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Research

Interaction between Maslinic acid and HSF1 enhances the ubiquitin degradation of HSF1, resulting in the inhibitory effect of pancreatic cancer

Li Cheng[1,2] · Mingyue Guo[3] · Ammad Ahmad Farooqi[4] · Linlin Wang[2] · Yuxi Zhang[2] · Hanlin Ye[5] · Gan Qiao[2] · Xiukun Lin[6]

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Abstract
Maslinic acid (MA), a pentacyclic triterpenoid, displays broad biological activity including anti-inflammatory, antihyperlipidemic, anti-tumor, cardiovascular protection etc. Our previous study has shown that MA inhibits the proliferation of pancreatic cancer cells via heat shock protein HSPA8-mediated autophagy pathway, but the detailed mechanism is still unclear. In the present study, we confirmed that MA downregulated the protein expression of the transcription factor of heat shock protein 1(HSF1), and knockdown of HSF1 was able to enhance the inhibitory effect of MA in pancreatic cancer cells. In contrast, high HSF1 expression was capable of partially reversing the MA-induced inhibitory effect and the ability of MA-induced autophagy in pancreatic cancer cells. Real-time quantitative PCR and Western blotting analysis indicated that MA reduced the protein expression of HSF1 but did not downregulate mRNA levels. Molecular docking and SPR analysis revealed that there was an specific interaction between MA and HSF1; MA was able to form hydrogen bonds with the amino acid residues, HIS-63 and GLN-72 on HSF1 molecules. Co-immunoprecipitation experiments confirmed that MA promoted the ubiquitination-mediated degradation of HSF1. Further study confirmed that the mutant of His-63 and Gln-72 amino acid residues of HSF1 partially reversed the inhibitory effect of MA on the autophagy and the growth of pancreatic cancer cells. Our study provides solid evidence that there is a specific interaction between MA and HSF1, and the interaction increases the ubiquitination-mediated degradation of HSF1, contributing to the inhibitory effect of pancreatic cancer cell growth. The study also suggests that targeting the binding sites of MA on HSF1 could be developed as a novel strategy for developing anticancer agents on the treatment of pancreatic cancer.

Keywords Maslinic acid · Antitumor · HSF1 · Ubiquitination-mediated degradation · Autophagy

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✉ Xiukun Lin, xiukunlin@126.com | [1]Drug Clinical Trial Institution Office, Suining Central Hospital, Suining 629000, Sichuan, China. [2]Department of Pharmacology, Southwest Medical University, Luzhou 646000, Sichuan, China. [3]Pharmacy Department, The People's Hospital of Jianyang City, Chengdu 641400, Sichuan, China. [4]Institute of Biomedical and Genetic Engineering (IBGE), Islamabad, Pakistan. [5]Department of Pharmacy, The Third People's Hospital of Yibin, Yibin 644000, Sichuan, China. [6]College of Marine Sci, Beibu Gulf University, 12, Binhai Rd, Qinzhou 535011, Guangxi, China.

Fig. 1 HSF1 played a critical role in MA-induced inhibitory effect on pancreatic cancer cells. **(A)** The chemical structure of MA. **(B)** Panc-28 ▶ cells were treated with certain concentration of MA, and the cell viability was analyzed by CCK8 approach. **(C, D)** Panc-28 cells were treated with certain concentration of MA for 48 h and the HSF1 expression was determined by Western blotting. **(E)** indicated the inhibitory effect of MA in cells downregulation of HSF1, while **(H)** showed the inhibitory effect of MA in cells overexpressing HSF1. **(F)** and **(G)** indicated inhibitory effect of MA on colony formation in cells downregulation of HSF1, while **(I)** and **(J)** showed that the effect of MA on colony formation in cells overexpression of HSF1

1 Introduction

Pancreatic cancer is a serious threat to human health; the 5-year survival rate of pancreatic patients is only 5% [1, 2]. Pancreatic cancer ranks seventh among all cancer death [1, 3, 4]. Although surgery, chemotherapy, radiotherapy and targeted therapy are often used in the treatment of pancreatic cancer, there is still no significant progress in the prognosis of pancreatic cancer. Gemcitabine combined with Erlotinib is a common approach in the chemotherapy of pancreatic cancer [5]. However, the efficacy is limited due to the drug resistance of the cancer cells [6]. Therefore, there is an urgent need to develop novel strategy to improve the therapeutic outcome, prolonging the survival of the pancreatic cancer patients.

Maslinic acid (MA, Fig. 1A), a pentacyclic triterpene acid, is widely distributed in dietary plants, especially abundant in olive fruit skins [7, 8]. MA displays broad biological activity, including antitumor, anti-inflammation, antivirus, antioxidation as well as cardiovascular protective effects [9, 10]. In recent years, the anticancer effects of MA attract great attention due to its safety and low toxicity [11, 12]. MA exhibits anticancer effect on a wide range of cancer cells, including human prostate cancer DU145 cells [13], human colon cancer cells HT29 [14], mouse prostate cancer cells [15] etc. However, the underlying mechanism of the inhibitory effects of MA on cancer cells is still unclear.

MA is able to suppress the migration and invasion of pancreatic cancer cell PANC-1[16]. Our previous study confirmed that heat shock protein A8 (HSPA8) plays a critical role in MA-induced inhibitory effect in pancreatic cancer; knockdown of HSPA8 enhanced the MA-induced inhibitory effect on autophagy and proliferation, while overexpression of HSPA8 reversed the effect of MA in cancer cells [17]. In the present study, we studied the effect of the transcription factor of HSPA8 (HSF1) on MA-induced inhibition in pancreatic cancer cells. Our results showed that the specific interaction between MA and HSF1 enhanced the ubiquitin-mediated degradation of HSF1, contributing to the suppression on the growth of pancreatic cancer.

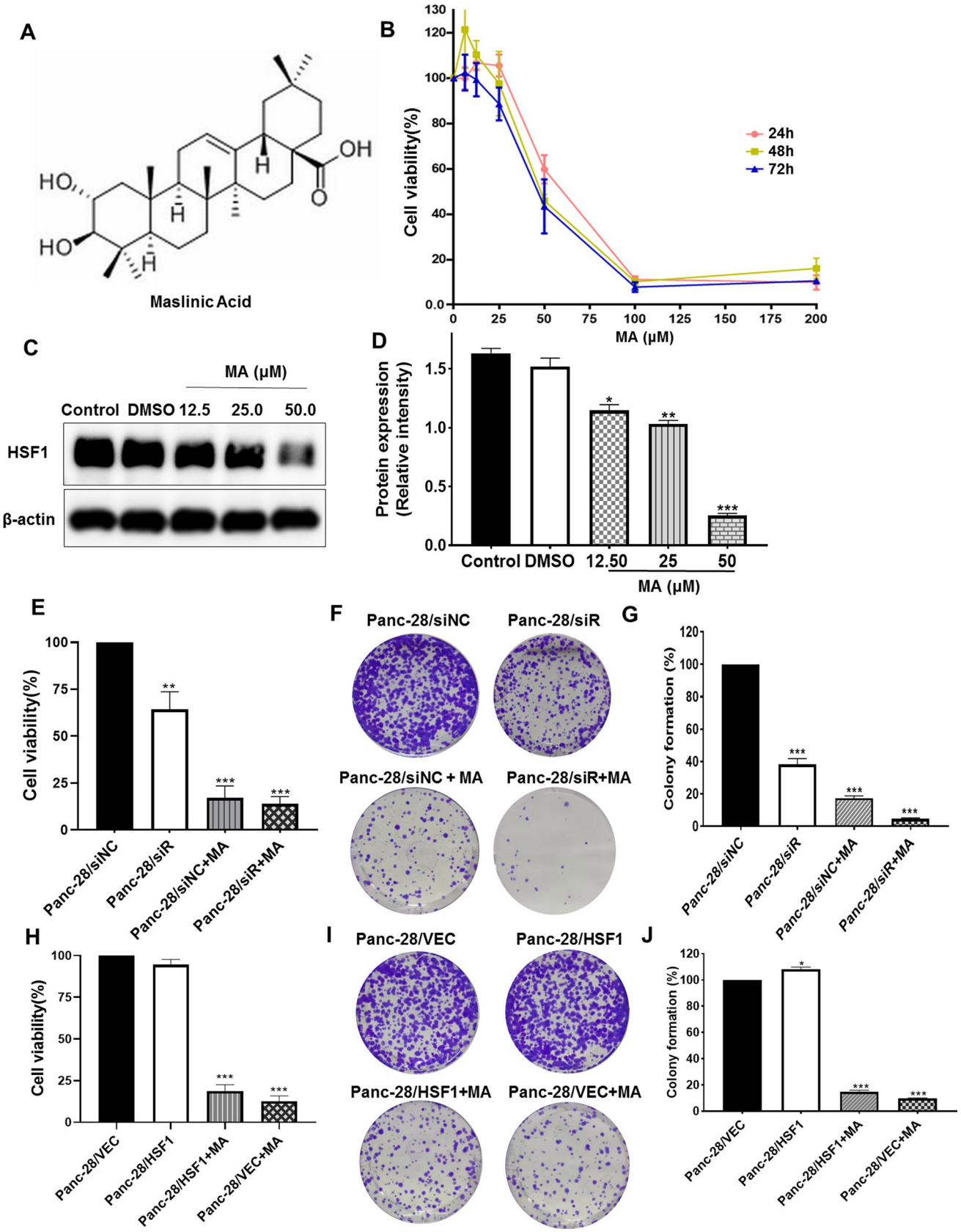
2 Materials and methods

2.1 Cell culture and reagents

The human embryonic kidney 293 T cells were purchased from the American Type Culture Collection (ATCC, MD, USA) and the cells were cultured in DMEM medium at 37 °C with 5% carbon dioxide (CO_2). The human pancreatic cancer Panc-28 cells were a gift from Dr D. Joshua Liao at the University of Minnesota, Austin, MN, USA and the cells were cultured in a modified RPMI 1640 medium (Gibco, CA, USA) with 10% fetal bovine serum (Gibco, CA, USA) at 37 °C with 5% carbon dioxide (CO_2). MA was obtained from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China).

2.2 Cell viability assay

Cell viability analysis was carried out using a CCK8 assay kit (CCK-8 assay kit, Beyotime Biotechnology, Jiangsu, China). Briefly, Panc-28 cells (1×10^4) were incubated in a 96-well plate. After being cultured for 24 h, the cells were treated with or without certain concentration of MA (0, 6.25, 12.5, 25, 50, 100 and 200 μM). CCK-8 solution was then added and the cells were incubated for an additional 1 h at 37 °C. The absorbance value of optical density (OD) at 450 nm was analyzed using an automatic microplate reader (Model 680, Bio-Rad, Hercules, CA, USA). All of the experiments were carried out for more than 3 times ($n \geq 3$).


A
HO
HO
H
H
H
OH
O
Maslinic Acid
B
Cell viability(%)
MA (μM)
24h
48h
72h
C
MA (μM)
Control DMSO 12.5 25.0 50.0
HSF1
β-actin
D
Protein expression (Relative intensity)
Control DMSO 12.50 25 50
MA (μM)
E
Cell viability(%)
Panc-28/siNC
Panc-28/siR
Panc-28/siNC+MA
Panc-28/siR+MA
F
Panc-28/siNC
Panc-28/siR
Panc-28/siNC + MA
Panc-28/siR+MA
G
Colony formation (%)
Panc-28/siNC
Panc-28/siR
Panc-28/siNC+MA
Panc-28/siR+MA
H
Cell viability(%)
Panc-28/VEC
Panc-28/HSF1
Panc-28/HSF1+MA
Panc-28/VEC+MA
I
Panc-28/VEC
Panc-28/HSF1
Panc-28/HSF1+MA
Panc-28/VEC+MA
J
Colony formation (%)
Panc-28/VEC
Panc-28/HSF1
Panc-28/HSF1+MA
Panc-28/VEC+MA

2.3 Construction of HSF1 down-regulated cancer cells

Three siRNA sequences targeting the HSF1 were designed as per the designer 2.0 (Shanghai GenePharma Co., Ltd, Shanghai, China) and their ability to downregulate HSF1 mRNA expression was evaluated using Western blot analysis. One siRNA designated as HSFsi was selected which displayed most obviously inhibitory effect on the expression of HSF1 mRNA; the sequence of the HSFsi is as follows; 5′GCAUGCCCAGCAACAGAAATT3′. HSFsi was transfected into cancer cells using the Lipofectamine 3000 (Invitrogen, CA, USA) reagent following the manufacturer' instructions.

2.4 Quantitative real-time PCR

Total RNA was extracted from cell lines with TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using TIANGEN FastQuant RT kit (TIANGEN BIOTECH Co., Ltd. Beijing, China) as per the manual instruction. Real-time quantitative PCR (qPCR) experiments were performed using the Super Real Premix (SYBR Green) kit (TIANGEN BIOTECH Co., Ltd. Beijing, China) according to the manufacture's instruction. The sequences of the primers used to amplify HSF-1 cDNA were as follows; sense 5′-CCATGAAGCATGAGAATGAGGC −3, antisense 5′-CTTGTT GACGACTTTCTGTTGC-3′. and the sequences of the primers used to amplify GAPDH cDNA were as follows; sense 5′- GGA GCGAGATCCCTCCAAAAT-3, antisense 5′- GGCTGTTGTCATACTTCTCATGG −3′. The amplifying conditions were 95 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min, followed by 40 cycles, with a final stage of 72 °C for 5 min. GAPDH was used as control. Each experiment was performed for more than 3 times.

2.5 Construction of stable overexpression HSF1 cell lines

The HSF1 cDNA was inserted into the lentivirus plasmid pHBLV-CMV-MCS-3 FLAG-EF1-ZsGreen-T2 A-PURO (Hanheng Biotechnology Co., Ltd. Shanghai, China) to construct a HSF1 expressing plasmid pHCM/HSF1, and pHCM/HSF1 was infected to the *E. Coli* for amplification. pHCM/HSF1 (7.3 μg), PaPAx2 (7.3 μg), PMD2.G (7.3 μg), h-HSF1 (7.3 μg) were co-transfected into 293 T cells for Lentivirus packaging, and the virus was collected and infected into Panc-28 cells. The cells were screened using puromycin (Shanghai McLin Biotech Co., Ltd. Shanghai, China) to obtain the HSF1-overessing cells, Pan-28/HSF1.

2.6 Colony formation assay

Both parent and HSF1 overexpression Panc-28 cells (Pan28/HSF1) (1×10^3) were plated in a 6-well plate. After incubation for 24 h, the cells were treated without or with HSF1 siRNA or MA (50 μM). the cells were stained with 0.05% crystal violet solution after incubation for 10 to 14 d at 37 °C, and the number of the colonies were counted under a microscope by manual.

2.7 Determination of autophagy

The human pancreatic Panc-28 cells (5.5×10^3) were cultured in a Laser confocal cell culture dish, After incubated for 24 h, the cells were infected with adenovirus harboring tandem fluo-rescent mRFP – GFP – LC3 (Hanbio Inc, China) at 1,000 multiplicity. After being incubated for another 24 h, the cells were treated with or without HSF1 siRNA or MA (50 μM) for additional 48 h. The autophagy flow was determined using a laser confocal microscope (Nikon, Tokyo, Japan).

2.8 Western blot analysis

The human pancreatic Panc-28 cells (2×10^5) were cultured in 6-well plates. After being incubated for 24 h, the cells were untreated or treated with certain concentration of MA or HSF1 siRNA after incubation for 24 h, and the cells were harvested by centrifugation at 1200 × g for 15 min at 4 °C and lysed with RIPA buffer (Beyotime Biological Co., Ltd. Shanghai, China). Protein concentrations were determined using the BCA protein assay kit (Beyotime Biological Co., Ltd. Shanghai, China). Equal amounts of proteins (30—40 μg) were separated by 10% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore). Non-specific

binds were blocked with 5% non—fat dry milk in 1 × TBST buffer (1 × TBS and 0.1% Tween 20) for 1 h at room temperature and incubated with the corresponding primary antibodies overnight at 4℃, including mTOR (Cell Signaling Technology, 1:1,000), HSF1(Cell Signaling Technology, 1:1,000), p-mTOR(Cell Signaling Technology, 1:1,000), Atg7(Proteintech, 1:1,000), ULK1(Cell Signaling Technology, 1:1,000), Atg5 (Cell Signaling Technology, 1:1,000), Beclin-1(Proteintech, 1:1,000), LC3 A/B(Cell Signaling Technology, 1:1,000), Atg3(Proteintech, 1:1,000), and β-Actin (Cell Signaling Technology, 1:1,000). The PVDF membranes were washed for three times with 1 × TBST (1 × TBS and 0.1% Tween-20) and incubated with HRP-labeled goat anti-Mouse IgG secondary antibody (Cell Signaling Technology, 1:2,000) or HRP-labeled goat anti-rabbit IgG secondary antibody (Cell Signaling Technology, 1:2,000) for 1 h at room temperature. The membranes were developed and visualized with chemiluminescence substrate solution (ECL Western detection reagents; Millipore, MA, Burlington, USA). The gray analysis of the protein bands were performed using ImageJ software version 1.52a (National Institutes of Health). All data were representatives of at least three independent experiments.

2.9 Co-immunoprecipitation

The human pancreatic cancer Panc-28 cells (1.5×10^6) were cultured in a 100 mm culture dish, After cultured for 24 h, the cells were treated without or with MA (50 μM) or MG132 (20 μM, Abcam, Cambridge, UK) for 48 h. Then, the cells were lysed with RIPA lysate buffer (Weak) supplemented with protease inhibitor cocktail (Abcam, Cambridge, UK). The total protein concentrations were determined using the BCA protein assay kit (Beyotime Biological Co., Ltd. Shanghai, China). Equivalent protein lysates (1000 μg) were immunoprecipitated with HSF1 antibody. After incubation overnight at 4 °C, 40 μL of protein A/G-Agarose mix (Bimake, PA, USA) were added and gently rotated at 4 °C for 16 h and eluted with PBST (NaCl, 136.89 mM; KCl, 2.67 mM; Na_2HPO_4, 8.1 mM; KH_2PO_4, 1.76 mM; 0.5% Tween20). The eluted samples were separated using 10% SDS-PAGE with the ubiquitin as the secondary antibody (Abcam, 1:500).

2.9.1 SPR assay

HSF1 protein (Abcam, Cambridge, UK) solution were directly immobilized on a NTA biosensor chip (Nicoya, Vancouver, Canada) and 8171 response units (RU) was applied. MA dissolved in a HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA and 0.05% (v/v) surfactant P20) was used as a mobile phase. Certain concentration of MA solution (0, 32, 64, 128, 256, 512 μM) was injected over the protein surface at a rate of 20 μL/min. The binding ability of MA with HSF1 was monitored using an OpenSPR (Nicoya, Vancouver, Canada). The parameter KD (equilibrium dissociation constant) was determined by the TraceDrawer (Ridgeview Instruments ab, Sweden) using the formulae: KD = Koff/Kon (Kon = association rate constant and Koff = dissociation rate constant).

2.9.2 Molecular docking

The 3D structure of MA was obtained from Pubchem Compound in NCBI and the relevant docking proteins used as receptors was obtained from the RCSB protein database (protein number: 7DCS). AutoDock Vina was used for molecular docking, and the LGA algorithm was used to predict the possible configuration of HSF1 docking with MA. The optimal molecular docking structure was selected by minimum energy theory using Pymol program.

2.9.3 Construction of stable HSF1 mutant cell lines

The H63 A and G72 A of HSF1 mutant plasmids were designed and synthesized by Hanheng Biotechnology Co., Ltd. (Shanghai, China). Briefly, the mutated fragments of HSF1 were inserted into the cloning vector (pCDH-CMV-MCS-EF1-Puro) to synthesize the mutant plasmids, and then infected into E. coli. After incubation for 24 h, the mutant plasmids were extracted using the mini plasmid kit (TIANGEN BIOTECH Co., Ltd., Beijing, China). The H63 A and G72 A mutant plasmids were transfected into Panc-28 cells using the Lipofectamine 3000 (Invitrogen, CA, USA) reagent following the manufacturer' instructions, and two stable cell lines, Panc-28/H63 A and Panc-28/G72 A which overexpress H63 A and G72 A of HSF1 mutant proteins separately were developed by screening with G418 (Shanghai McLin Biotech Co., Ltd. Shanghai, China).

2.9.4 Statistical analysis

All of the experiments were repeated for more than 3 times and the data were analyzed by Graphpad Prism software version 8.0 (Graphpad Software, San Diego, CA) and presented as mean ± standard deviation (SD). The comparison between two sets of univariate data was conducted using t-test, and the one-way ANOVA test was used between multiple groups. $P < 0.05$ indicates a significant statistical difference between groups.

3 Results

3.1 HSF1 played a critical role in MA-induced inhibitory effect on pancreatic cancer cells

Panc28 cancer cells were treated with or without MA and the viability of the cells were determined using CCK8 assay kit. The results showed that MA inhibited the growth of Panc-28 cells in a concentration dependent manner (Fig. 1B, Table 1). Our previous study showed that MA inhibited the growth of Panc-28 cells via down-regulation of heat shock protein A8 (HSPA8) [17]. To further reveal the underlying mechanism of MA-induced inhibitory effect on pancreatic cancer cells, the regulation of HSF1, a transcription factor HSPA8 was studied using Western blot and the results showed that treatment of pancreatic cancer cells with MA resulted in downregulation of HSF1 (Fig. 1C, D). We next studied the inhibitory effect of MA in cells downregulated HSF1. The results indicated that knockdown of HSF1 inhibited the growth of cancer cells significantly with an inhibitory rate of 35.7 ± 4.9% at 72 h. However, the inhibitory effect of MA increased in cells downregulated HSF1; the inhibitory rate enhanced from 81.80 ± 6.22% in parent Panc28 cancer cells to 85.92 ± 3.68% at 72 h in cells downregulated HSF1 (Fig. 1E). In contrast, overexpression of HSF1 partially reversed the inhibitory effect of MA on pancreatic cancer cells; the cell viability increased from 12% in the control vector transfected cells (panc28/vec + MA) to 18% in cells overexpressing HSF1 (Panc28/HSF1 + MA) (Fig. 1H). Similar results of colony formation analysis were found in cells treated with MA; the inhibitory effect of MA on colony formation was decreased significantly in cells overexpressing HSF1, while downregulation of HSF1 increased the inhibitory effect on colony formation by MA (Fig. 1F, G, I, J). The results confirmed that HSF1 plays a critical role in MA-induced inhibitory effect on the growth of pancreatic cancer cells.

3.2 MA-induced autophagy is associated with the HSF1 level in pancreatic cancer cells

Previous study has shown that MA was able to induce autophagy in Panc-28 cancer cells [17]. In the present study, the effect of HSF1 in MA-induced autophagy was studied in pancreatic cancer cells. The results showed that downregulation of HSF1 resulted in autophagy in the cancer cells (Fig. 2A). Furthermore, compared with the parent Panc28 cells, the autophagy induced by MA was increased significantly in cells downregulated expression of HSF1; the number of autophagosomes increased from 4.275 in parent Panc28 cancer cells to 14.404 in cells downregulated HSF1 (Fig. 2C). Western blot analysis indicated that the ratio of LC3 II/LC3I was significantly increased in HSF1 knockdown cells treated with MA compared with that in the parent Panc28 cells (Fig. 2B, D), Furthermore, the expression of p-ULK1, Atg3, Atg5 and Atg7 increased significantly, while the expression of p-mTOR declined in HSF1 knockdown cells treated with MA. (Fig. 2B, D). These results indicated that HSF1 also plays important role in MA-induced autophagy in pancreatic cancer cells.

Table 1 The antiproliferative effect of MA

Time (hr)	IC_{50}(μM)
24	56.5 ± 5.52
48	46.8 ± 3.55
72	47.2 ± 6.7

The Panc-28 cells were treated with certain concentrations (0, 6.25, 12.5, 25, 50, 100 and 200 μM) of MA for 24, 48 and 72 h, respectively. The data are representative of three independent experiments (n = 3) in triplicate and expressed as mean ± SD.

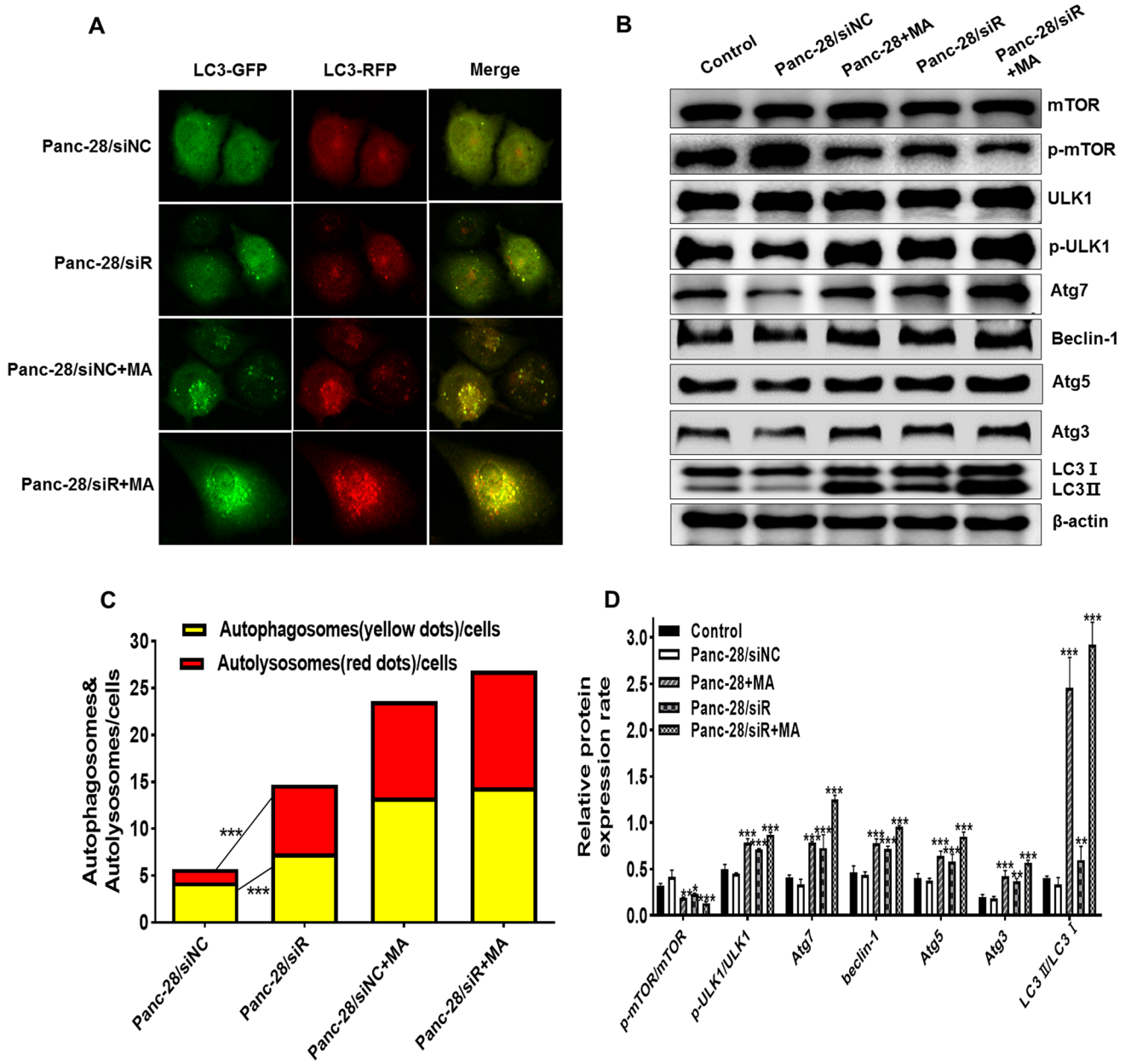


Fig. 2 HSF1 expression was important for MA-induced autophagy in pancreatic cancer cells. **(A)** Pancreatic cancer cells were infected with adenovirus harboring tandem fluo-rescent mRFP-GFP-LC3 at 1,000 multiplicity. After incubation for another 24 h, the cells were treated without or with HSF1 siRNA (Panc-28/siR), scramble siRNA (Panc-28/NC) or the combination of HSF1 siRNA and MA (Panc-28/siR + MA) as well as the combination of scramble siRNA and MA (Panc-28/NC + MA). After cultured for 48 h, the cells were photographed using a laser confocal microscope (Nikon, Tokyo, Japan), and the red and green fluorescence spot was merged. **(C)** Quantitative of the results of (**A**), the autophagy flow. **(B)** The expression of autophagy-related proteins. Wild-type pancreatic cancer cells and HSF1 knock-down cancer cells were treated with MA, the expression of autophagy-related proteins were detected by Western Blot and the gray value was calculated by Image J software **(D)**; Mean ± SD, n = 3, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs* Control group

3.3 MA promoted HSF1 degradation via ubiquitination

The mRNA levels of HSF1 was determined using qRT-PCR, and the results showed that the expression of HSF1 mRNA remained unchanged in Panc-28 cancer cells treated with MA (Fig. 3A), this result suggested that MA-induced down-regulation of HSF1 was occurred at translational level. It is very established that ubiquitination-induced degradation plays an important role in protein degradation in cells. The results showed that in the presence of MG132, a

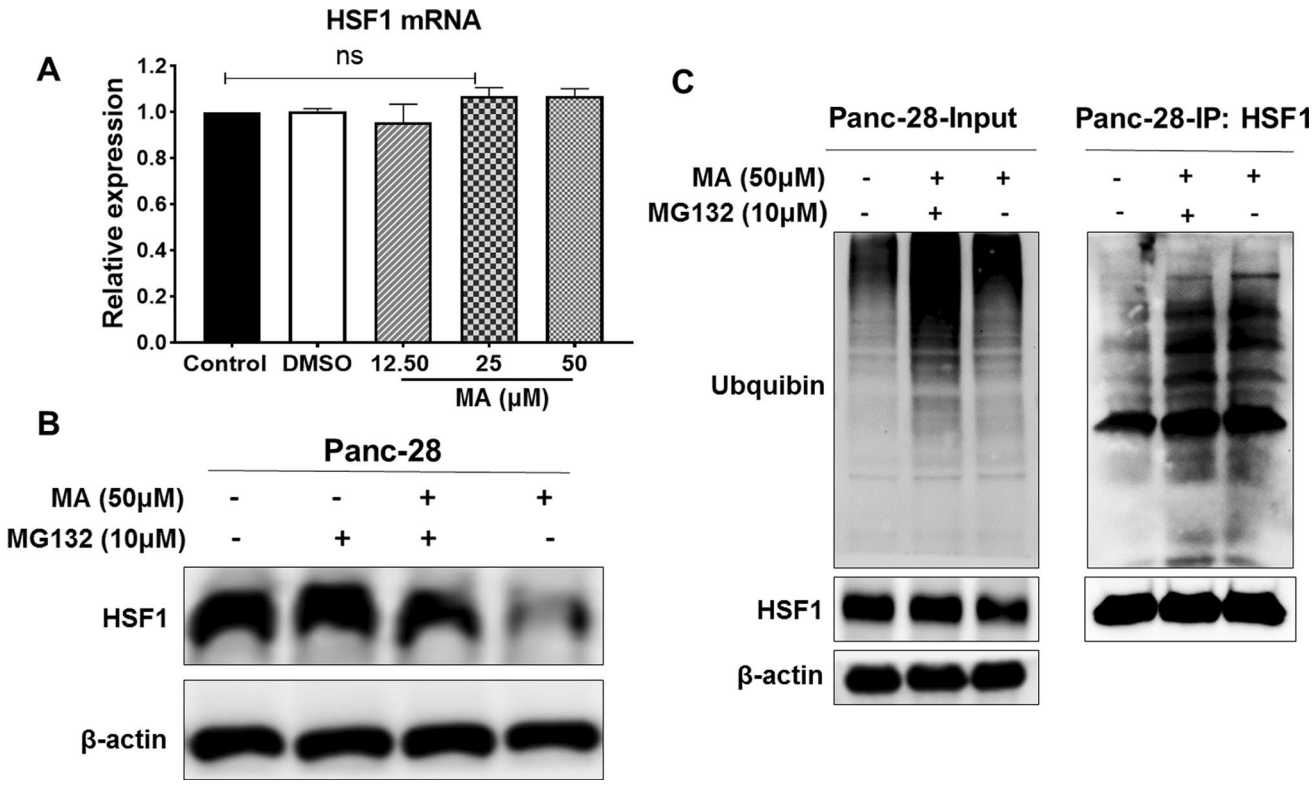


Fig. 3 MA increased the ubiquitination level of HSF1 in pancreatic cancer cells. Panc-28 cells were treated with certain concentrations of MA for 48 h, and the mRNA level of HSF1 was detected by qRT-PCR **(A)**, while the protein expression level of HSF1 was detected by Western blotting in the presence or absence of MG132 **(B)**. **(C)** indicated the ubiquitination level of HSF1 as determined by co-immunoprecipitation. Mean ± SD, n = 3, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs* Control group

proteasome inhibitor, the inhibitory effect of MA on HSF1 expression was attenuated (Fig. 3B). Furthermore, the results of co-immunoprecipitation experiments showed that the ubiquitination levels of HSF1 were increased in cells treated with the combination of MA and MG132 (Fig. 3C). This result provided solid evidence that MA is able to promote the ubiquitination of HSF1 protein.

3.4 Interaction between MA and HSF1

The 3D structure of MA (Fig. 4A) was obtained from Pubchem Compound in NCBI and the relevant docking proteins (Fig. 4B) used as receptors was obtained from the RCSB protein database (protein number: 7DCS). Molecular docking analysis was performed to determine the interaction between MA and HSF1 using Autodock Vina, and the results showed that the binding energy was 6.4 kcal/mol, suggesting there is a strong interaction between MA and HSF1. As shown in Fig. 4C, MA was able to form hydrogen bonds with the amino acid residues, HIS-63 and GLN-72 on HSF1.

SPR (plasma resonance) analysis was performed to determine the interaction between MA and HSF1. The binding and dissociation forces of HSF1 and MA at concentration of 0、32、64、128、256、512 μM were determined using an OpenSPRTM biomolecular interaction analyzer. Our result showed that the dissociation constant KD of MA and HSF1 is 7.69 M, which indicates a strong interaction between MA and HSF1 (Fig. 4D).

3.5 Interaction between MA and HSF1 contributed to the inhibitory effect of MA on the growth of pancreatic cancer cells

In order to elucidate the biological relevance of the interaction of MA and HSF1, two mutant Panc-28 cells, Panc-28/H63 A and Panc-28/G72 A, in which the His and Gln amino acid residues were mutated to Ala separately. A control HSF1 overexpressing Panc-28 cells, Panc-28/HSF1 cells were also constructed, in which the wild type HSF1 was infected into Panc-28 cells. The effect of interaction between HSF1 and MA on the ubiquitination-mediated HSF1

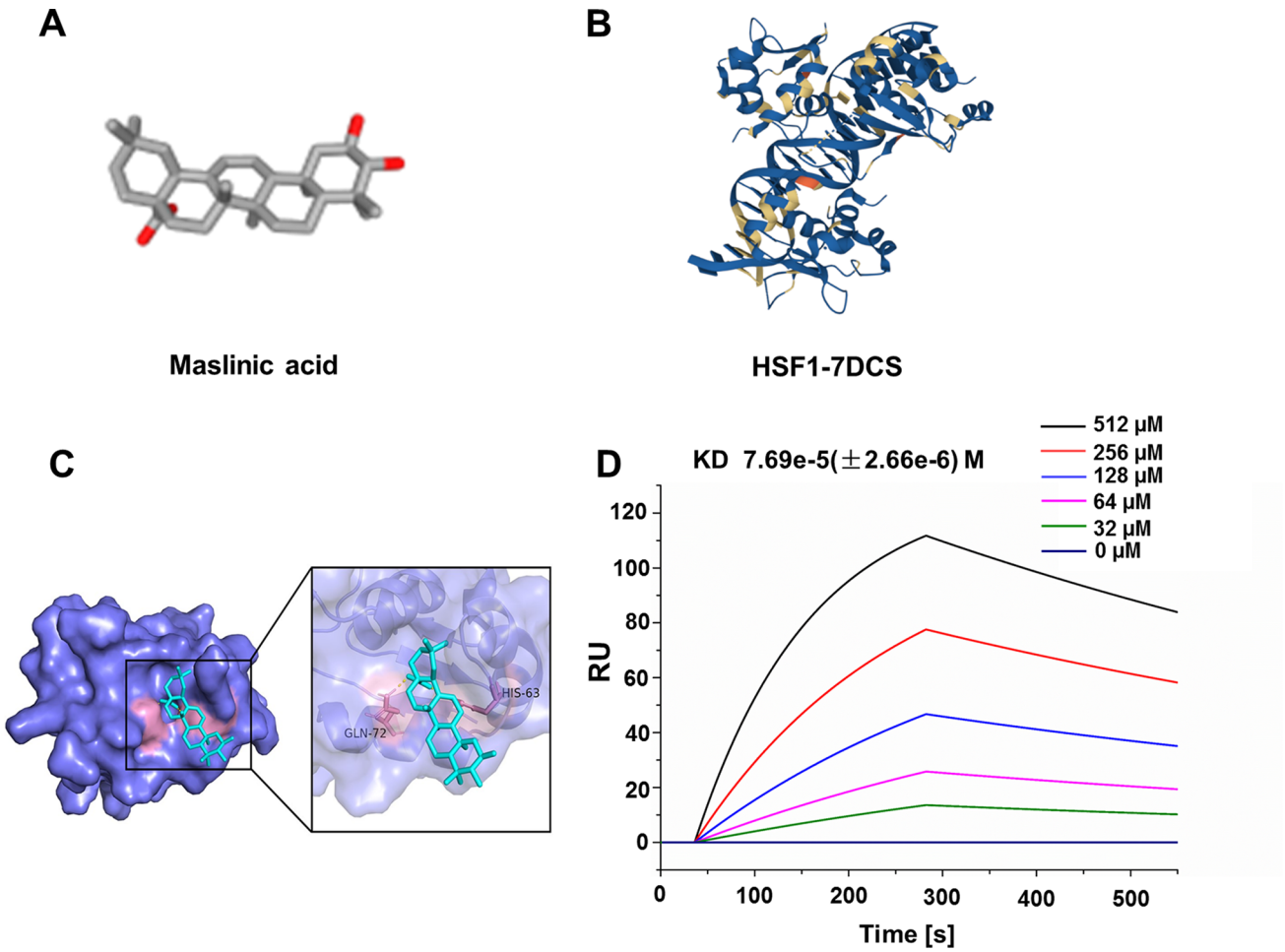


Fig. 4 Interaction between MA and HSF1.The 3D structure of MA was obtained from Pubchem Compound in NCBI **(A)**. AutoDock Vina was used for molecular docking, and the LGA algorithm was used to predict the possible configuration of HSF1 docking with MA. The optimal molecular docking 3D structure of MA and HSF1 was selected by minimum energy theory using Pymol program **(B)**, **(C)** indicated the best docking results of MA and HSF1. SPR analysis was performed to determine the interaction between MA and HSF1 **(D)**

degradation was studied using Western blot analysis. The results showed that in the presence of proteinase inhibitor MG132, the inhibitory effect of MA on the expression of HSF1 was significantly suppressed (Fig. 5A); the inhibitory rate of MA on HSF1 expression decreased from 78 to 12%. However, the inhibitory effect of MA on the HSF1 expression was almost reversed in cells (Pan-28/HSF1) overexpressing HSF1; the expression rate of HSF1 decreased only from 78 to 57% (Fig. 5B). Additionally, the effect of MA on HSF1 expression was almost vanished in the two HSF1 mutant cells. (Fig. 5C, D). The results suggested that the interaction between HSF1 and MA is important for the ubiquitination-mediated degradation of HSF1.

The inhibitory effect of MA on the two mutants Panc-28 (Panc-28/H36 A, Pan-28/G72 A), HSF1 overexpressing (Panc-28/HFS1) and wild-type HSF1 (Parent Panc-28) cells were studied using CCK8 analysis, and the results showed that overexpressing HSF1 partially suppressed the MA's inhibitory effect on the growth of pancreatic cancer cells. However, there were almost no effect of MA on the growth in both mutant pancreatic cancer cells (Fig. 6A); the inhibitory rates were 88.85 ± 3.24 86.48 ± 2.15, 84.25 ± 3.95, and 80.65 ± 4.52 in the parent Panc-28, two mutant Panc-28/H63 A and Panc-28/G72 A cells as well as the HSF1 overexpressing Panc-28/HSF1 cells at 72 h. The expression of autophagic related genes were studied using Western blot analysis and the results showed that overexpression of HSF1 was able to reverse the effect of MA on the expression of p-mTOR, p-ULK1, Atg3,5, 7, Beclin-1, and the ratio of LC3I/LC3II. In contrast, there were almost no effect on the expression of p-mTOR, p-ULK1, Beclin-1, Atg3, 5, 7 in the two HSF1 mutant cells. In addition, the ratio of LC3I/LC3II was also partially declined in cells overexpressing the mutant HSF1 cells compared with that in the cells overexpressing the wild-type HSF1 (Fig. 6B). The results suggested that the interaction between MA and HSF1 played a critical role in MA-induced inhibitory effect on pancreatic cancer cells.

Fig. 5 The interaction between MA and HSF1 played an important role in MA-induced HSF1 ubiquitination. Panc-28 parent cells were ▶ treated with MA in the presence or absence of MG132 (**A**). HSF1 overexpressing Panc28 cells (Panc-28/HSF1) were treated with MA in the presence or absence of MG132 (**B**). The two HSF1 mutant cells, Panc-28/H63 A and Panc-28/G72 A were treated with MA in the presence or absence of MG132 (**C**, **D**). The level of HSF1 was determined by Western blotting. Mean ± SD, n = 3, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs Control group

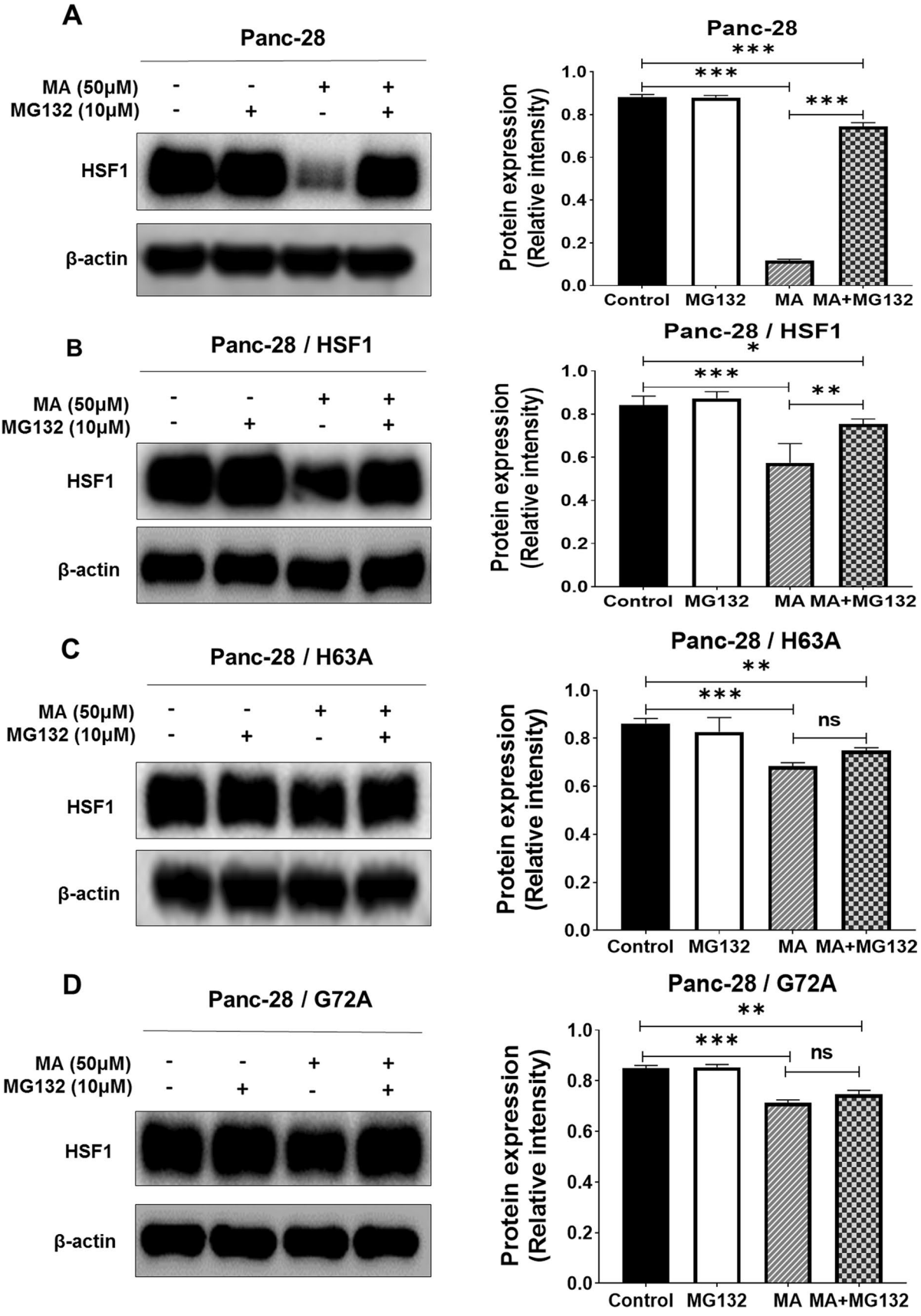
4 Discussion

In the present study, we confirmed that MA downregulated the protein expression of HSF1, and knockdown of HSF1 enhanced the inhibitory effect of MA on pancreatic cancer cells. In contrast, high expression of HSF1 was able to partially reverse the MA-induced inhibitory effect on the growth of pancreatic cancer cells. Our further study showed that there is an interaction between MA and HSF1, and the interaction enhanced the ubiquitin-mediated degradation of HSF1, resulting in the augmentation of autophagy and the inhibitory effect on the growth of pancreatic cancer cells (Fig. 7).

Compared with most anticancer agents, the advantage of MA is its low toxicity; study has shown that repeated daily oral administration of 50 mg/kg of MA in Swiss CD-1 male mice for 28 d did not induce any sign of toxicity as analyzed by hematology, clinical biochemistry, and histopathology evaluation [11, 12]. It is promising to develop MA as an adjuvant or a scaffold to design novel anticancer agents due to its high safety and potent anticancer effect. Studying is ongoing in our laboratory to synthesize novel MA derivatives targeting HSF1 as novel anticancer agents for the treatment of pancreatic cancer.

Our study confirmed that the interaction between HSF1 with the two amino acid residues increased the ubiquitin-mediated degradation of HSF1. It is possible that the change of 3D structure of HSF1 as well as acetylation and the phosphorylation status of some amino acids residues on HSF1 molecules contributes the increased ubiquitination of HSF1. It is well established that ubiquitination as a main posttranslational modification, the ubiquitin moiety is covalently attached to a target protein to affect protein stability and biological function [18, 19]. Further study is needed to address if there are a change of 3D structure of HSF1 and if the status of the phosphorylation of some amino acid residues on HSF1 molecules have been changed.

Heat Shock Factor 1 (HSF1) is a master regulator of heat shock responsive signaling. In addition to playing critical roles in cellular heat shock response, emerging evidence suggests that HSF1 also regulates a non-heat shock responsive transcriptional network to handle metabolic, chemical, and genetic stress. The function of HSF1 in cellular transformation and cancer development has been extensively studied and HSF1-targeted therapy for cancer has attracted great attention in recent years. Several strategies have been developed via targeting HSF1 as anticancer targets including interference with HSP90-HSF1 dissociation or HSF1 translocation and trimerization. Other mechanisms involve inhibition of HSF1 by targeting post translational modifications or intramolecular interactions with multichaperone complexes or cellular proteins [20]. Quercetin, a flavonoid isolated from Tripterygium wilfordii induced apoptosis effectively via suppressing the expression of Hsp27, Hsp70 and Hsp90 in MCF-7 breast cancer cells [21]. Cantharidin, a terpenoid isolated from blister beetles and other insects displayed broad anticancer activity on several cancer cells, including hepatoma, multiple myeloma. Pancreatic cancer and colon cancer cells. Recent studies showed that Cantharidin was able to block the binding of HSF1 with the promotor of HSP70.resulting in apoptosis of HCT116 colon cancer cells [22]. However, it is widely accepted that developing HSF1 inhibitor is difficult due to a lack of potential target sites in its tertiary structure[20]. In our present study, we confirmed that MA was able to interact with HSF1 specifically via two amino acid residues, and the interaction enhanced the ubiquitin-mediated HSF1 degradation. Our study provides a novel strategy for developing anticancer agents via targeting the MA-binding site of HSF1.


A
Panc-28
MA (50μM) - - + +
MG132 (10μM) - + - +
HSF1
β-actin
Panc-28
***
***
***
Protein expression (Relative intensity)
1.0
0.8
0.6
0.4
0.2
0.0
Control MG132 MA MA+MG132
B
Panc-28 / HSF1
MA (50μM) - - + +
MG132 (10μM) - + - +
HSF1
β-actin
Panc-28 / HSF1
*
***
**
Protein expression (Relative intensity)
Control MG132 MA MA+MG132
C
Panc-28 / H63A
MA (50μM) - - + +
MG132 (10μM) - + - +
HSF1
β-actin
Panc-28 / H63A
**
***
ns
Protein expression (Relative intensity)
Control MG132 MA MA+MG132
D
Panc-28 / G72A
MA (50μM) - - + +
MG132 (10μM) - + - +
HSF1
β-actin
Panc-28 / G72A
**
***
ns
Protein expression (Relative intensity)
Control MG132 MA MA+MG132

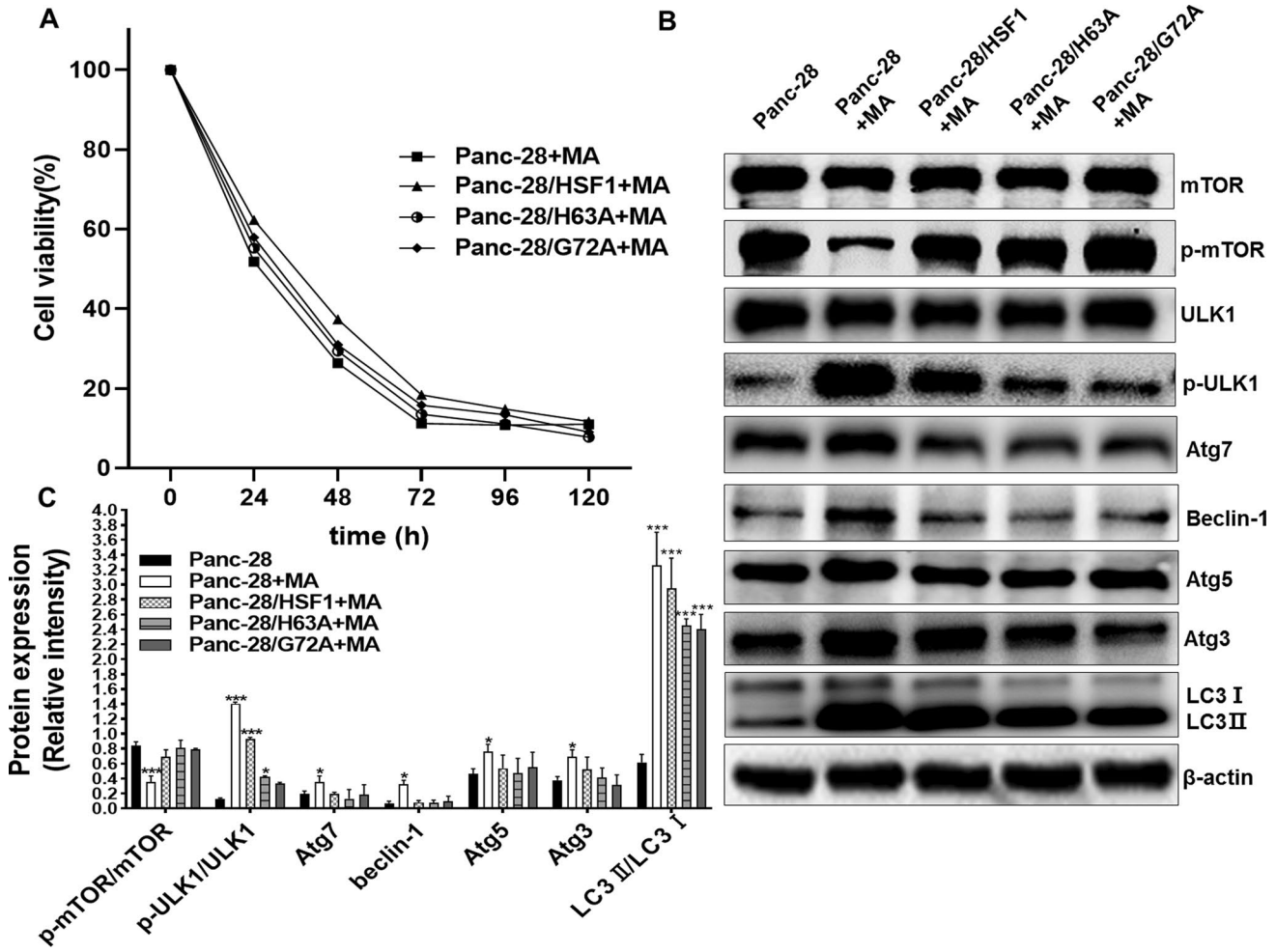


Fig. 6 The interaction between MA and HSF1 played a critical role in MA-induced growth inhibition and autophagy in pancreatic cancer cells. Cells were treated with MA for 48 h, and the cell viability was determined using CCK8 assay **(A)**. The expression level of autophagic related genes was determined using Western blotting analysis **(B, C)**. Each experiment was performed triplicates. Mean ± SD, n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* Control group

5 Conclusion

There is a specific interaction between MA and HSF1 in pancreatic cancer cells, and the interaction enhanced the ubiquitin-mediated degradation of HSF1, which contributed to the suppression on the growth of pancreatic cancer. The study provides evidence that targeting the binding sites of MA on HSF1 could be developed as a novel strategy for developing anticancer agents for the treatment of pancreatic cancer.

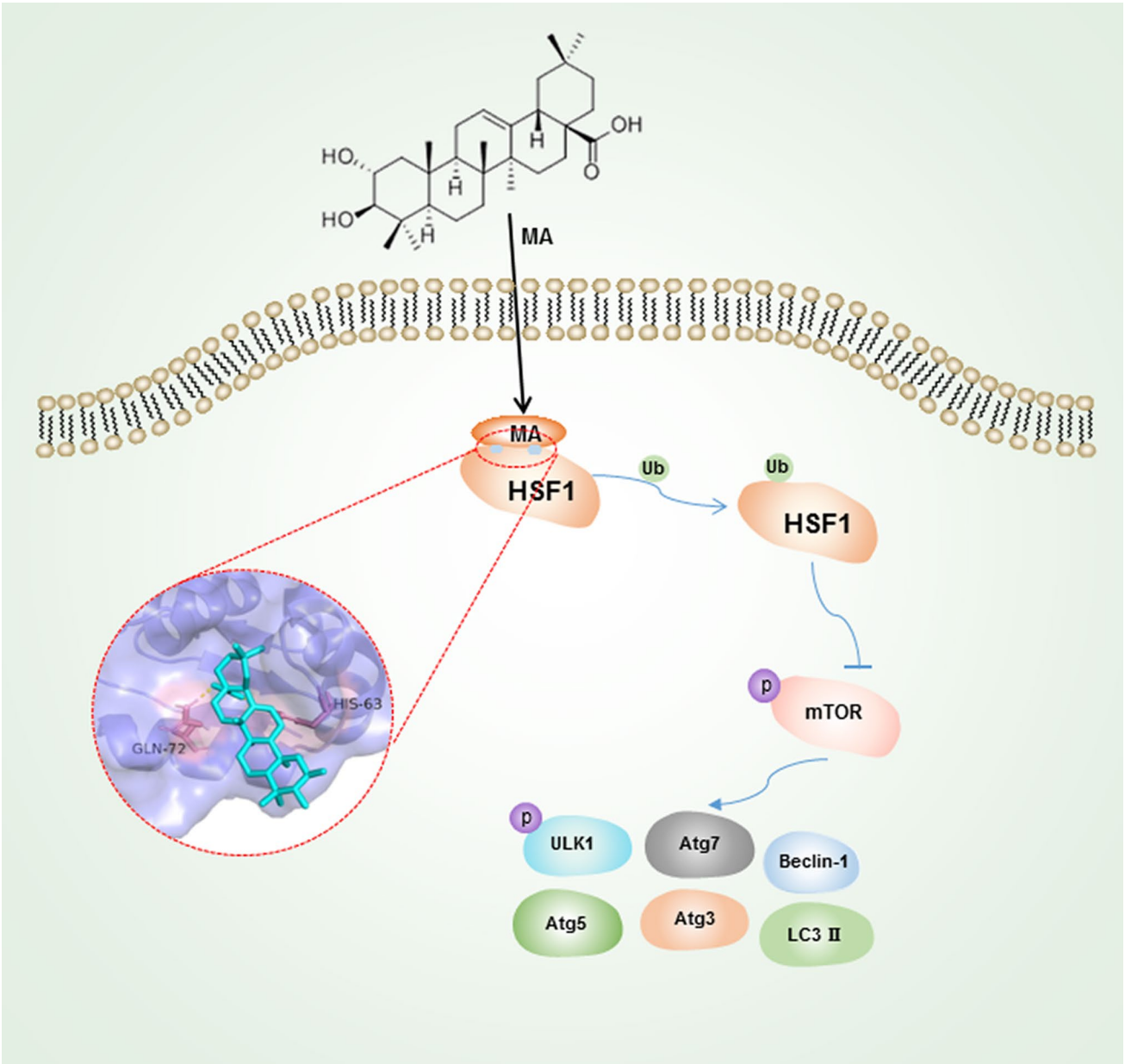


Fig. 7 The schematic diagram of MA-induced inhibitory effect on pancreatic cancer cells. MA binds with the GLN72 and HIS63 on HSF1 molecule, and the interaction between MA and HSF1 enhanced the ubiquitin-mediated degradation of HSF1, resulting in increased autophagy, resulting in the inhibitory effect of pancreatic cancer cells

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Author contributions Conceptualization, X.L.; data curation, L.C. and M.G.; formal analysis, L.C. and L.W.; investigation, L.C., M.G., Y.Z., H.Y. and X.L.; methodology, L.C., M.G., A.F., L.W. and G.Q.; resources, X.L.; supervision, A.F. and X.L.; validation M.G., L.W., Y.Z., H.Y. and G.Q.; visualization, L.C; writing-original draft, L.C., and M.G.; writing—review & editing, A.F., L.W., Y.Z., H.Y., G.Q. and X.L.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Competing interests The authors declare no competing interests.

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