Inducible HSP70 antagonizes cisplatin-induced cell apoptosis through inhibition of the MAPK signaling pathway in HGC-27 cells

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Abstract. Inducible heat shock protein 70 (HSP70; also known as HSPA1 or HSP72) is implicated in cancer. As a stress-inducible heat shock protein, HSP70 is highly expressed in a variety of cancers and correlates with metastasis, chemotherapy resistance and tumor prognosis. The present study demonstrated that suppression of HSP70 through the specific inhibitor pifithrin-μ or by HSP70 knockdown enhanced cisplatin-induced apoptosis in HGC-27 gastric cancer cells. By contrast, upregulation of HSP70 through transfection of a HSP70 overexpressing plasmid decreased cisplatin-induced HGC-27 cell apoptosis. In exploring the underlying molecular mechanisms, the present results revealed that HSP70 antagonized cisplatin-induced HGC-27 cell apoptosis by regulating the mitogen-activated protein kinase (MAPK) signaling pathway. In addition, suppressing the MAPK pathway enhanced cisplatin-induced HGC-27 cell apoptosis. Collectively, the present findings suggest that inhibition of HSP70 expression enhanced the sensitivity of HGC-27 cells to cisplatin via the MAPK signaling pathway, and that HSP70 may serve as a potential therapeutic target in gastric cancer.

Introduction

Gastric cancer is a common and frequently occurring malignancy worldwide. At present, systemic chemotherapy is the most common clinical treatment for gastric cancer. Cisplatin (DDP) is a well-known chemotherapeutic drug, that is widely used in chemotherapy of multiple types of cancer, including gastric cancer (1). However, development of chemotherapy resistance significantly reduces its therapeutic effect in gastric cancer. The regulatory molecules involved in resistance in gastric cancer remain not fully elucidated and require further study.

The heat shock proteins (HSPs) are a highly conserved protein family consisting of several members. As molecular chaperones, HSPs have been reported to be involved in the folding, modification and degradation of intracellular proteins (2,3). The stress-inducible HSP70 (also known as HSPA1 or HSP72) is an important member of the HSP family. Recent studies have demonstrated that HSP70 is expressed at extremely low levels in unstressed normal cells, but is highly expressed in a variety of cancers and correlated with tumor grade, metastasis, chemotherapy resistance and poor prognosis (4-6). Because of its tumor-related expression and important role in cancer progression, HSP70 has become an attractive target for cancer therapy (7,8).

The primary aim of the present study was to investigate the effect of HSP70 in the cisplatin-induced apoptosis of gastric cancer cells and to explore its molecular mechanism. The results demonstrated that downregulating HSP70 expression enhanced cisplatin-induced gastric cancer apoptosis via regulation of the mitogen-activated protein kinase (MAPK) signaling pathway. The present study provided an experimental basis and novel insights for HSP70 as a target for cancer therapy.

Materials and methods

Reagents and antibodies. Cisplatin (purity, ≧99.9) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), and pifithrin-μ (PES), SB203580, SP600125 and U0126 were purchased from Selleck Chemicals (Houston, TX, USA). All of the above reagents were dissolved in DMSO and then diluted with sterilized PBS. The final concentration of DMSO in cultures was <0.1%. Rabbit monoclonal antibodies

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against p38 (cat. no. 9219s; 1:1,000), phosphorylated (p)-p38 (Thr180/Tyr182; cat. no. 9215s; 1:1,000), extracellular signal-regulated kinase (ERK, cat. no. 9102s; 1:1,000), p-ERK (Thr202/Tyr204; cat. no. 4376s; 1:1,000), c-Jun N-terminal kinase (JNK; cat. no. 9252s; 1:1,000), p-JNK (Thr183/Tyr185; cat. no. 4671s; 1:1,000), p-SRC proto-oncogene non-receptor tyrosine kinase (Src, Tyr416; cat. no. 6943s; 1:500), p-AKT serine/threonine kinase 1 (Akt, Ser473; cat. no. 4060s; 1:500), p-inhibitor of κB (IκB, Ser32; cat. no. 2859s; 1:500), poly-ADP-ribose-polymerase (PARP, cat. no. 9532s; 1:1,000), pro-caspase-3 (cat. no. 9662s; 1:500), cleaved caspase-3 (cat. no. 9661s; 1:500), β-actin (cat. no. 4970s; 1:1,000), GAPDH (cat. no. 5174s; 1:1,000), and rat monoclonal antibody against HSP70 (cat. no. 4573s; 1:1,000) were all from Cell Signaling Technology, Inc. (Beverly, MA, USA). All secondary antibodies (cat. no. 926-32210 or 926-32211; 1:5,000) were purchased from LI-COR Biosciences (Lincoln, NE, USA).

Cell culture. The human gastric cancer HGC-27 cell line was obtained from GuangZhou Cellcook Biotech Co., Ltd. (Guangzhou, China). Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 10% non-essential amino acids, 100 μg/ml streptomycin and 100 U/ml penicillin and incubated at 37˚C in an atmosphere of 5% CO_2.

Plasmids and transfection. Green fluorescent protein (GFP)-labeled HSP70 overexpression plasmids and negative plasmids (empty vector control plasmid; CMV-MCS-EGFP-SV40-Neomycin), and HSP70 short hairpin (sh) RNA and negative plasmids (non-targeting shRNA plasmid; hU6-MCS-CMV-GFP-SV40-Neomycin), were purchased from GeneChem Co., Ltd. (Shanghai, China). HGC-27 cells were seeded into 12-well cell culture plates and cultured to 60-70% confluence, prior to transfection with plasmids for the indicated times using Lipofectamine® 3000 Reagent (Thermo Fisher Scientific, Inc.). Operations were performed according to the manufacturers instructions.

Annexin V/propidium iodide (PI) double staining assay. Apoptosis rate was detected by Annexin V/PI double staining (Jiancheng Bioengineering Institute, Nanjing, China). Briefly, following treatment, HGC-27 cells were collected and washed two times with PBS. Cells were resuspended in 500 μl of binding buffer, and 5 μl PI and 5 μl Annexin V-fluorescein isothiocyanate were added to the cells. Apoptosis rate was detected by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

DAPI staining. Following treatment, HGC-27 cells were washed three times with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. After rising with PBS, cells were stained with DAPI for 3 min in the dark. The morphology of apoptotic cells was examined using an inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Western blotting. HGC-27 cells were washed three times with cold PBS and then lysed in RIPA buffer containing protease inhibitors (Beyotime Institute of Biotechnology, Haimen, China) for 30 min on ice. Lysates were centrifuged (14,300 x g) at 4˚C for 15 min, and the supernatant was collected. The bicinchoninic acid assay was used to measure protein concentration in the supernatant, and equal amounts of total protein (50 μg) were loaded onto 12% gels for SDS-PAGE. Separated proteins were transferred onto nitrocellulose membranes (Pall Corporation, Port Washington, NY, USA), which then were blocked with 5% skim milk for 1 h at room temperature and then incubated with the indicated primary antibodies overnight at 4˚C. After washing three times with TBST, membranes were incubated with IRDye800 fluorophore-conjugated secondary antibody for 1 h at room temperature in the dark. Antigen-antibody complexes were detected using a LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences) and LI-COR Odyssey analysis software was used to quantify the proteins.

Cell Counting Kit-8 (CCK-8) assay. HGC-27 cells were seeded into 96-well cell culture plates, and the following day they were treated with different doses of PES for 24 h. CCK-8 was then added at 10 μl/well. Following incubation for 2 h at 37˚C and 5% CO_2, absorbance at 450 nm was measured using a Multiskan GO plate reader (Thermo Fisher Scientific, Inc.). Each experiment was repeated three times.

Statistical analysis. Data were expressed as mean ± standard deviation. Statistical analysis was performed using SPSS17.0 software (SPSS, Inc., Chicago, IL, USA). The results were compared using one-way analysis of variance followed by a post hoc Tukey test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HSP70 overexpression antagonizes cisplatin-induced HGC-27 cell apoptosis. HGC-27 cells were transfected with GFP-HSP70 or control plasmid, and 24 h post-transfection they were stimulated with cisplatin (5 μg/ml) for 24 h. The morphology of apoptotic cells was observed with DAPI staining and the apoptosis rate was determined by flow cytometry. The expression of apoptosis-related proteins, including PARP, pro-caspase-3 and cleaved caspase-3, were detected by western blotting. HGC-27 cells transfected with control plasmid and not treated with cisplatin had light blue, round nuclear morphology as evident by DAPI staining (Fig. 1A). However, following cisplatin treatment, the nuclei exhibited morphological changes typical of apoptosis, including nuclear condensation and nuclear fragmentation (Fig. 1A). These typical cisplatin-induced morphological changes were clearly reversed in HSP70-overexpressing HGC-27 cells (Fig. 1A). In addition, cisplatin-induced expression of cleaved PARP and cleaved caspase-3 was decreased, while pro-caspase-3 levels were increased, in GFP-HSP70-overexpressing cells compared with cells transfected with control plasmid (Fig. 1B). Flow cytometry was also used to determine the apoptotic rate of HGC-27 cells induced by cisplatin treatment. The apoptotic rate was 31.38% in control plasmid-transfected cells, while it was reduced to 21.2% in HSP70-overexpressing cells (Fig. 1C).

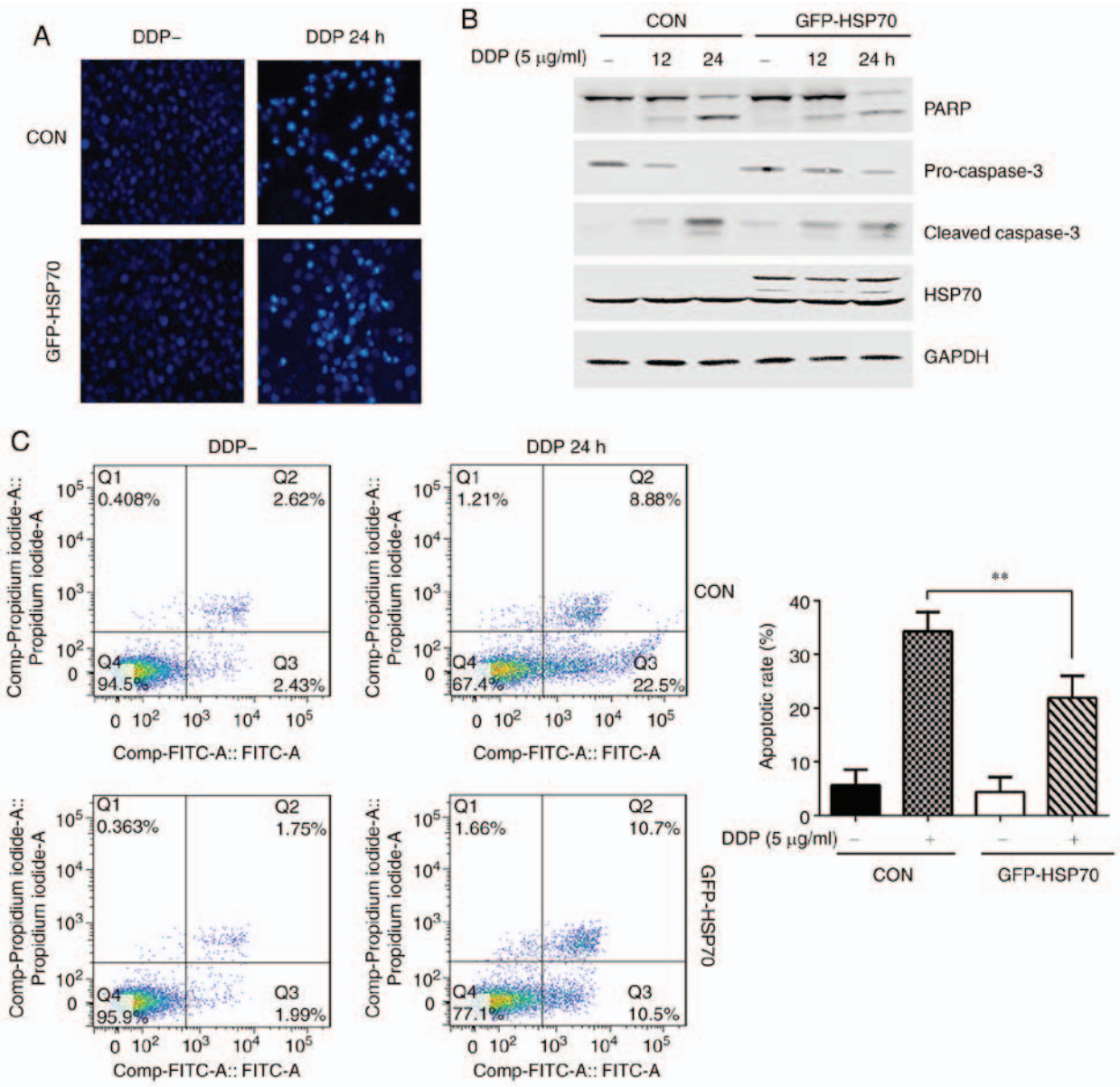

Figure 1. HSP70 overexpression antagonizes cisplatin-induced HGC-27 cell apoptosis. HGC-27 cells were transfected with GFP-HSP70 plasmid or control plasmid. After 24 h, cells were stimulated with 5 μg/ml cisplatin for the indicated times. (A) Nuclear morphology of apoptotic cells was detected by DAPI staining (magnification, x100). (B) Expression levels of apoptosis-related proteins PARP, pro-caspase-3 and cleaved caspase-3 were detected by western blotting. (C) Apoptosis rate was determined by flow cytometry (representative plots and quantification is shown). **P<0.01, with comparisons indicated by lines. HSP70, heat shock protein 70; GFP, green fluorescent protein; PARP, poly-ADP-ribose-polymerase; DDP, cisplatin; CON, control.

Downregulation of HSP70 or suppression of its function enhances cisplatin-induced HGC-27 cell apoptosis. To further explore the role of HSP70 in cisplatin-induced apoptosis, a HSP70 inhibitor, PES, was used to treat the cells. PES interacts selectively with the stress-inducible HSP70 protein and inhibits its function (2,9,10). Firstly, the cytotoxicity of PES was determined using the CCK-8 assay. HGC-27 cells were treated with different concentrations of PES (1, 2, 4, 6, 8, 10 or 20 μM) for 24 h, and cell viability was analyzed by CCK-8 assay. PES did not affect cell viability, even at a dose of 10 μM (Fig. 2A); the dose of 8 μM was therefore selected for subsequent experiments. Secondly, the effects of PES on cisplatin-induced cell apoptosis were examined. Western blot analysis indicated that PES pretreatment increased the expression of cleaved PARP and cleaved-caspase-3, and decreased the levels of pro-caspase-3, compared with cells treated with cisplatin alone (Fig. 2B). An Annexin V/PI double staining assay indicated that the apoptotic rate in cisplatin-stimulated cells was 18.2%, while inhibition of HSP70 by PES increased this to 41.2% (Fig. 2C).

As a second method to suppress HSP70 function and explore its effect on cisplatin-induced apoptosis, HSP70-specific shRNA was transfected in the cells to downregulate its expression. The nuclear condensation and nuclear fragmentation induced by cisplatin in HSP70 shRNA-transfected cells were obviously enhanced compared with control plasmid-transfected HGC-27 cells (Fig. 3A). The levels of cleaved PARP and cleaved caspase-3 following cisplatin stimulation were increased, while pro-caspase-3 levels were decreased, in the HSP70 shRNA-transfected cells compared

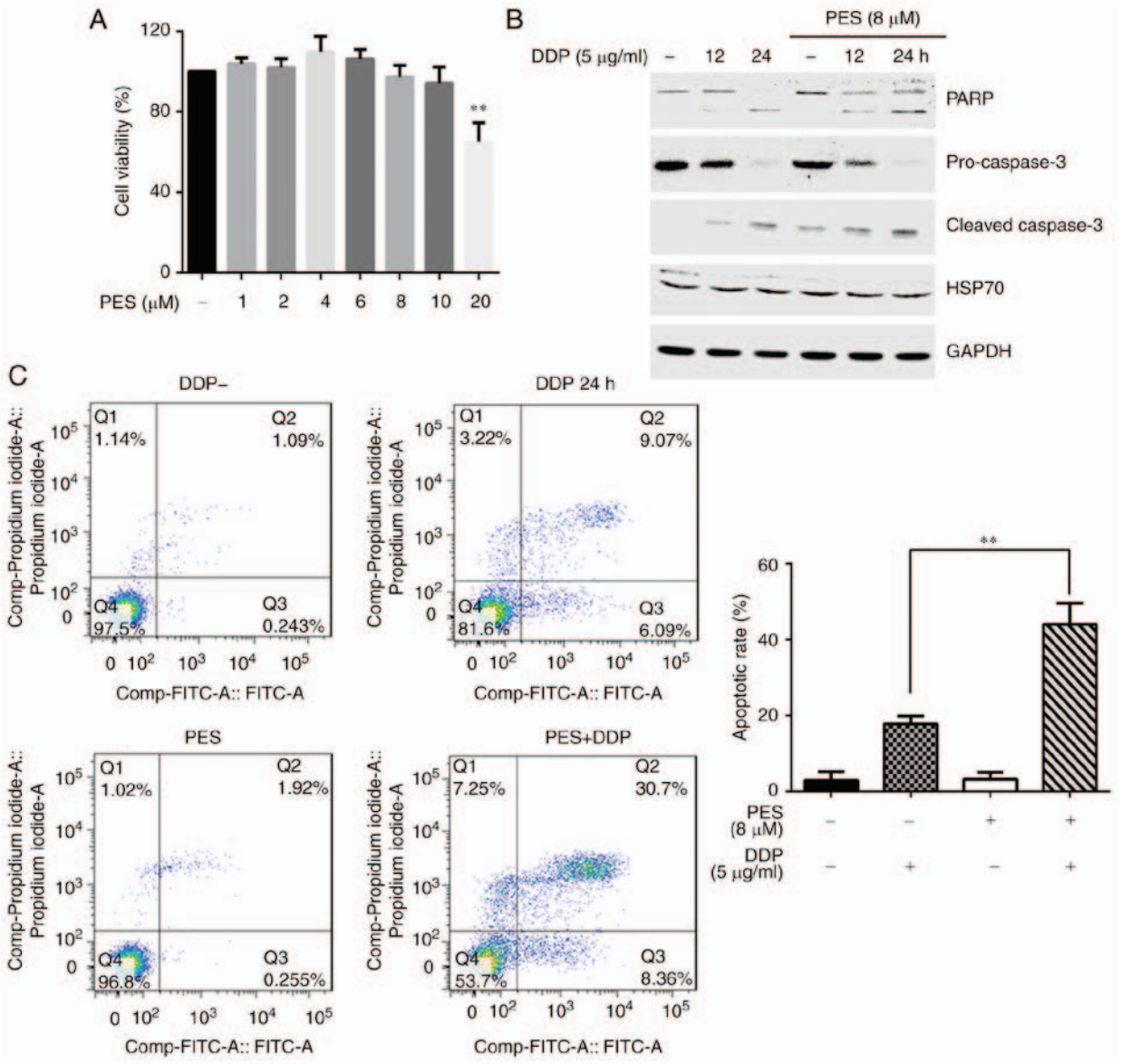

Figure 2. Pretreatment with an HSP70 inhibitor enhances cisplatin-induced HGC-27 cells apoptosis. (A) HGC-27 cells were treated with different concentrations of PES for 24 h, and cell viability was measured using a Cell Counting Kit-8 assay. **P<0.01 compared with untreated cells. (B) HGC-27 cells were pretreated with PES (8 μM) for 2 h and then stimulated with cisplatin (5 μg/ml) for 24 h. Expression levels of apoptosis-related proteins PARP, cleaved-caspase-3 and pro-caspase-3 were detected by western blotting. (C) Apoptosis rate was determined by flow cytometry (representative plots and quantification is shown). **P<0.01 with comparisons indicated by lines. HSP70, heat shock protein 70; PES, pifithrin-μ; PARP, poly-ADP-ribose-polymerase; DDP, cisplatin.

with control (Fig. 3B). Concurrently, the apoptotic ratio was 45.8% in HSP70 shRNA-transfected cells and 27.15% in the control (Fig. 3C). Taken together, these results suggest that HSP70 serves a protective role in cisplatin-induced HGC-27 cell apoptosis.

HSP70 affects cisplatin-induced MAPK signaling pathway activation. MAPK and survival-related signaling pathways, such as Akt and nuclear factor (NF)-κB, have an important role in the proliferation, differentiation and apoptosis of tumor cells (11-13). To investigate whether the molecular mechanism of HSP70 regulation of cisplatin-induced apoptosis is modulated by the MAPK, Akt or NF-κB signaling pathways, HGC-27 cells were treated with cisplatin for different amounts of time, and the phosphorylation of p38, ERK, JNK, Src, Akt and IκB were monitored by western blotting. Cisplatin stimulation resulted in a time-dependent increase in the phosphorylation of p38, ERK and JNK, but had no effect on the total levels of p38, ERK and JNK proteins (Fig. 4A). Phosphorylation of Src, Akt and IκB was also detected, but this was unaffected by cisplatin treatment (Fig. 4B). These results suggested that the MAPK signaling pathway may be involved in cisplatin-induced apoptosis.

To further investigate the effects of HSP70 on cisplatin-induced phosphorylation in the MAPK signaling pathway, HSP70-overexpressing plasmids were transfected into HGC-27 cells. HSP70 overexpression did not exert any effects on p38, ERK or JNK phosphorylation compared with empty vector controls (Fig. 4C). Conversely, PES was used to inhibit HSP70 function, and HSP70 inhibition resulted in a striking reduction of cisplatin-induced phosphorylation of p38, ERK and JNK (Fig. 4D). HSP70 shRNA transfection

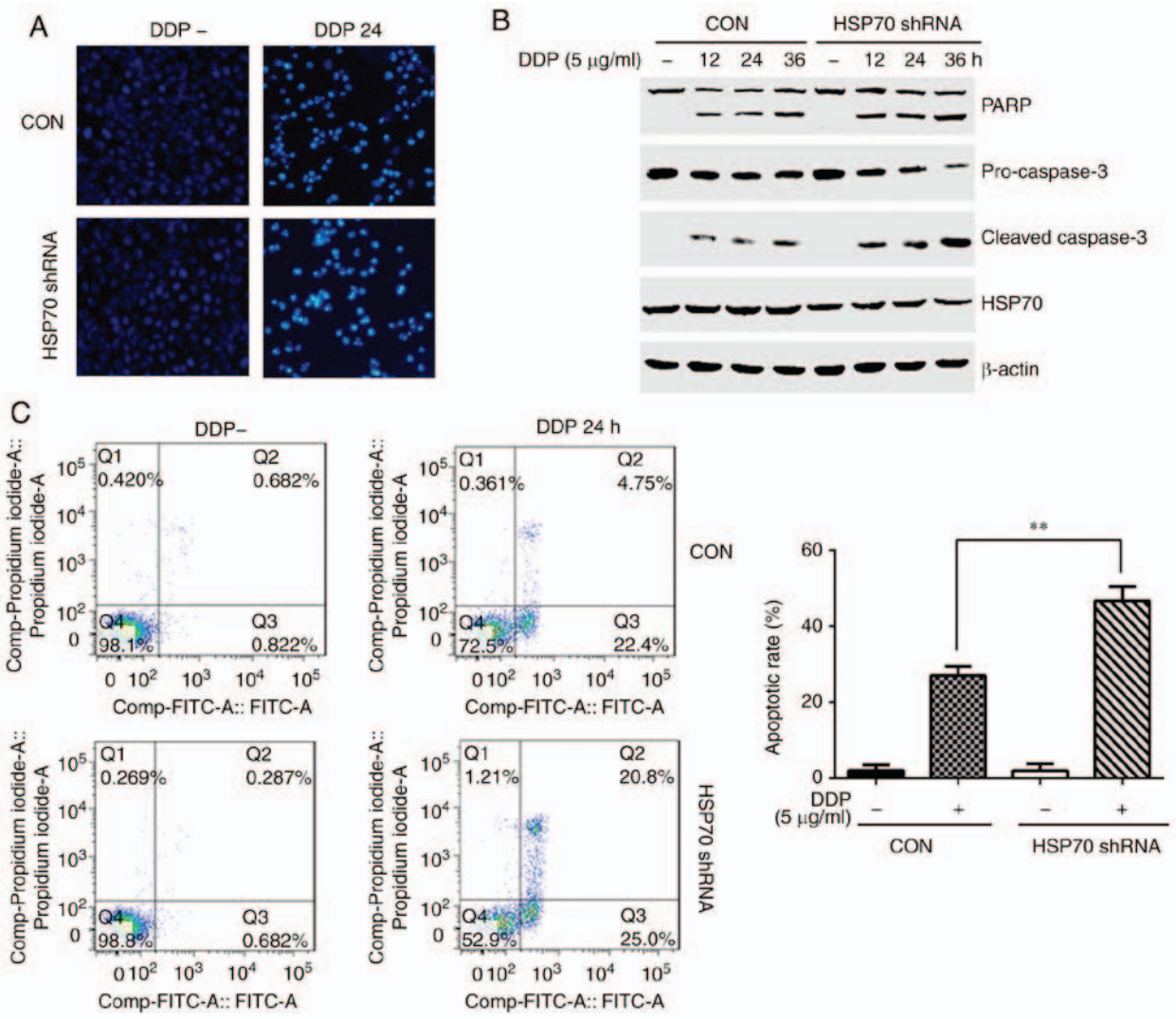

Figure 3. HSP70 downregulation enhances cisplatin-induced HGC-27 cell apoptosis. HGC-27 cells were transfected with HSP70 shRNA plasmid and control plasmid, and at 48 h post-transfection, cells were stimulated with 5 μg/ml cisplatin for the indicated times. (A) The morphology of apoptotic cell nuclei was detected by DAPI staining (magnification, x100). (B) Expression levels of apoptosis-related proteins PARP, pro-caspase-3 and cleaved caspase-3 were detected by western blotting. (C) Flow cytometry was used to determine apoptosis rates (representative plots and quantification is shown). **P<0.01 with comparisons indicated by lines. HSP70, heat shock protein 70; sh, short hairpin; PARP, poly-ADP-ribose-polymerase; DDP, cisplatin; CON, control.

also suppressed cisplatin-induced phosphorylation of p38, ERK and JNK (Fig. 4E). Collectively, these results suggest that HSP70 facilitates the activation of p38, ERK and JNK in HGC-27 cells.

MAPK pathway inhibition enhances cisplatin-induced HGC-27 cell apoptosis. To investigate the role of MAPK signaling in the process of cisplatin-induced HGC-27 cell apoptosis, cells were stimulated with cisplatin or cisplatin and a p38-specific inhibitor (SB203580), a JNK specific inhibitor (SP600125) or an ERK1/2 inhibitor (U0126) for 24 h. All inhibitor pretreatments enhanced the expression of cleaved PARP and cleaved caspase-3 and decreased the levels of pro-caspase-3 induced by cisplatin (Fig. 5A). Additionally, compared with cisplatin-only treated cells, apoptosis levels were significantly elevated in cells stimulated with cisplatin and the inhibitors (Fig. 5B). Finally, in order to confirm the inhibitory effects of each inhibitor, the phosphorylation levels of p38, ERK or JNK were respectively detected. Fig 5C illustrates that each inhibitor treatment suppressed its corresponding molecule, while having little effect on HSP70 protein expression levels. Collectively, these findings suggest that the MAPK signaling pathway antagonized cisplatin-induced apoptosis in HGC-27 cells.

Discussion

HSPs are chaperone proteins, which are induced by a variety of different stresses. As a member of this family, HSP70 has been reported to be highly expressed in different types of cancer (6) and is considered to be a negative prognostic factor in breast cancer, osteosarcoma and bladder cancer. By contrast, it has been reported as a positive prognostic factor in esophageal, pancreatic and renal cancers (14-16). Recently, multiple studies have demonstrated that HSP70 is involved in resistance to anticancer agents in tumors (14,17,18); however, the molecular mechanism of its action in resistance to chemotherapy remains unclear.

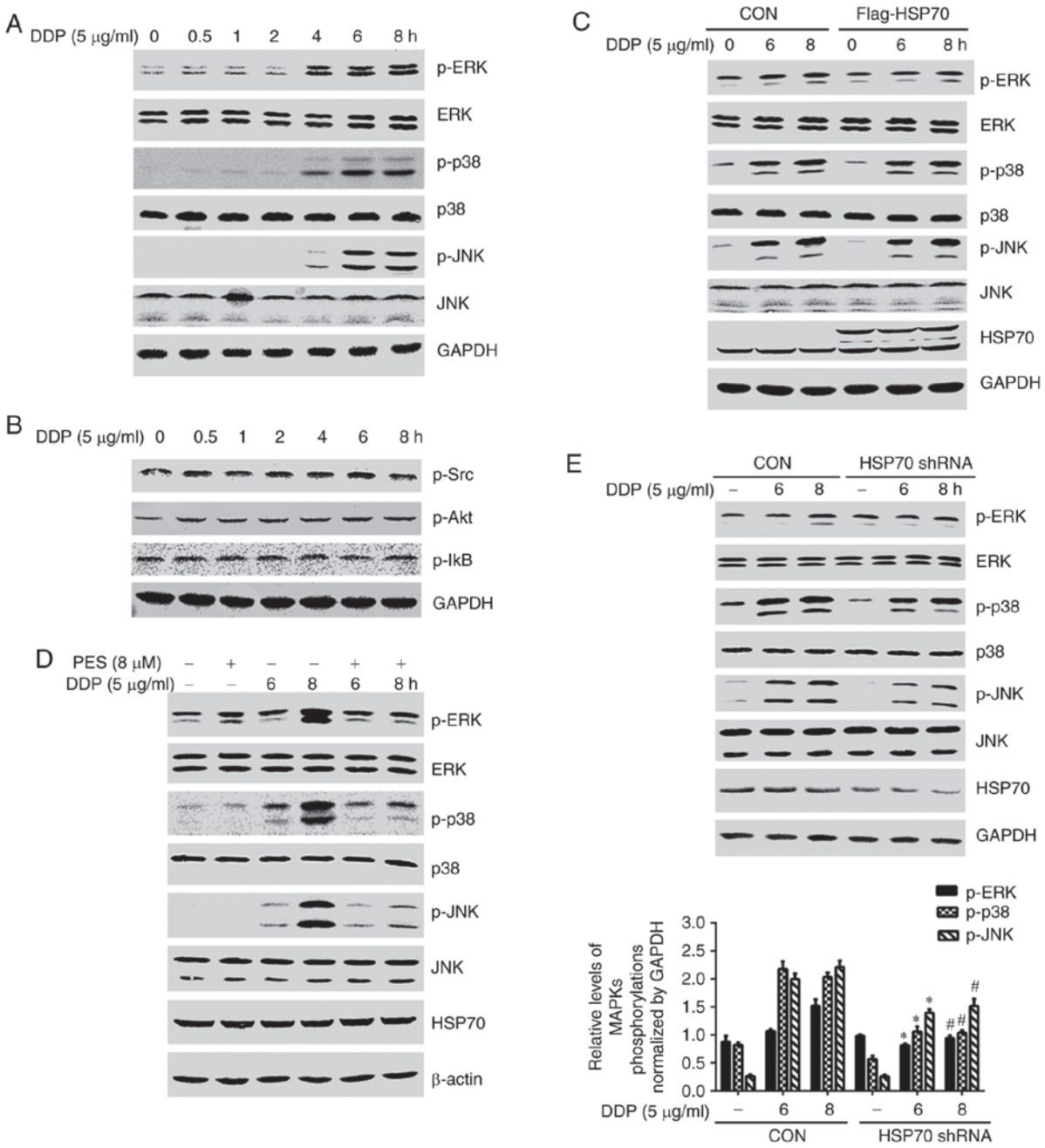

Figure 4. HSP70 affects cisplatin-induced MAPK signaling pathway activation. (A) HGC-27 cells were treated with cisplatin (5 μg/ml) for different amounts of time and the phosphorylation and total protein levels of (A) p38, ERK and JNK, and (B) Src, Akt and IκB, were monitored by western blotting. (C) HSP70-overexpressing and control plasmids were transfected into HGC-27 cells. (D) HGC-27 cells were pretreated with PES for 2 h and then stimulated with cisplatin for 6 or 8 h. (E) HSP70-shRNA and control plasmids were transfected into HGC-27 cells. After transfection, the cells were treated with cisplatin for 6 or 8 h and the phosphorylation and total protein levels of p38, ERK and JNK were monitored by western blotting. *P<0.05 compared with control cells at 6 h; $^{\#}$P<0.05 compared with control cells at 8 h. HSP70, heat shock protein 70; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; Src, SRC proto-oncogene non-receptor tyrosine kinase; Akt, AKT serine/threonine kinase 1; IκB, inhibitor of κB; sh, short hairpin; DDP, cisplatin; CON, control; p-, phosphorylated; PES, pifithrin-μ.

Several signaling pathways are reported to be involved in the regulation of tumor cells apoptosis, such as the MAPK, Akt and NF-κB signaling pathways (11-13). Ding *et al* (19) demonstrated that HSP70 expression decreases the sensitivity of osteosarcoma cells to baicalein via activation of the PI3K/Akt and MAPK/ERK signaling pathways. Other studies have also demonstrated that HSP70 regulates tumor cell apoptosis by inhibiting the activation of JNK and NF-κB signaling (20,21). Cisplatin, a commonly used chemotherapeutic drug, is used clinically to treat cancer, and HSP70 expression decreases the sensitivity of osteosarcoma cells to cisplatin (14). However, the question of whether HSP70 regulates the sensitivity of gastric cancer cells to cisplatin and its underlying molecular mechanism still remain to be explored.

Cisplatin induces cell death by activating the apoptosis pathway (22,23); however, HSP70 expression has been demonstrated to exert a strong anti-apoptotic effect (15). In the present study, the effect of HSP70 on cisplatin-induced

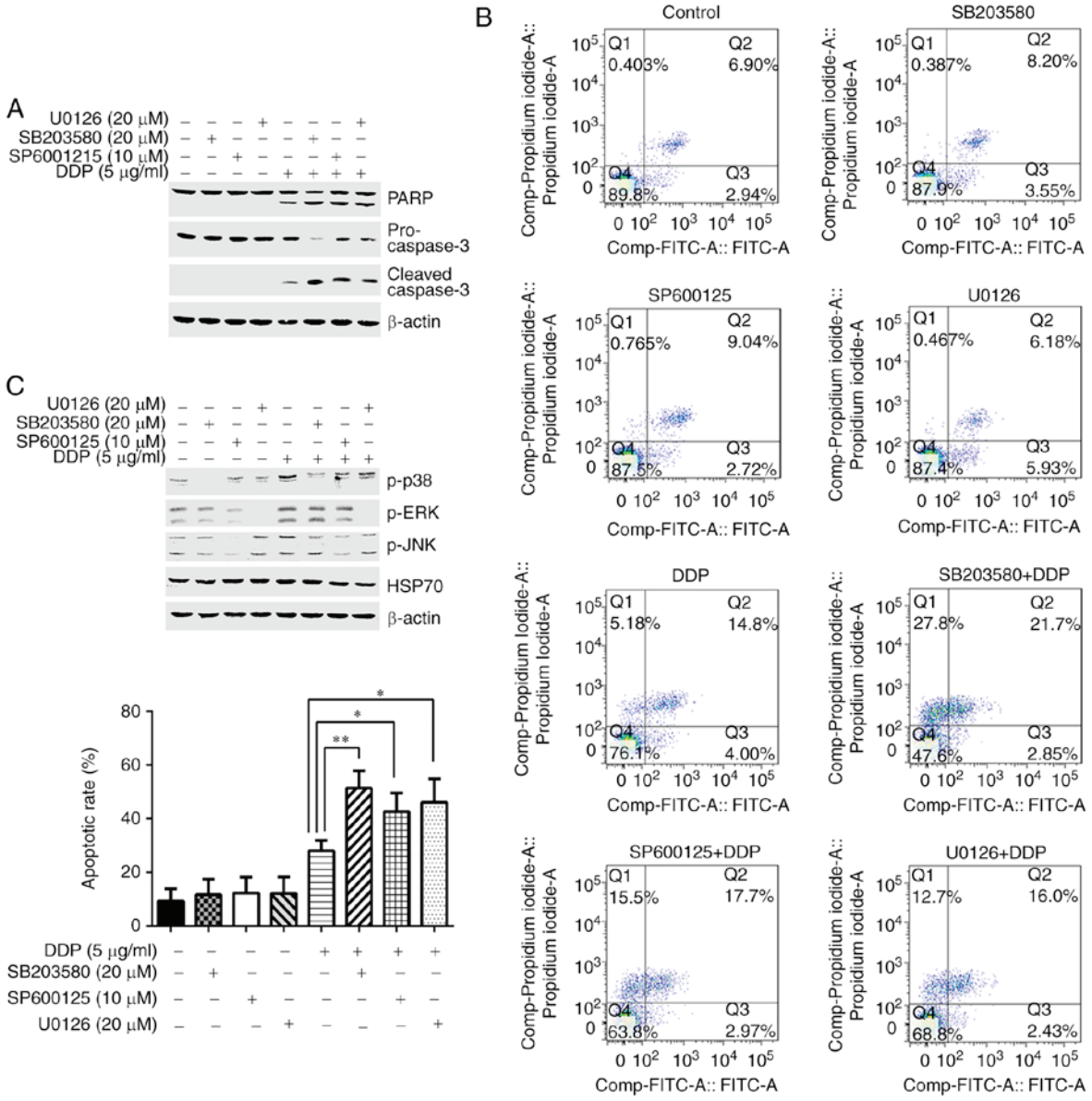

Figure 5. MAPK pathway inhibition enhances cisplatin-induced HGC-27 cell apoptosis. (A) HGC-27 cells were pretreated with specific inhibitors for p38, ERK or JNK for 2 h and then treated with cisplatin for 24 h. Expression levels of PARP, cleaved caspase-3 and pro-caspase-3 were detected by western blotting. (B) Apoptotic rate was determined by flow cytometry (representative plots and quantification is shown). (C) HGC-27 cells were pretreated with specific inhibitors for p38, ERK or JNK for 2 h and then treated with cisplatin for 6 h. Phosphorylation of p38, ERK, JNK and the levels of HSP70 were detected by western blotting. $^{*}P<0.05$ and $^{**}P<0.01$, with comparisons indicated by lines. MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PARP, poly-ADP-ribose-polymerase; HSP70, heat shock protein 70; DDP, cisplatin; p-, phosphorylated.

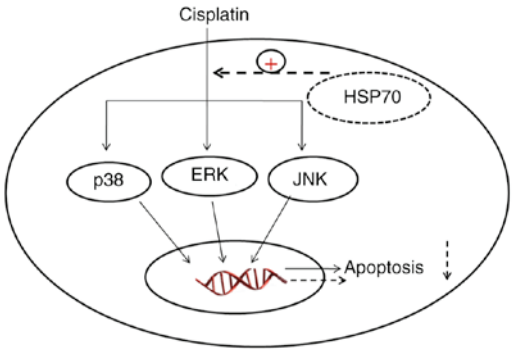

Figure 6. Schematic diagram illustrating the signaling pathway involved in the regulation of HSP70 in cisplatin-induced HGC-27 cell apoptosis. HSP70, heat shock protein 70; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

HGC-27 gastric cancer cell apoptosis was examined. Western blotting was used to detect the expression levels of cleaved PARP, cleaved caspase-3 and pro-caspase-3, an Annexin V/PI assay was used to determine apoptotic rates, and DAPI staining was used to monitor the morphology of apoptotic nuclei. As illustrated in Figs. 1-3, HSP70 overexpression decreased cisplatin-induced HGC-27 cell apoptosis, while inhibition of HSP70 using a specific inhibitor or by transfection of specific shRNA enhanced the cellular apoptosis induced by cisplatin. The present results are consistent with the study of Mori *et al* (14). In addition, Liu *et al* (2) reported that an HSP70 inhibitor combined with cisplatin suppresses cervical cancer cell proliferation and transplanted tumor growth. Collectively, these results suggest that inhibition of HSP70 is beneficial to the antitumor activity of cisplatin.

The present study also investigated the potential molecular mechanism of HSP70 antagonism of cisplatin-induced apoptosis. HGC-27 cells were treated with cisplatin for different amounts of time, and the phosphorylation states of p38 MAPK, ERK, JNK, Src, Akt and IκB were monitored by western blotting. The phosphorylation of p38, ERK and JNK were enhanced following cisplatin treatment, but Src, Akt and IκB were unaffected. This finding suggested that the MAPK signaling pathway is involved in cisplatin-induced apoptosis in gastric cancer cells. Therefore, to determine whether HSP70 regulates the activation of MAPK signaling, HSP70 was overexpressed in HGC-27 cells, and the results revealed no effect on the cisplatin-induced phosphorylation of p38, ERK and JNK compared with the empty plasmid-only transfected cells. However, knockdown of HSP70 by HSP70 shRNA transfection attenuated the phosphorylation of p38, ERK and JNK, similar to the HSP70 inhibition by PES treatment (Fig. 4). Taken together, these results demonstrated that HSP70 facilitates the activation of MAPK signaling in response to cisplatin in HGC-27 cells. Notably, overexpression of HSP70 in HGC-27 cell did not affect the phosphorylation of p38, ERK and JNK induced by cisplatin. A possible reason for this phenomenon might be the high expression levels of HSP70 in gastric cancer cells; the phosphorylation of p38, ERK and JNK induced by cisplatin may already be at a maximum level in these cells and therefore additional HSP70 expression may have no further effect on the activation of MAPK signaling pathway.

To establish that the MAPK pathway is involved in cisplatin-induced gastric cell apoptosis, specific inhibitors were used to suppress the activation of p38, ERK and JNK, and then cisplatin-induced apoptosis was examined. As expected, suppression of p38, ERK and JNK phosphorylation enhanced cisplatin-induced apoptosis (Fig. 5). It has been reported that ERK regulates cell apoptosis by promoting cell proliferation, while p38 and JNK are typically described as stress-activated kinases that mediate apoptotic signals (24-26). However, there are also some reports demonstrating that JNK and p38 act as anti-apoptotic signals (27,28). The present study tested the hypothesis that the MAPK signaling pathway acts as an anti-apoptotic signal in cisplatin-induced gastric cell apoptosis.

Collectively, the current results demonstrated that HSP70 protected cisplatin-induced gastric cancer cells from apoptosis by regulating the MAPK signaling pathway (Fig. 6). These findings present a novel insight into the anti-apoptotic mechanism of HSP70 and provide a theoretical basis for targeting HSP70 in clinical therapy of cancer drug resistance.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

ZQ and YZ conceived and designed the experiments. LS, TT, HT and YM performed the experiments. ZW, YL analyzed the data. LS and ZQ wrote the paper. All authors read and approved the final manuscript.

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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