and membrane cytochrome C (Figure 3F,G). Mff knockdown further enhanced the cytotoxic effect of cisplatin treatment (Figure 3F,G). These data indicated that Mff plays a critical role in the chemoresistance of HCC cells.

Knockdown of Mff inhibits cell migration and invasion of cisplatin-resistant Huh-7/DDP cells treated with cisplatin

Transwell assay was performed to assess the function of Mff in cell migration and invasion. Cisplatin treatment or knockdown of Mff suppressed Huh-7/DDP cell migration and invasion, and Huh-7/DDP cells with Mff knockdown and cisplatin treatment showed the lowest cell migration and invasion capability (Figure 4A). Would healing assay further confirmed the synergistic effect of Mff knockdown and cisplatin treatment on cell migration (Figure 4B). In addition, cisplatin treatment or Mff knockdown enhanced the expression of E-cadherin and inhibited the expressions of N-cadherin, MMP-2 and MMP-9, indicating the inhibition of cell migration (Figure 4C,D). However, knockdown of Mff, together with cisplatin treatment, further enhanced the upregulation of E-cadherin and downregulation of N-cadherin, MMP-2, and MMP-9 (Figure 4C,D). To validate the migration capacity of the cells after Mff knockdown, we examined the migration-related proteins and found that both CXCR4 and CXCR7 were significantly reduced after Mff knockdown (Figure 4E). These findings suggest that knockdown of Mff inhibits cell migration and invasion of cisplatin-resistant Huh-7/DDP cells treated with cisplatin.

Knockdown of Mff inhibits mitochondrial fission and downregulates the expression of mitochondrial Drp1 in cisplatin-resistant Huh-7/DDP cells

To explore the effect of Mff knockdown on mitochondria, we analyzed the mitochondria mass by fluorescence staining. In comparison with the negative control, knockdown of Mff or treatment with cisplatin decreased mitochondrial mass and showed less mitochondrial fission in Huh-7/DDP cells (Figure 5A,B). Knockdown of Mff further inhibited mitochondrial mass and mitochondrial fission in cisplatin-treated Huh-7/DDP cells (Figure 5A,B). It has been reported that mitochondrial fission requires Drp1 but not dynamins [25]. Intriguingly, cisplatin treatment did not affect Drp1 expres-

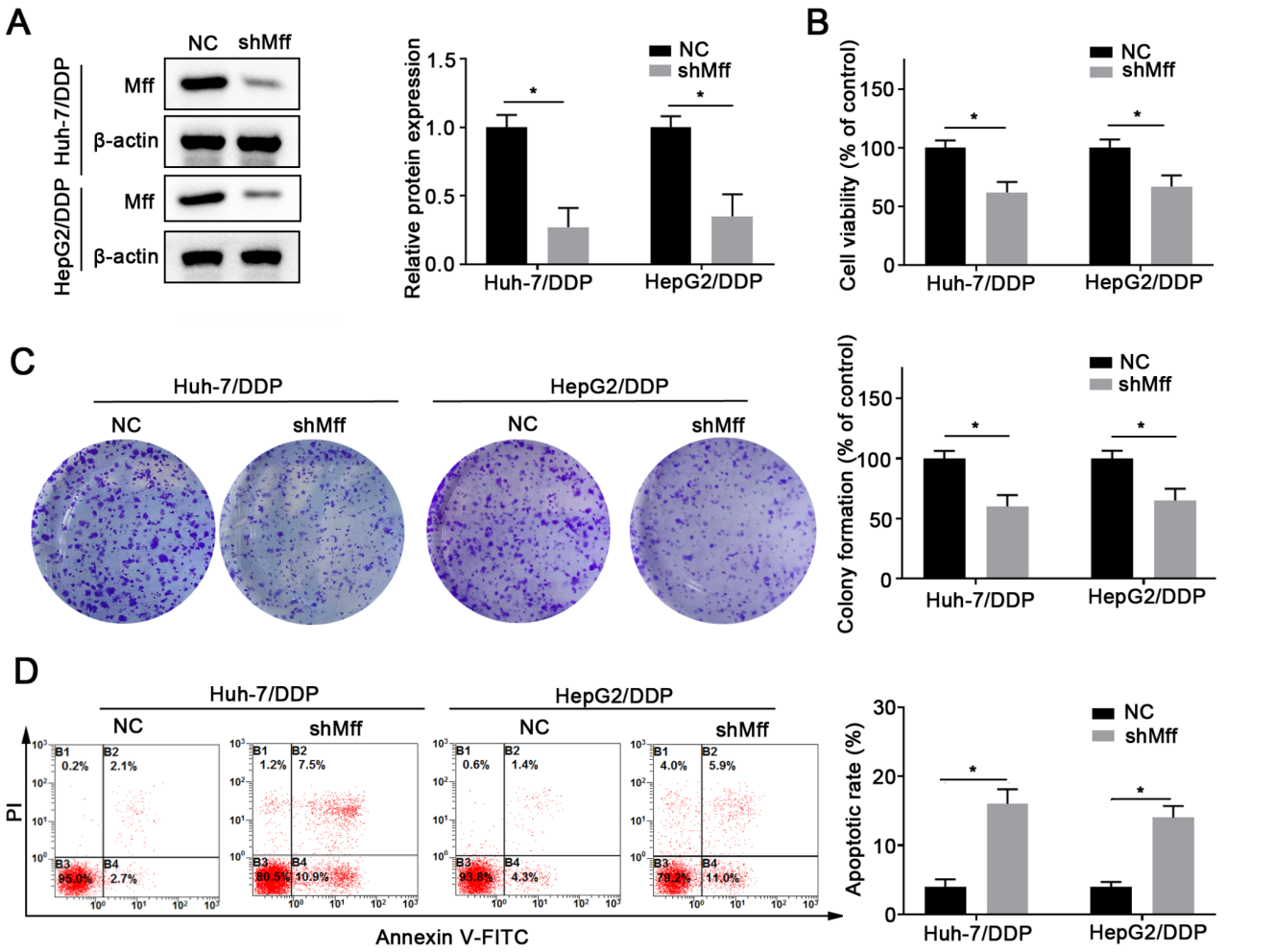

Figure 2. Knockdown of Mff suppresses cell proliferation and promotes cell apoptosis of HCC/DDP cells Huh-7/DDP or HepG2/DDP cells were transfected with negative control (NC) or shMff. (A) The protein expression of Mff was analyzed by western blot analysis 48 h later. β-Actin was used as an internal control. (B) Cell viability was analyzed by CCK-8 assay 48 h later. (C) Cell colonies were analyzed 14 days later by colony formation assay. (D) Cell apoptosis was analyzed by Annexin-V/Propidium Iodide staining 48 h post transfection. Each experiment was repeated three times. $*P < 0.05$.

sion, while knockdown of Mff significantly inhibited the expression of mitochondrial Drp1 (Figure 5C). Moreover, we found that Mff knockdown markedly suppressed membrane Drp1 expression, but not cytosol Drp1 expression (Figure 5D,E). These data indicated that Mff induces mitochondrial fission by regulating the level of mitochondrial Drp1 IN Huh-7/DDP cells.

Knockdown of Mff sensitizes cisplatin-resistant Huh-7/DDP xenograft tumor to cisplatin treatment *in vivo*

To further study the function of Mff *in vivo*, we established the xenograft tumor model by implanting Huh-7/DDP cells into nude mice and treated the mice with cisplatin. As shown in Figure 6A,B, xenograft tumors were resistant to DDP treatment, while knockdown Mff significantly inhibited tumor growth. Mice bearing shMff-transduced Huh-7/DDP cells with cisplatin treatment further suppressed the tumor development, with the lowest tumor weight. DDP treatment enhanced Mff expression in xenograft tumors, and xenograft tumors derived from shMff-transduced Huh-7/DDP cells showed significantly less expression of Mff (Figure 6C). In addition, immunohistochemical staining revealed decreased expression of Ki-67 in tumors from Mff-knockdown group and lowest Ki-67 expression in the shMff + DDP group (Figure 6D). In contrast, cisplatin treatment only had no effect on Ki-67 expression. Moreover, Mff knockdown promoted cell apoptosis, showing higher cleaved-Caspase 3 expression (Figure 6E). Xenograft tumors from the shMff + DDP group had the highest level of cleaved-Caspase 3 expression (Figure 6E). Overall, our data suggest that Mff knockdown sensitizes cisplatin-resistant HCC/DDP xenograft tumor to cisplatin treatment *in vivo*.

Discussion

Resistance to chemotherapy remains a major hindrance for HCC treatment, and mitochondria play a critical role in tumor-resistant to cisplatin therapy [4,26]. In the current study, we identified that Mff regulates mitochondrial Drp1 expression and exerts important function in the resistance to cisplatin therapy in HCC. Knockdown of Mff sensitized the anti-tumor effect of cisplatin in cisplatin-resistant HCC both *in vitro* and *in vivo*, demonstrating an essential link between mitochondrial dysfunction and cisplatin resistance in HCC therapy.

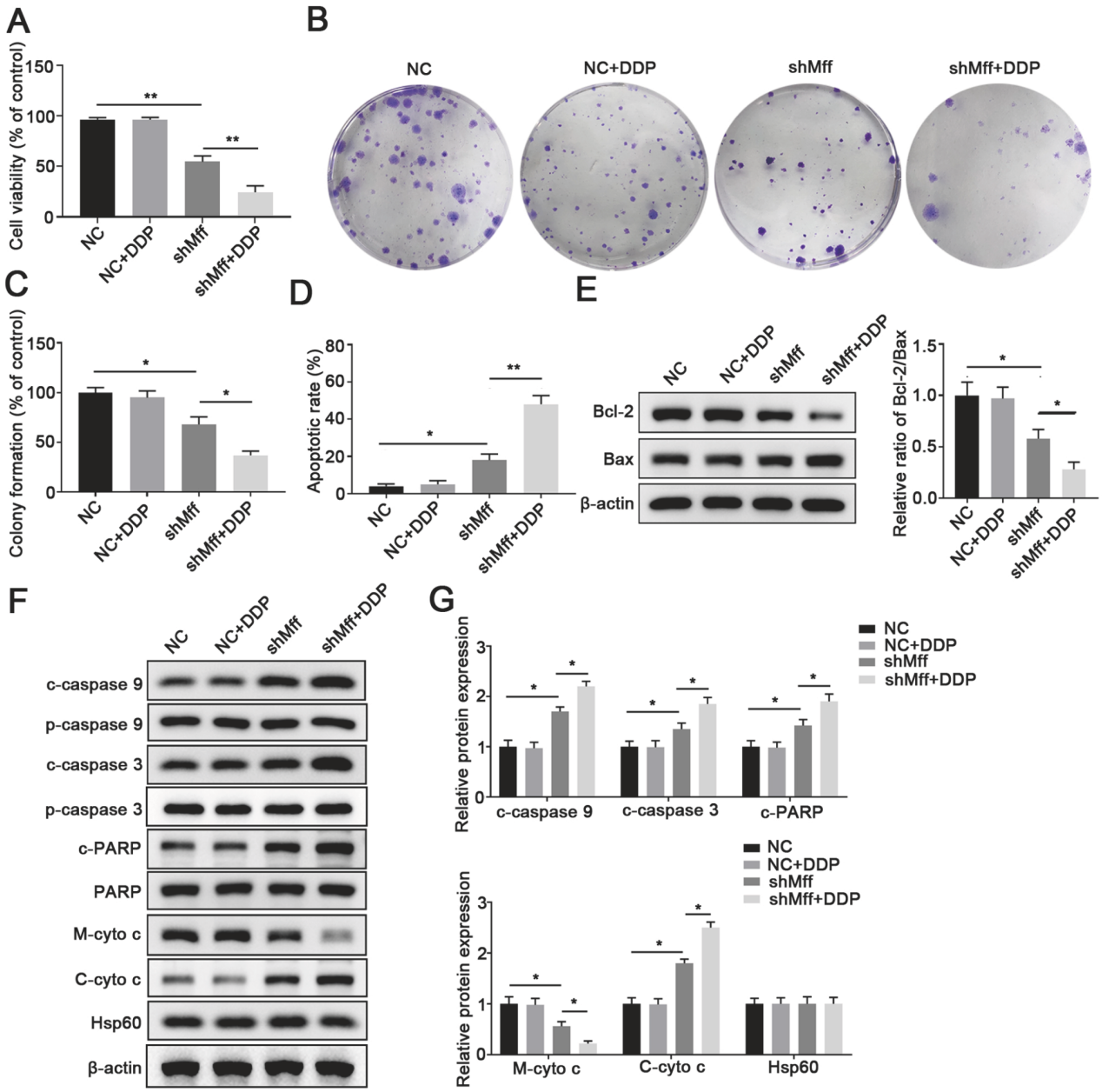

Figure 3. Knockdown of Mff sensitizes cisplatin-resistant Huh-7/DPP cells to cisplatin treatment *in vitro* Huh-7/DDP cells were transfected/treated with negative control, shMff, cisplatin, or shMff + cisplatin. (A) Cell viability was analyzed by CCK-8 assay 48 h later. (B,C) Cell colonies were analyzed by colony formation assay 2 weeks later. (D) Cell apoptosis was analyzed by Annexin V/Propidium Iodide staining 48 h later. (E) The protein expressions of Bcl-2 and Bax in Huh-7/DDP cells were analyzed by western blot analysis. The ratio of Bcl-2/Bax was calculated. (F,G) The protein expressions of caspase 9, cleaved-caspase 9, caspase 3, cleaved-caspase 3, PARP, cleaved-PARP, mitochondrial cytochrome c (M-cyto c), HSP60, and cytosol cytochrome c (C-cyto c) were analyzed by western blot analysis. β-Actin was used as an internal control. Each experiment was repeated three times. $*P < 0.05$, $**P < 0.01$.

Mff plays essential roles during mitochondrial fission via recruiting Drp1 in mammalian cells [27,28]. Multiple microRNAs such as miR-761 and miR-27 have been demonstrated to regulate the mitochondrial network via modulating Mff expression [29,30]. Seo *et al.* [31] reported that Mff is a novel target of oncogenic Myc and knockdown of Mff enhances mitochondrial outer membrane permeability and leads to the suppression of tumor cell proliferation. The Mff and voltage-dependent anion channel-1 (VDAC1) complex was identified as a novel therapeutic target in multiple patient-derived xenograft tumor models, including breast and lung cancer and glioblastoma [32]. Mff has been studied as a novel marker for HCC [21]. Emerging evidence has demonstrated that Mff may regu-late the cisplatin sensitivity in different tumors, such as tongue squamous cell carcinoma and ovarian cancer [33,34]. Consistently, we found that Mff was highly expressed in cisplatin-resistant HCC tissue and cell lines. Knockdown of Mff inhibited cell proliferation and promoted cell apoptosis of Huh-7/DDP cells. Moreover, knockdown of Mff improved the sensitivity to cisplatin treatment of Huh-7/DDP both *in vitro* and *in vivo*, showing inhibited cell proliferation, migration and invasion, as well as sup-

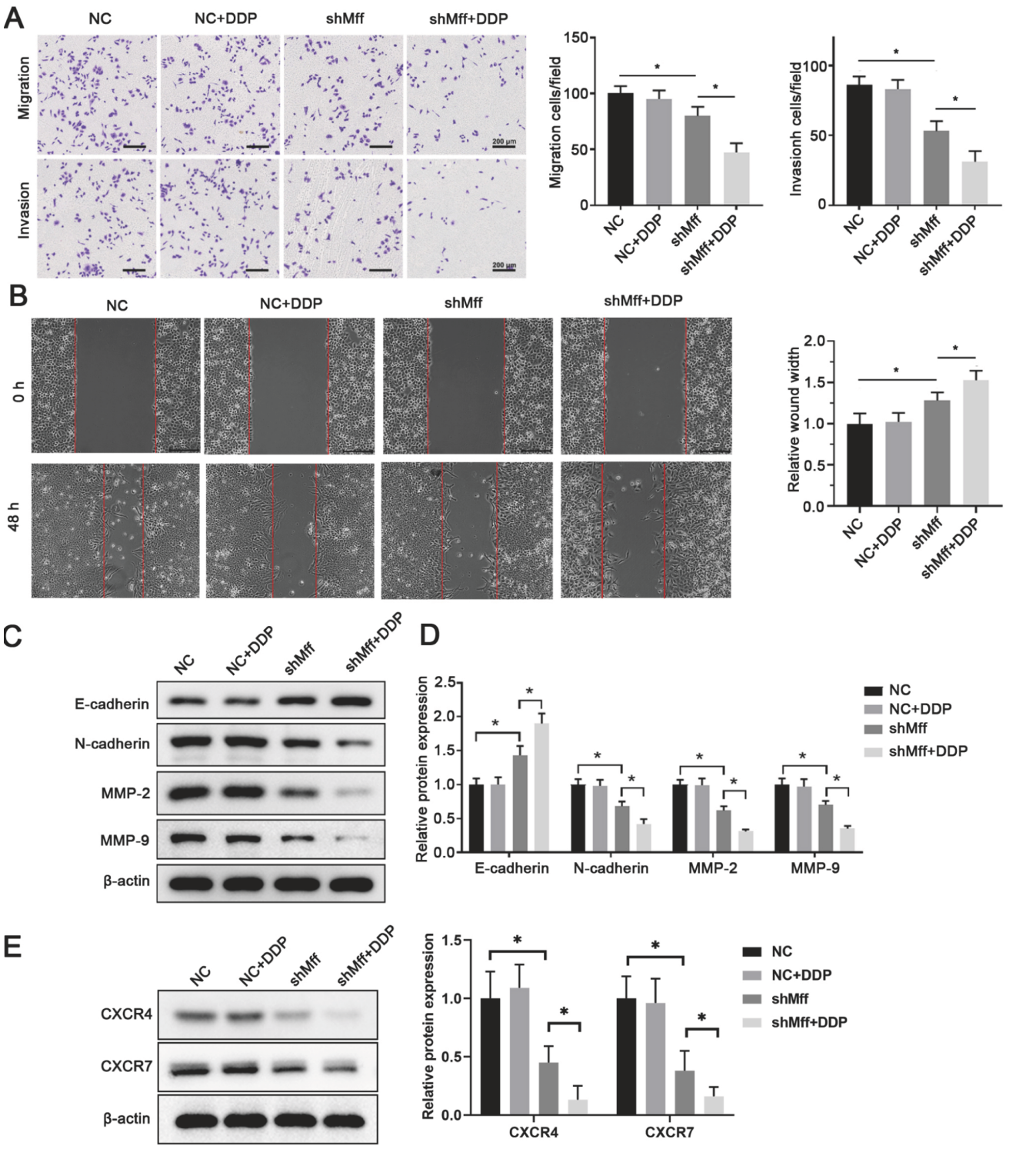

Figure 4. Knockdown of Mff inhibits cell migration and invasion of cisplatin-resistant Huh-7/DDP cells treated with cisplatin Huh-7/DDP cells were transfected/treated with negative control, shMff, cisplatin, or shMff + cisplatin. (A) Cell migration and invasion were analyzed by transwell assay. (B) Cell migration was analyzed by wound-healing assay. (C–E) The expressions of E-cadherin, N-cadherin, MMP-2, MMP-9, CXCR4, and CXCR7

pressed development of HCC xenograft tumor.

Mitochondrial fission is trigged by Drp1, and Mff-Drp1 interaction is important for mitochondrial mobility and function [35]. Intriguingly, our results showed that knockdown of Mff inhibited Drp1 expression. Mitochondrial fractionation assay further demonstrated that knockdown of Mff mainly affected the membrane expression of Drp1. This finding is consistent with previous reports that Mff is essential for mitochondrial recruitment of Drp1 [27,36]. Moreover, Akane Inoue-Yamauchi *et al.* [37] reported that depletion of Drp1 enhanced cell apoptosis in human colon cancer through controlling

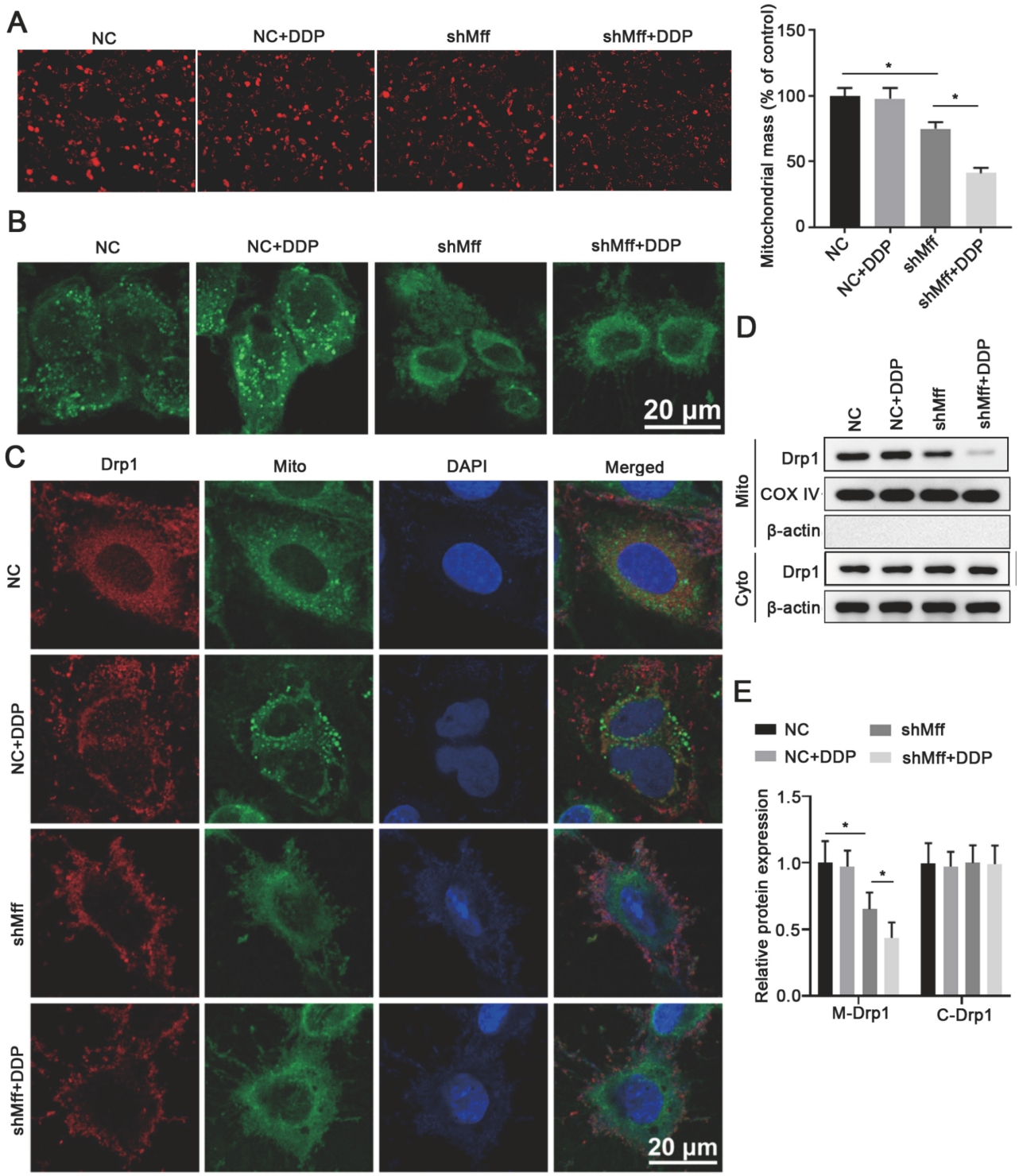

Figure 5. Knockdown of Mff inhibits mitochondrial fission and downregulates the expression of membrane Drp1 in cisplatin-resistant Huh-7/DDP cells Huh-7/DDP cells were transfected/treated with negative control, shMff, cisplatin, or shMff + cisplatin. (A) Mitochondrial mass in different groups was analyzed by fluorescence staining. (B) Mitochondrial fission in different groups was visualized by confocal microscopy. (C) The mitochondrial expression of Drp1 in Huh-7/DDP cells was analyzed by immunofluorescence staining. (D,E) The mitochondrial localization of Drp1 was analyzed by western blot analysis. Each experiment was repeated three times. $*P < 0.05$.

cytochrome c release and mitochondrial membrane integrity. Another study demonstrated that Drp1 promoted cell proliferation in HCC via interaction with p53 and NF-κB signaling pathway [38,39]. These findings, together with our results, indicate that Mff/Drp1 may be potential therapeutic targets to improve the cisplatin treatment of chemo-resistant HCC patients.

However, how Mff/Drp1 is regulated in cisplatin-resistant HCC remains illusive. The signaling pathway involved in Mff/Drp1-

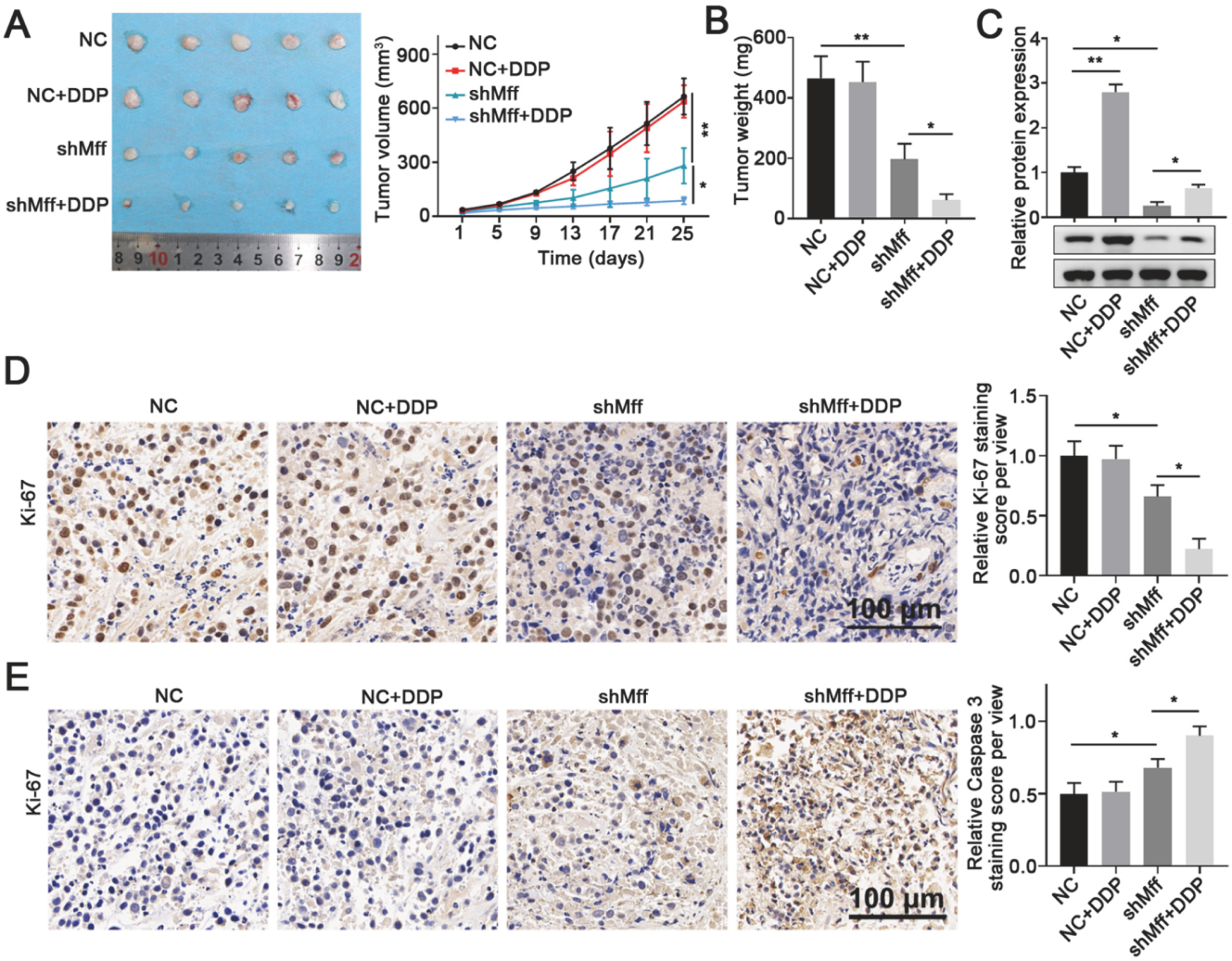

Figure 6. Knockdown of Mff sensitizes cisplatin-resistant Huh-7/DDP xenograft tumor to cisplatin treatment *in vivo* Huh-7/DDP cells were stably transfected with shMff or negative control. Cells were inoculated into nude mice to establish xenograft tumor and mice were treated with 5 mg/kg cisplatin every 4 days. (A) Tumor growth was monitored at indicated time points and the xenograft tumors were extracted at the end of the experiment. (B) Mice were euthanized at day 25 and the tumor weights in different groups were examined. (C) The Mff expression in xenograft tumors was analyzed by western blot analysis. (D) Ki-67 expression in xenograft tumors was analyzed by immunohistochemical staining. (E) Cleaved-Caspase 3 expression in xenograft tumors was analyzed by immunohistochemical staining. Each experiment was repeated five times. $*P<0.05$, $**P<0.01$.

mediated cisplatin-resistance needs further investigation. In addition, the physiological and pathological roles of Mff/Drp1 in normal liver, HCC, and cisplatin-resistant HCC should be well understood to develop novel therapy strategies.

In summary, we demonstrate that Mff/Drp1 facilitates cisplatin resistant in HCC chemotherapy. Knockdown of Mff sensitizes HCC to cisplatin treatment, with inhibited cell proliferation, migration, and invasion and suppressed tumor development. Our findings suggest that Mff may be a potential target for cisplatin-resistant HCC therapy.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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