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Gingerenone A attenuates gastric cancer progression *via* PI3K/Akt/FOXO1 signaling pathway, autophagy, and apoptosisYang Zhou^{1#}, Rui Mu^{2#}, Tong Wu¹, Xiao-Hui Mu¹, Hui Wan¹, Liang-Yu Zhao^{1✉}¹Department of Gastrointestinal Surgery, People's Hospital of Ningxia Hui Autonomous Region, No. 301, Zhengyuan North Street, Jinfeng District, Yinchuan 750000, Ningxia, China²Department of Gastroenterology, People's Hospital of Ningxia Hui Autonomous Region, No. 301, Zhengyuan North Street, Jinfeng District, Yinchuan 750000, Ningxia, China

ABSTRACT

Objective: To investigate anti-gastric cancer effects of gingerenone A (GA) and explore its association with the PI3K/Akt/FOXO1 signaling pathway, autophagy, and apoptosis.

Methods: Cell viabilities were detected *via* CCK-8 assay. Proliferation, migration, invasion, and apoptosis were assessed by colony formation, EdU, Transwell assays, and flow cytometry. Autophagy, mitochondrial dysfunction, and apoptosis were detected *via* Western blot, immunofluorescence, and staining assays. Inhibitors and siFOXO1 were used for molecular mechanism studies. An MKN-45 xenograft model was used to evaluate *in vivo* efficacy and safety.

Results: GA reduced gastric cancer cell proliferation, migration, and invasion, and promoted apoptosis, with MKN-45 cells being most sensitive. GA induced autophagy-related changes, mitochondrial dysfunction, and apoptosis-related changes, with suppression of PI3K/Akt signaling and reduced inhibitory phosphorylation of FOXO1; these effects were attenuated by the pharmacological and genetic interventions. *In vivo*, GA inhibited tumor growth without notable histopathological damage to major organs at the tested doses.

Conclusions: GA may exert anti-gastric cancer effects *via* PI3K/Akt/FOXO1 signaling pathway, with autophagy-related changes and apoptosis-related responses. It could be a candidate compound for gastric cancer therapy.

KEYWORDS: Gingerenone A; Gastric cancer; PI3K/Akt/FOXO1 axis; Autophagy; Mitochondria-associated apoptosis; Diarylheptanoids; Oxidative stress; DNA damage

1. Introduction

Gastric cancer is a leading cause of cancer-related deaths worldwide[1]. The prognosis of gastric cancer patients remains poor, despite the availability of multimodal treatments such as surgical resection, chemotherapy, targeted therapy, and immunotherapy[2]. This is particularly evident in advanced-stage gastric cancer, where patients often suffer local invasion, distant metastasis, high

Summary

Question: Does gingerenone A suppress gastric cancer progression, and is this effect associated with the PI3K/Akt/FOXO1 signaling axis, autophagy, and apoptosis?

Findings: Gingerenone A inhibited tumor growth, and led to autophagy-related changes, mitochondrial dysfunction, and apoptosis. These observations may be related to inhibition of PI3K/Akt signaling pathway and reduced inhibitory phosphorylation of FOXO1.

Meaning: Gingerenone A may be a candidate natural compound for gastric cancer, and the effects are associated with modulation of the PI3K/Akt/FOXO1 signaling axis and related cell death programs.

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recurrence rates, and therapeutic resistance, all of which seriously threaten their survival and health[3]. The pathogenesis of gastric cancer is a complex, multistep process involving uncontrolled cell proliferation, defective apoptosis, metabolic rewiring, elevated oxidative stress, and aberrant activation of key signaling pathways[4]. Improving the prevention and treatment of gastric cancer requires a deep understanding of its molecular mechanisms and the identification of effective natural anticancer compounds with low toxicity.

Aberrant regulation of programmed cell death is a hallmark of both the onset and progression of gastric cancer, with the mitochondrial apoptotic pathway acting as a primary determinant of tumor cell fate[5]. Mitochondria are central regulators of cellular energy metabolism and important integrators of oxidative stress, DNA damage, and apoptotic signaling[6]. Mitochondrial dysfunction leads to a series of detrimental cellular events, including loss of membrane potential, excessive accumulation of oxygen-derived free radicals, and an imbalance between pro- and anti-apoptotic proteins. This disruption of cellular homeostasis can trigger apoptosis, as indicated by altered Bax/Bcl-2 expression and caspase-3 activation[7]. Targeting mitochondria to induce dysfunction and subsequently apoptosis has emerged as a potent therapeutic approach to counteract the malignant progression of gastric cancer[8]. Additionally, the DNA damage response is closely associated with mitochondrial dysfunction, as excessive reactive oxygen species (ROS) production can further upregulate DNA damage markers such as γ -H2AX, thereby promoting tumor cell death[9].

Autophagy, a highly conserved catabolic process for bulk intracellular degradation, is essential for preserving cellular homeostasis, clearing dysfunctional organelles, and mediating cellular responses to stress[10]. Studies have shown that excessive autophagy activation can promote tumor cell apoptosis by disrupting mitochondrial function, enhancing ROS accumulation, and modulating the Bax/Bcl-2 balance[11]. However, the precise interplay between autophagy and mitochondria-associated apoptosis in gastric cancer cells remains poorly understood, and elucidating this relationship is crucial for a more comprehensive understanding of the mechanisms governing gastric cancer cell death.

The PI3K/Akt signaling pathway is central to gastric cancer pathogenesis, driving a wide range of malignant behaviors from proliferation and motility to metabolic reprogramming and apoptosis evasion, and is frequently aberrantly activated in this disease[12]. Activation of PI3K leads to the phosphorylation of Akt and subsequently regulates multiple downstream target proteins, thereby suppressing apoptosis and enhancing tumor cell survival[13]. As a pivotal terminal mediator of the PI3K/Akt signaling cascade, the Forkhead box O (FOXO) protein family, particularly FOXO1, orchestrates a diverse array of physiological programs, including redox homeostasis and autophagic activity, growth arrest, and

programmed cell death[14]. Sustained PI3K/Akt signaling primarily targets FOXO1 for inhibitory phosphorylation, thereby silencing its transcriptional activity. Consequently, FOXO1 loses its tumor-suppressive transcriptional function, thereby facilitating cell survival and malignant progression[15]. Therapeutically targeting the PI3K/Akt axis may relieve the inhibitory control on FOXO1. Under such conditions, FOXO1 has been reported to participate in tumor-suppressive programs involving autophagy-related gene expression, mitochondrial stress, and apoptosis-related responses[16]. The PI3K/Akt/FOXO1 signaling axis is now recognized as a central regulatory node in gastric cancer, orchestrating various malignant phenotypes from proliferation and metastasis to chemoresistance, suggesting its potential as a target for therapeutic intervention[17]. Therefore, clarifying the link between the PI3K/Akt/FOXO1 axis, autophagy, and apoptosis is important to gaining insights into the molecular basis of gastric cancer malignancy and identifying new vulnerabilities.

Owing to their pleiotropic mechanisms, minimal systemic toxicity, and diverse biological potencies, natural compounds serve as a vital reservoir for the discovery of novel anti-neoplastic agents[18]. Ginger (*Zingiber officinale*), a traditional medicinal and edible plant, is rich in a variety of bioactive phenolic and ketonic compounds and has shown broad pharmacological effects, particularly in anti-inflammatory, antioxidant, and antitumor activities[19]. Studies have shown that standardized ginger extract can alleviate hepatocellular carcinoma progression by suppressing oxidative stress- and inflammation-related pathways, thereby reducing tumor cell proliferation and inducing apoptosis[20]. In addition, 8-paradol, a bioactive compound derived from ginger, has been reported to inhibit gastric cancer progression and induce apoptosis in gastric adenocarcinoma cells by enhancing PINK1/Parkin-mediated mitochondrial processes[21]. These findings suggest that ginger-derived bioactive compounds may have potential therapeutic value in gastric cancer intervention, and that some of these constituents may exert antitumor effects by regulating mitochondria-related processes and cell death programs.

Gingerenone A (GA), a key bioactive constituent of ginger, has attracted attention because of its anti-inflammatory and antioxidant activities[22]. In recent years, its antitumor potential has attracted increasing attention. The antitumor properties of GA have been previously described in breast cancer, where its mechanism of action involves the induction of cell cycle arrest and senescence[23]. GA exhibits versatility as an anticancer agent through distinct mechanisms of action. On one hand, it can directly induce cell death, as demonstrated in colorectal cancer by targeting the SLC7A11 pathway[24]. On the other hand, it functions as a potent chemosensitizer, for example, restoring sunitinib sensitivity in renal cell carcinoma *via* suppression of LDHA-mediated glycolysis[25]. Although the anticancer potential of GA has been preliminarily

demonstrated in several tumor types, its role in gastric cancer has not yet been systematically investigated. A critical knowledge gap remains regarding whether GA can inhibit gastric cancer progression by regulating the PI3K/Akt/FOXO1 pathway, autophagy-related changes, and mitochondria-associated apoptotic changes.

To address this gap, we evaluated the anti-gastric cancer effects of GA using both *in vitro* assays and an *in vivo* xenograft model and explored the associated signaling mechanisms. Particular attention was given to whether GA suppresses PI3K/Akt signaling and alters FOXO1 phosphorylation in association with autophagy-related changes, mitochondrial dysfunction, and apoptotic changes, ultimately accompanying suppression of the proliferation, migration, and invasion of gastric cancer cells. This study may provide new evidence supporting the anti-gastric cancer potential of GA. In addition, it may offer mechanistic insight into how natural compounds targeting the PI3K/Akt/FOXO1 signaling axis contribute to gastric cancer suppression.

2. Methods

2.1. Reagents and chemicals

GA (purity $\geq 98\%$; MedChemExpress, USA; Cat# HY-120912) was dissolved in dimethyl sulfoxide (DMSO; Gibco, USA; Cat# D12345) to prepare a stock solution and stored at -20°C in the dark. For *in vitro* experiments, the stock solution was diluted with complete culture medium to the required final concentrations. For animal dosing, GA was formulated in phosphate-buffered saline (PBS) and administered intragastrically at the predetermined dose concentrations.

Autophagy inhibitor 3-methyladenine (3-MA; Cat# HY-19312), the PI3K activator 740 Y-P (Cat# HY-P0175), and the autophagy inducer sirolimus (Cat# HY-10219) were all purchased from MedChemExpress (USA). Stock solutions were prepared and subsequently used in strict accordance with the manufacturer's protocols.

2.2. Cell culture and treatments

The human gastric cancer cell lines MKN-45 (Cat# SNL-173), AGS (Cat# SNL-103), and NCI-N87 (Cat# SNL-106), along with the non-malignant human gastric mucosal epithelial cell line GES-1 (Cat# SNL-304), were all obtained from SUNNCELL (Wuhan, China). Short tandem repeat fingerprinting was employed to authenticate the genetic identity of each cell line, and periodic diagnostic screenings were performed to ensure the absence of mycoplasma contamination. All cells were cultured in a humidified incubator at 37°C with a $5\% \text{CO}_2$ atmosphere. The culture medium was supplemented with

10% fetal bovine serum (Gibco, USA; Cat# A5256701) and 1% penicillin-streptomycin (Gibco, USA; Cat# 15140122) to provide essential nutrients and prevent microbial contamination.

MKN-45, AGS, NCI-N87, and GES-1 cells were exposed to serial dilutions of GA (from 0 to $160 \mu\text{M}$) for 24 h to identify the most responsive cellular model. Cell viability was determined using CCK-8 assay, with GA concentrations of 0, 2.5, 5, 10, 20, 40, 80, and $160 \mu\text{M}$ [23,24]. This screening aimed to evaluate the inhibitory efficacy of GA across different gastric cancer cell backgrounds. Based on the criterion that the selected concentrations showed no obvious toxicity to the normal cell line, MKN-45 cells, which exhibited the highest sensitivity to GA, were chosen for subsequent experiments. Unless otherwise stated, GA was used at concentrations of 20, 40, and $80 \mu\text{M}$ for 24 h in the following cell-based experiments.

For autophagy-related experiments, MKN-45 cells were divided into the control, GA, GA + 3-MA, and sirolimus groups. Cells were pretreated with 3-MA for 1 h before GA administration. Meanwhile, sirolimus was administered alone as a positive control for autophagy activation. GA, 3-MA, and sirolimus were used at concentrations of $40 \mu\text{M}$, 4 mM, and $50 \mu\text{M}$, respectively.

In the signaling pathway intervention experiments, MKN-45 cells were treated with different concentrations of PI3K activator 740 Y-P, and the optimal concentration was determined based on CCK-8 assay results and protein expression levels of p-PI3K/PI3K. Both 740 Y-P and GA were used at $40 \mu\text{M}$ in subsequent experiments. Small interfering RNA (siRNA) transfection was completed before drug treatment. In the PI3K/FOXO1 pathway intervention experiments, MKN-45 cells were assigned to the control, GA, GA + 740 Y-P, GA + 740 Y-P + siNC (negative control siRNA), and GA + 740 Y-P + siFOXO1 (FOXO1-targeting siRNA) groups. Cells were pretreated with 740 Y-P for 1 h before GA exposure, and siRNA transfection was performed 24–48 h prior to drug treatment.

2.3. Xenograft tumor model in nude mice

BALB/c nude mice (age: 4–6 weeks; weight: 16–20 g) were purchased from HFK Bioscience (Beijing, China) and acclimatized for 7 d in a specific-pathogen-free environment prior to any experimental interventions. The housing conditions were maintained at $(22 \pm 2)^{\circ}\text{C}$ and a relative humidity of $(50 \pm 10)\%$, with a 12-hour light/dark cycle. All mice had *ad libitum* access to a sterilized standard diet and water.

For the establishment of subcutaneous xenografts, log-phase MKN-45 cells were harvested and resuspended in sterile PBS to prepare a single-cell suspension, which was then subcutaneously injected into the murine axillary region of nude mice. Each mouse received 1×10^6 MKN-45 cells in $100 \mu\text{L}$ to establish a subcutaneous xenograft model. Intragastric administration of GA was initiated when tumors had formed and reached a volume of approximately 100mm^3 [26],

and was then continued once daily during the 15-day overall experimental period. Mice were randomly divided into 4 groups: a vehicle control group receiving PBS and 3 intervention groups administered with GA at increasing dosages (5, 10, and 20 mg/kg, respectively)[24].

To monitor tumor progression, tumor size was measured at regular intervals using a digital caliper, with the volume as calculated using the standard ellipsoidal formula $V=0.5 \times L \times w^2$, where L represents the longest diameter of the tumor, and w represents the shortest diameter.

At the end of the experiment, mice were humanely euthanized, and the net tumor weight was measured. Subsequently, tumor tissues and a complete set of visceral specimens, including cardiac, hepatic, splenic, pulmonary, and renal tissues, were collected for subsequent molecular detection and histopathological analysis.

2.4. siRNA transfection

Prior to genetic silencing, MKN-45 cells were seeded in 6-well plates at a density of 2×10^5 cells/well. This seeding density was used to ensure appropriate cell confluency at the time of transfection. When the cells reached a sub-confluent state (50%-60%), siRNA transfection was performed using Lipofectamine™ RNAiMAX (Invitrogen, USA; Cat# 13778075) in strict adherence to the optimized laboratory protocols provided by the manufacturer. Cells were transfected with either negative control siRNA (siNC) or FOXO1 siRNA (siFOXO1) at a final concentration of 50 nM. After 24-48 h of transfection, cells were harvested, and FOXO1 protein expression was detected by Western blotting to verify the knockdown efficiency. Only after confirming successful FOXO1 knockdown were subsequent drug treatment and mechanistic experiments conducted. FOXO1 siRNA was purchased from Guangzhou IGE Biotechnology Co., Ltd. (Guangzhou, China), and the sequence was 5'-UUAUCUCACACAUGGGTT-3'[27]. The sequence of siNC was 5'-UUCUCCGAACGUGUCACGUTT-3'.

2.5. Cell viability assay (CCK-8)

For cell viability assessment, MKN-45 cells were seeded in 96-well microplates at a density of 5.0×10^3 cells/100 μ L. After overnight incubation to allow complete cell adherence, cells were subjected to the corresponding experimental treatments. To evaluate survival rates, 10 μ L of CCK-8 reagent (Dojindo, Japan; Cat# CK04) was added to each well, and plates were incubated at 37 °C for 1-2 h. Optical density (OD) at 450 nm was measured using a Multiskan FC spectrophotometer (Thermo Fisher Scientific, USA). Cell viability was finally calculated in accordance with the manufacturer's instructions.

2.6. Colony formation assay

For the colony formation assay, MKN-45 cells were seeded into

6-well plates at a low density of 500 cells/well to enable single-cell colony formation. After 24 h of incubation to allow complete cell adherence, cells were treated with various concentrations of GA. The culture medium was refreshed every 3 days. After 14 days, colonies (defined as clusters containing more than 50 cells) were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution. The number of colonies was manually counted under a stereomicroscope.

2.7. EdU proliferation assay

Cell proliferation was quantified by measuring DNA synthesis using an EdU incorporation assay (Invitrogen, USA; Cat# E10415). MKN-45 cells were seeded onto coverslips in 24-well plates at a density of 3×10^4 cells/well. After overnight adherence and subsequent experimental treatment, the cells were pulse-labeled with 10 μ M EdU for 2 h. Cells were fixed with 4% paraformaldehyde for 15 min, then permeabilized with 0.3% Triton X-100 for 15 min. A Click-iT reaction was carried out to visualize EdU-incorporated DNA, followed by DAPI counterstaining to label cell nuclei. To quantify the proliferative index, EdU-positive nuclei were counted in 5 randomly selected, non-overlapping fields of view for each replicate. Fluorescent images were captured using an Olympus IX73 imaging system (Japan), and the cell proliferation capacity was expressed as the percentage of EdU-positive cells relative to total number of DAPI-stained nuclei.

2.8. Transwell migration and invasion assays

Cell migration and invasion abilities of MKN-45 cells were assessed using Transwell inserts with an 8 μ m pore polycarbonate membrane (Corning, USA; Cat# CLS3422). After treatment, cells were harvested and resuspended in serum-free medium. For the migration assay, 5×10^3 cells were seeded into the upper chamber. For the invasion assay, inserts precoated with reconstituted basement membrane matrix (Matrigel; Corning, USA; Cat# CLS354248) were used. Medium supplemented with 10% fetal bovine serum was added to the lower chamber as a chemoattractant. Following a 24-h incubation, non-migratory cells on the upper membrane surface were carefully removed. Cells that successfully migrated or invaded the lower membrane surface were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Finally, five randomly selected visual fields per insert were photographed and counted.

2.9. Analysis of apoptosis by flow cytometry

For apoptotic assessment, MKN-45 cells were stained using an Annexin V-FITC/PI kit (Invitrogen, USA; Cat# V13242) as per the manufacturer's instructions. Harvested cells were washed, resuspended in binding buffer, and followed by dual staining with Annexin V-FITC and PI for 15 min at room temperature in the dark.

Cell apoptosis was analyzed by flow cytometry using a FACSCanto II instrument (BD Biosciences, USA), with no fewer than 2×10^5 events collected for each sample. Early apoptotic cells (Annexin V⁺/PI⁻) and late apoptotic cells (Annexin V⁺/PI⁺) were distinguished and quantified. FlowJo software was used for gating and data analysis, and the total apoptotic rate was calculated as the sum of early and late apoptotic cell proportions.

2.10. Western blot analysis

To analyze protein expression, cells and homogenized tumor tissues were lysed with RIPA lysis buffer (Abcam, UK; Cat# ab288006) supplemented with protease and phosphatase inhibitors. Lysates were centrifuged at $12\,000 \times g$ at 4 °C for 15 min, and protein concentrations were quantified using a BCA protein assay kit. Subsequently, 30 µg of each protein sample was separated by SDS-PAGE and transferred electrophoretically onto PVDF membranes. To minimize non-specific binding, membranes were blocked with 5% skim milk or BSA (for phosphoprotein detection) for 1 h. Membranes were incubated with primary antibodies overnight at 4 °C. Membranes were rinsed with TBST and probed with HRP-linked secondary antibodies (Abcam, UK; ab6728, 1:2 000) for 1 h. Immunoreactive bands were visualized using ECL reagents and imaged with a digital imaging system. Band gray values were quantified using ImageJ, with GAPDH as the internal reference for normalization. Relative expression of target proteins was calculated as the gray value ratio of target proteins to GAPDH. For specific proteins, activation or modification levels were assessed by the ratio of phosphorylated to total protein, cleaved caspase-3 to total caspase-3, or LC3-II to LC3-I, respectively.

The primary antibodies used in this study were as follows: LC3 A/B (Abcam, UK, Cat# ab62721, 1:1 000), p62 (Abcam, UK, Cat# ab109012, 1:1 000), p-PI3K (Abcam, UK, Cat# ab191606, 1:1 000), PI3K (Abcam, UK, Cat# ab302958, 1:1 000), p-Akt (Abcam, UK, Cat# ab81283, 1:1 000), Akt (Abcam, UK, Cat# ab8805, 1:1 000), p-FOXO1 (Abcam, UK, Cat# ab131339, 1:1 000), FOXO1 (Abcam, UK, Cat# ab179450, 1:1 000), γ -H2AX (Abcam, UK, Cat# ab81299, 1:1 000), Bcl-2 (Abcam, UK, Cat# ab182858, 1:1 000), Bax (Abcam, UK, Cat# ab32503, 1:1 000), cleaved caspase-3 (Abcam, UK, Cat# ab32042, 1:500), caspase-3 (Abcam, UK, Cat# ab32351, 1:1 000), COX4 (Abcam, UK, Cat# ab317183, 1:1 000), GAPDH (Abcam, UK, Cat# ab181602, 1:10 000).

2.11. Immunofluorescence staining in cells

Following experimental treatments, MKN-45 cells cultured on sterile coverslips were subjected to immunofluorescence staining. Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 5% BSA for 1 h at room temperature. Cells were incubated

overnight at 4 °C with primary antibodies against LC3B (Abcam, UK; ab48394; 1:200) or γ -H2AX (Abcam, UK; ab81299; 1:200). After thorough rinsing, cells were incubated with fluorescence-conjugated secondary antibodies for 1 h at room temperature in the dark. Cell nuclei were counterstained with DAPI for 10 min, and fluorescence images were acquired using a fluorescence microscope. For quantitative analysis, LC3-positive foci per cell were counted in 5 randomly selected fields. Genotoxic stress was similarly evaluated by calculating the proportion of γ -H2AX-positive cells from the same number of microscopic fields.

2.12. Measurement of mitochondrial membrane potential (JC-1 staining)

Mitochondrial membrane potential ($\Delta\Psi_m$) was assessed using a JC-1 assay kit (Abcam, UK; Cat# ab113850). Briefly, treated MKN-45 cells were incubated with the JC-1 working solution for 20 min at 37 °C. After washing with the dedicated assay buffer, cellular fluorescence intensity was analyzed by flow cytometry. The shift from red fluorescence (JC-1 aggregates) to green fluorescence (JC-1 monomers) was monitored to reflect mitochondrial depolarization. The relative $\Delta\Psi_m$ level was calculated as red/green intensity ratio using FlowJo software.

2.13. Detection of intracellular ROS

Intracellular ROS was quantified using a commercial assay kit (KeyGEN BioTECH, China; Cat# KGA7308-100). After the treatments, the MKN-45 cells were incubated with the DCFH-DA probe for 20 min at 37 °C to facilitate cellular uptake of the probe. To ensure the accuracy of ROS detection, cells were thoroughly rinsed with PBS to eliminate residual non-internalized fluorophores. Subsequently, the samples were analyzed by flow cytometry, with green fluorescence emission (corresponding to ROS-bound DCFH-DA) specifically captured using the FL1 detector. The resulting cytometric data were processed using the FlowJo software, where the mean fluorescence intensity (MFI) served as a direct indicator for evaluating the level of intracellular oxidative stress. The obtained values were normalized to the control group, and the results are expressed as relative ROS levels.

2.14. Detection of mitochondrial ROS

Mitochondria-derived ROS levels were assessed by co-staining with MitoSOX Red Mitochondrial Superoxide Indicator (Invitrogen, USA; Cat# M36008) and MitoTracker Green FM (Invitrogen, USA; Cat# M7514). Following the designated treatments, MKN-45 cells were sequentially incubated with MitoTracker Green (100 nM, 30 min) and MitoSOX Red (5 µM, 10 min) at 37 °C in the dark to label mitochondria and mitochondrial ROS, respectively. After PBS

washing, images were acquired using fluorescence microscopy. The MitoSOX Red fluorescence intensity was quantified using ImageJ, and the results were normalized to the control group to calculate relative mitochondrial ROS levels.

2.15. Hematoxylin and eosin (H&E) staining

To assess the systemic safety of GA *in vivo*, visceral specimens, including cardiac, hepatic, splenic, pulmonary, and renal tissues, were collected and fixed in 4% paraformaldehyde. Following an overnight fixation, 4- μ m-thick paraffin-embedded sections were prepared. Hematoxylin and eosin (H&E) staining was performed to visualize histological changes: paraffin sections were deparaffinized, rehydrated, and stained with hematoxylin for 5 min, differentiated in acid-alcohol for 5 s, and blued with running tap water. After counterstaining with eosin for 2 min, sections were dehydrated, cleared, and mounted. Histological changes were examined under a light microscope.

2.16. Immunohistochemistry for Ki-67

Formalin-fixed, paraffin-embedded tumor tissues were cut into 4- μ m sections for immunohistochemical staining. After deparaffinization and graded rehydration, antigen retrieval was performed by heating sections in sodium citrate buffer for 15 min. Endogenous peroxidase activity was blocked with 3% H₂O₂ for 10 min, and non-specific binding was inhibited using 5% BSA for 1 h. The sections were then incubated overnight at 4 °C with a primary antibody against Ki-67 (Abcam, UK; Cat# ab15580, 1:200). After incubation with secondary HRP-conjugated antibody for 1 h, positive immunoreactivity was visualized through DAB chromogenic reaction. Cell nuclei were counterstained with hematoxylin, and the proliferation index was determined by calculating the percentage of Ki-67-reactive nuclei in 5 randomly selected high-power fields per section.

2.17. TUNEL fluorescence assay

To detect apoptotic cells in tumor tissues, 4- μ m paraffin-embedded sections were prepared. Following de-waxing and graded rehydration, tissue permeability was enhanced through proteolytic digestion with proteinase K (20 min) as per the TUNEL kit's protocol (Invitrogen, USA; Cat# C10625). Cleaved DNA fragments were then labeled with the TUNEL reaction mixture for 1 h at 37 °C in a light-restricted environment. Cell nuclei were counterstained with DAPI for 10 min, and fluorescence images were subsequently captured. The apoptotic index was quantified by calculating the percentage of TUNEL-positive nuclei in 5 randomly selected fields for each sample.

2.18. Immunofluorescence staining of tumor tissues

The paraffin-embedded tumor tissues were cut into 4- μ m-thick sections. For epitope retrieval, dewaxed and rehydrated sections were subjected to heat-induced retrieval in sodium citrate buffer for 15 min. Following permeabilization with 0.1% Triton X-100 for 10 min, tissue sections were blocked with a 5% BSA blocking buffer at room temperature for 1 h. The slides were finally incubated overnight at 4 °C with primary antibodies: anti-LC3B (Abcam, UK; Cat# ab48394; 1:200) and anti- γ -H2AX (Abcam, UK; Cat# ab81299; 1:200). Following overnight primary antibody incubation and buffer washing, sections were incubated with corresponding fluorescently-labeled secondary antibodies at room temperature for 1 h. All staining procedures were conducted in strict darkness to prevent potential photobleaching. Cell nuclei were counterstained with DAPI for 10 min. Fluorescence images were subsequently acquired under a fluorescence microscope, and five random visual fields were selected for quantitative analysis. LC3B staining was used to evaluate autophagic changes, and γ -H2AX staining was applied to detect DNA damage in tumor tissues. The number of LC3 fluorescent puncta per cell was counted to calculate the number of LC3 puncta per cell. The proportion of γ -H2AX-positive cells was determined and expressed as a percentage (%) of the total cell number.

2.19. Detection of ROS in tumor tissues by dihydroethidium (DHE) staining

The ROS levels in the tumor tissues were detected using a DHE fluorescent probe (KeyGEN BioTECH, China; Cat# KGA7502-5). Fresh tumor tissues were cut into 8- μ m frozen sections. After equilibration to room temperature and rinsing with PBS, sections were incubated with a DHE working solution at 37 °C for 30 min in complete darkness to visualize intracellular superoxide anions. Following buffer-mediated washing, the red fluorescent ethidium signals were promptly captured using a fluorescence imaging system. For quantitative analysis, five random microscopic fields per sample were selected. The percentage of DHE-positive cells was calculated and normalized to the total cell count in each field.

2.20. Statistical analysis

All quantitative data are presented as mean $\pm$ SD. Unless otherwise stated, all *in vitro* experiments were performed with at least 3 independent biological replicates ($n = 3$). For multi-well assays (*e.g.*, CCK-8), technical triplicates from each independent experiment were averaged to generate a single data point. For *in vivo* experiments, six mice were included in each group. All statistical analyses were performed using GraphPad Prism 9.0. Two-tailed

unpaired Student's *t*-tests were applied for comparison between two groups, and one-way ANOVA followed by Tukey's *post-hoc* test was applied for multigroup comparisons. A *P*-value of 0.05 was defined as the threshold for statistical significance.

2.21. Ethical statement

All animal experimental protocols were reviewed and approved by the People's Hospital of Ningxia Hui Autonomous Region Ethics Committee (Approval No. 2023-NZR-148; approval date: November 30, 2023).

3. Results

3.1. GA inhibits the malignant phenotype of gastric cancer cells

The chemical structure of GA is shown in Supplementary Figure 1. To assess the antitumor potential of GA against gastric cancer cells, a CCK-8 assay was conducted to determine the viability of various gastric cancer cell lines and normal gastric mucosal epithelial cells treated with a range of GA concentrations. Our findings revealed that GA significantly inhibited the viability of MKN-45, AGS, and NCI-N87 cells to different extents ($P < 0.05$), among which MKN-45 cells were the most sensitive to GA. In contrast, the viability of normal gastric mucosal epithelial GES-1 cells showed no obvious change within the concentration range of 0–80 μ M, suggesting that GA exerts a relatively selective inhibitory effect on gastric cancer cells (Figure 1A). Based on these findings, MKN-45 cells were chosen for subsequent mechanistic studies.

Clonogenic capacity reflects the long-term proliferative and survival potential of cancer cells. GA treatment induced a significant dose-dependent inhibition of MKN-45 clonogenicity, with a significant reduction in colony numbers ($P < 0.05$) (Figure 1B–C). EdU incorporation assays further showed that GA reduced DNA synthesis activity, indicating an inhibition of cell proliferation (Figure 1D–E). Cellular migration and invasion are key features of tumor progression and metastasis. Transwell assays showed that the migratory and invasive capacities of MKN-45 cells were significantly suppressed following GA treatment ($P < 0.05$) (Figure 1F–I). Additionally, flow cytometry analysis revealed that GA exposure significantly increased the proportion of apoptotic cells ($P < 0.05$) (Figure 1J–K).

3.2. GA induces autophagy-related changes and apoptosis in gastric cancer cells

To determine whether GA affects autophagy in gastric cancer cells, the abundance of autophagy-associated proteins was

detected by Western blot analysis. The findings demonstrated that GA treatment significantly enhanced the LC3-II/LC3-I ratio and significantly attenuated p62 expression in MKN-45 cells ($P < 0.05$), a trend consistent with that observed in cells treated with the autophagy inducer sirolimus (Figure 2A–B). Consistently, LC3B immunofluorescence staining further revealed that GA significantly increased the number of LC3B puncta, which is a characteristic feature of autophagy activation (Figure 2E–F).

Flow cytometric analysis revealed that GA treatment significantly decreased the red-to-green fluorescence ratio ($P < 0.05$), indicating a decrease in mitochondrial membrane potential (MMP) in MKN-45 cells (Figure 2G–H). The data further demonstrated that GA treatment led to a significant increase in intracellular total ROS levels ($P < 0.05$) (Figure 2C–D). Further co-staining with MitoTracker and MitoSOX Red revealed a significant increase in mitochondria-derived ROS in the GA group, with obvious colocalization with mitochondrial signals ($P < 0.05$) (Figure 2I–J).

Both immunofluorescence and Western blot analyses showed that GA significantly increased γ -H2AX expression ($P < 0.05$) (Figure 2K–L). The results showed that GA significantly upregulated Bax and cleaved caspase-3 expression while significantly downregulated Bcl-2 and COX4 levels ($P < 0.05$) (Figure 2M–N), suggesting that GA induced apoptosis-related changes associated with mitochondrial dysfunction.

3.3. Autophagy inhibition partially attenuates the effects of GA on malignant phenotypes and apoptosis in gastric cancer cells

To elucidate whether autophagy contributes to the antitumor effects of GA, the classical autophagy inhibitor 3-MA was utilized for targeted intervention. Clonogenic analysis revealed that GA significantly suppressed clonogenic ability of MKN-45 cells ($P < 0.05$), whereas 3-MA treatment partially restored colony formation. The sirolimus group further confirmed the association between autophagy activation and suppression of malignant phenotypes (Figure 3A–B). EdU assays similarly showed that GA inhibited cell proliferation, and this effect was partially reversed by 3-MA treatment (Figure 3C–D).

Transwell assays were further performed to evaluate cell migration and invasion. GA treatment significantly impeded the migratory and invasive potential of MKN-45 cells ($P < 0.05$), which was partially reversed by 3-MA (Figure 3E–H). Flow cytometric analysis showed that GA promoted cell apoptosis, whereas inhibition of autophagy by 3-MA reduced the apoptotic rate (Figure 3I–J).

3.4. GA inhibits PI3K/Akt signaling and is associated with reduced inhibitory phosphorylation of FOXO1

To determine whether the effects of GA are associated with

PI3K/Akt signaling, key markers of this pathway were assessed by immunoblotting. The findings indicated that GA exposure significantly reduced p-PI3K and p-Akt levels in MKN-45 cells ($P < 0.05$), whereas total PI3K and Akt expression remained unchanged. GA treatment led to a significant decrease in the levels of p-FOXO1 at Ser256 ($P < 0.05$) (Figure 4A-D).

In order to confirm functional role of PI3K, the optimal concentration of 740 Y-P for experimental intervention was

determined through CCK-8-based cytotoxicity screening. The proliferative capacity of MKN-45 cells remained largely unaffected by 740 Y-P treatment throughout the tested dosage range (0–40 μM) (Figure 4E). Western blot assay showed that 740 Y-P significantly increased p-PI3K abundance, with the most prominent effect observed at 40 μM ($P < 0.05$); therefore, this concentration was selected for subsequent co-treatment assays (Figure 4F).

Further investigation revealed that, compared with the GA group,

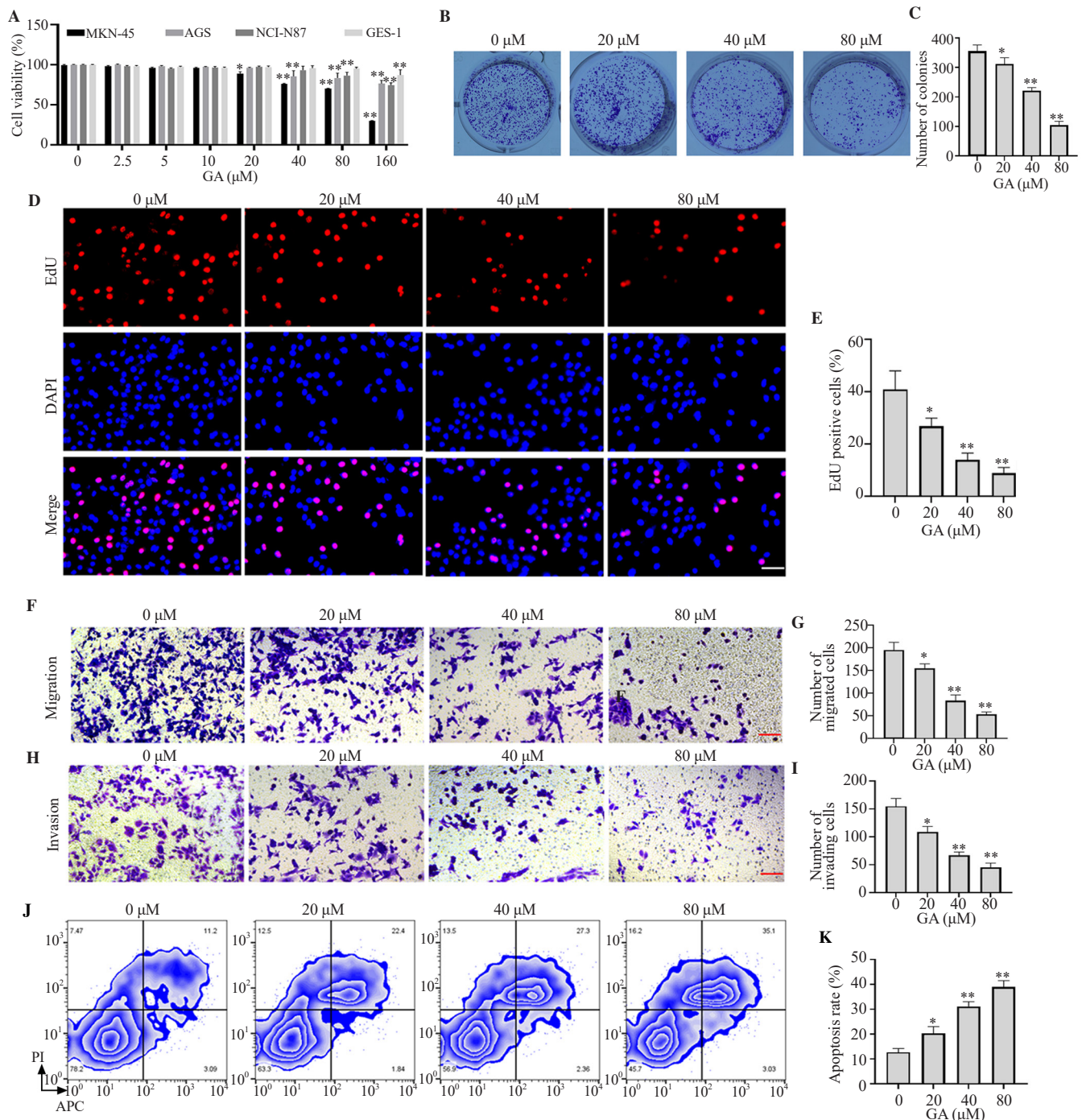


Figure 1. Effects of gingerenone A (GA) on gastric cancer cells. A: Cell viability of MKN-45, AGS, NCI-N87, and GES-1 cells following exposure to GA for 24 h determined by CCK-8 assay. B-C: Representative images of colony formation and colony numbers in MKN-45 cells treated with GA. D-E: Evaluation of MKN-45 cell proliferation *via* EdU incorporation (scale bar = 50 μm) and the percentage of EdU-positive cells. F-G: Migratory potential of MKN-45 cells assessed by Transwell assay (scale bar = 100 μm) and migrated cell counts. H-I: Invasive ability of MKN-45 cells (scale bar = 100 μm) and the quantitative results of invasive cell numbers. J-K: Apoptosis induction in MKN-45 cells visualized by flow cytometry and percentage of the total apoptotic rate. Data are presented as mean $\pm$ SD from 3 independent experiments ($n = 3$). * $P < 0.05$, ** $P < 0.001$ vs. GA 0 μM group.

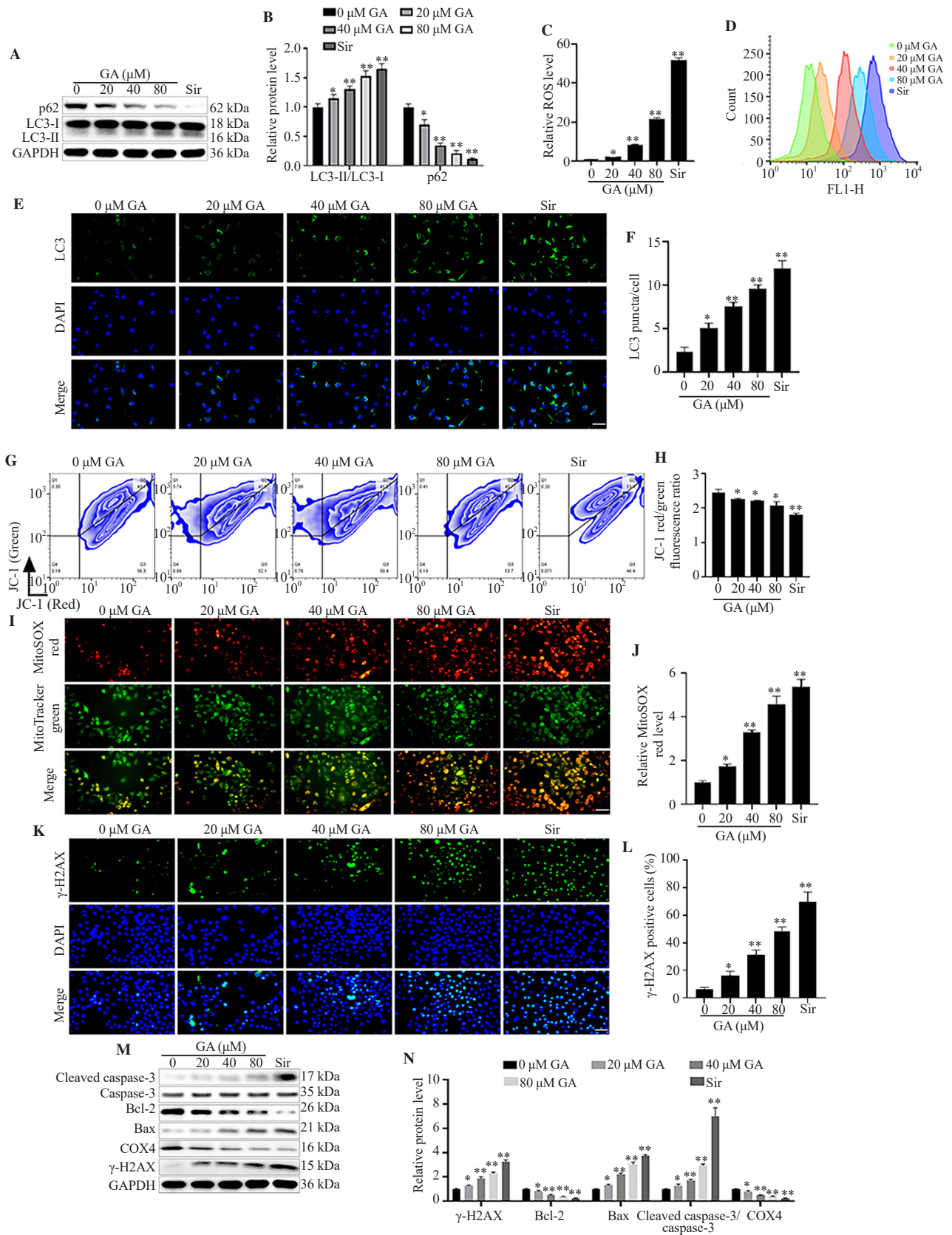


Figure 2. GA induces autophagy-related changes and apoptosis in gastric cancer cells. A-B: Immunoblotting results of LC3-II/LC3-I and p62 in MKN-45 cells. C-D: Total intracellular reactive oxygen species (ROS) production. E-F: LC3B punctate distribution visualized by immunofluorescence (scale bar = 50 μm) and the average count of LC3B puncta per cell. G-H: Flow cytometric evaluation of mitochondrial membrane potential (MMP) variations and the ratio of JC-1 aggregates to monomers. I-J: Representative images of mitochondrial ROS via MitoTracker and MitoSOX Red co-labeling (green: mitochondria; red: mtROS; scale bar = 50 μm) and the integrated MitoSOX fluorescence intensity. K-L: Nuclear γ-H2AX foci distribution determined by immunofluorescence (scale bar = 50 μm) and the percentage of γ-H2AX-positive cells. M-N: Western blot analysis of γ-H2AX, Bcl-2, Bax, cleaved caspase-3, and COX4. Data are presented as mean ± SD from 3 independent experiments ($n = 3$). * $P < 0.05$, ** $P < 0.001$ vs. GA 0 μM group. Sir: Sirolimus.

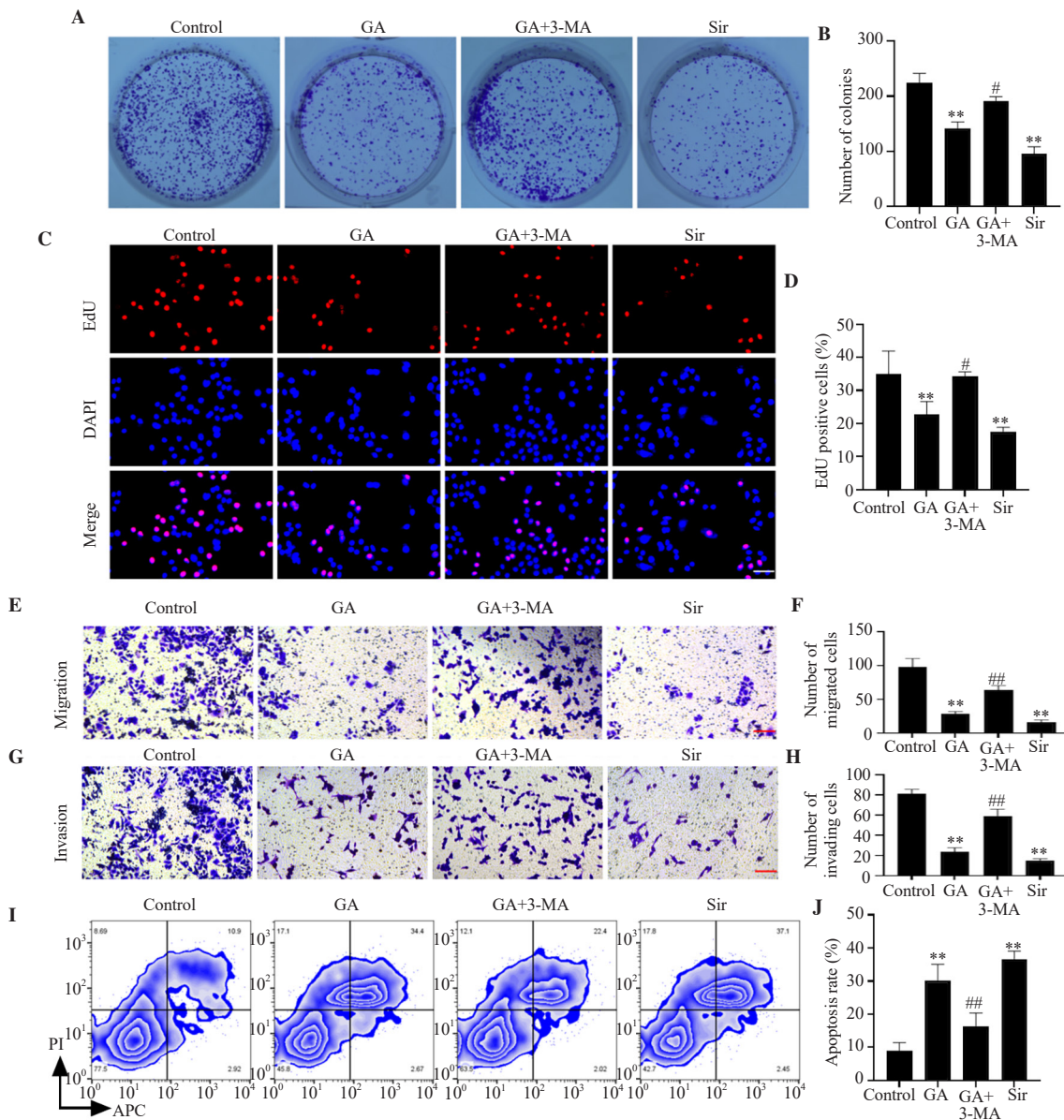


Figure 3. GA suppresses the malignant progression of gastric cancer cells in association with autophagy-related changes. A-B: Clonogenicity of MKN-45 cells and colony numbers. C-D: EdU-based detection of cell proliferation (scale bar = 50 μ m) and the relative proportion of EdU-positive cells. E-F: Migratory ability of MKN-45 cells determined by Transwell assay (scale bar = 100 μ m) and migrated cell counts. G-H: Invasive potential of MKN-45 cells assessed via Transwell invasion assay (scale bar = 100 μ m), and number of invaded cells. I-J: Apoptotic profiles of MKN-45 cells by flow cytometry and apoptotic rates. Data are presented as mean $\pm$ SD from 3 independent experiments ($n = 3$). ** $P < 0.001$ vs. the control group; # $P < 0.05$, ## $P < 0.001$ vs. the GA group. MA: methyladenine.

740 Y-P treatment partially restored the p-FOXO1/FOXO1 ratio (Figure 4G). In addition, transfection with siFOXO1 significantly reduced the protein levels of both FOXO1 and p-FOXO1, indicating efficient knockdown ($P < 0.05$) (Figure 4H). The results from 740 Y-P and siFOXO1 intervention experiments, particularly the corresponding changes in downstream protein expression, further supported the involvement of the PI3K/Akt/FOXO1 axis in the antitumor activity of GA (Figure 4I-K).

3.5. GA induces autophagy-related changes and apoptosis-related changes are associated with PI3K/Akt inhibition and FOXO1 modulation

To investigate the role of the PI3K/Akt/FOXO1 axis in GA-mediated autophagy activation and apoptosis, intervention experiments were performed using 740 Y-P and siFOXO1. Western blot analysis showed that GA significantly increased the LC3-II/LC3-I ratio and reduced p62 expression ($P < 0.05$), whereas these

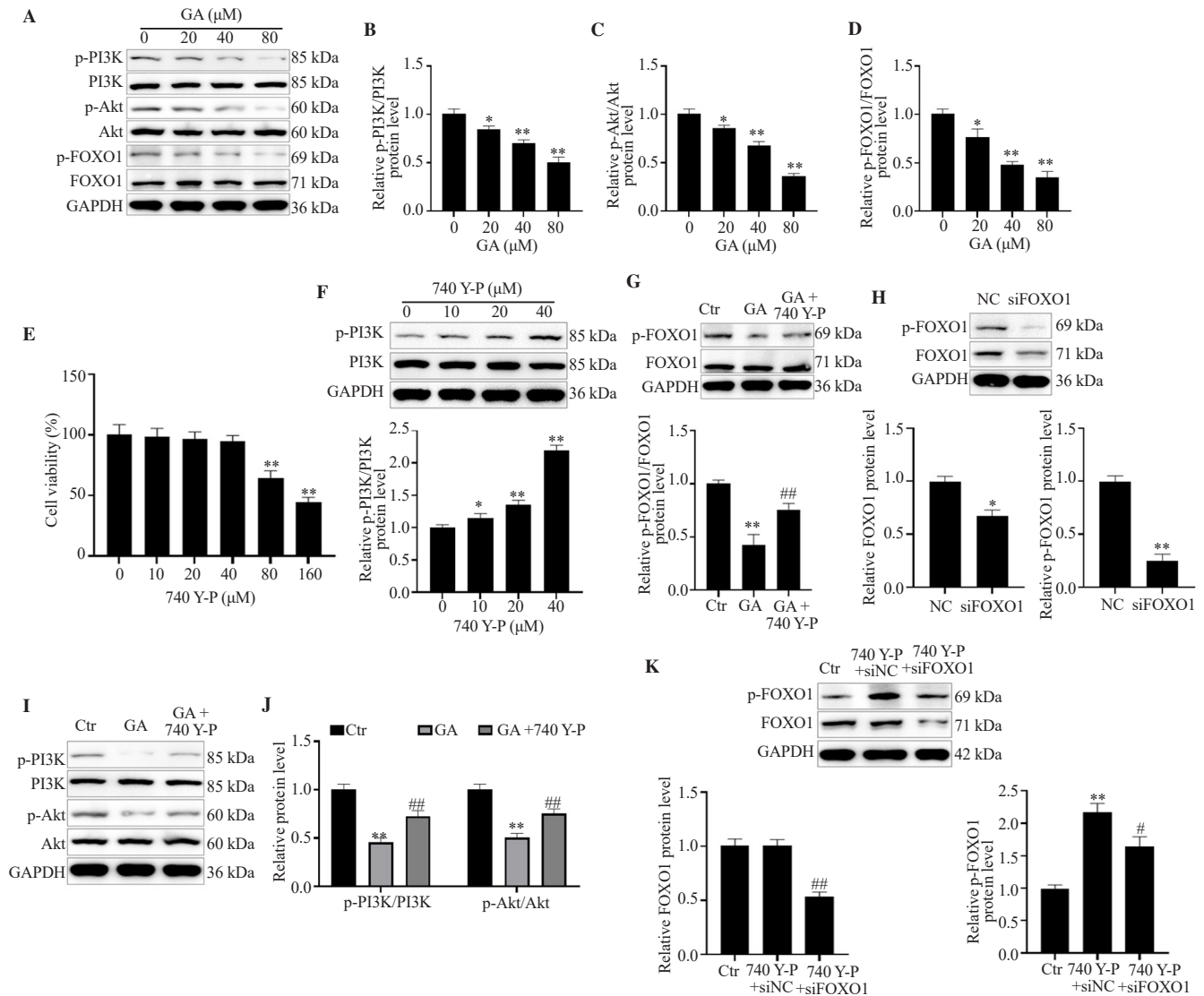


Figure 4. GA suppresses PI3K/Akt signaling and modulates FOXO1 phosphorylation. A-D: Immunoblotting results of p-PI3K, PI3K, p-Akt, Akt, p-FOXO1, and FOXO1 levels in MKN-45 cells. E: Viability of MKN-45 cells assessed by CCK-8 assay after exposure to the PI3K activator 740 Y-P. F: p-PI3K and PI3K expression in MKN-45 cells after 740 Y-P treatment. G: Western blot analysis of p-FOXO1 and FOXO1 abundance in MKN-45 cells treated with GA alone or combined with 740 Y-P. H: Protein levels of p-FOXO1 and FOXO1 in MKN-45 cells transfected with siNC or siFOXO1. I-K: Immunoblotting analysis of the phosphorylation and total expression of PI3K, Akt, and FOXO1. Data are presented as mean $\pm$ SD from 3 independent experiments ($n = 3$). * $P < 0.05$, ** $P < 0.001$ vs. the control group; # $P < 0.05$, ## $P < 0.001$ vs. the GA group in Figure G and J, or vs. the 740 Y-P + siNC group in Figure K. Ctrl: control.

changes were partially reversed by 740 Y-P. FOXO1 knockdown further counteracted the effect of 740 Y-P, as shown by a rebound in the LC3-II/LC3-I ratio and a decrease in p62 expression. These findings suggest that GA-triggered autophagy is mediated, at least in part, via the PI3K/Akt/FOXO1 signaling pathway (Figure 5A-B).

Intracellular ROS assays showed that GA significantly increased total ROS levels ($P < 0.05$), which were partially attenuated by 740 Y-P, while siFOXO1 transfection diminished the effect of 740 Y-P and restored high ROS levels (Figure 5C-D). Flow cytometric analysis showed that GA treatment significantly reduced the JC-1 red/green fluorescence ratio ($P < 0.05$), indicating a decrease in MMP. This effect was partially reversed by 740 Y-P, whereas FOXO1

silencing effectively abrogated its effect and further aggravated mitochondrial depolarization (Figure 5E-F).

γ -H2AX immunofluorescence staining showed that GA significantly increased DNA damage foci ($P < 0.05$). Although this effect was partially mitigated by 740 Y-P, siFOXO1 treatment increased the proportion of γ -H2AX-positive cells again (Figure 5G-H). Western blot results further showed that GA upregulated γ -H2AX, Bax, and the cleaved caspase-3/caspase-3 ratio, and downregulated Bcl-2 and COX4 protein levels. 740 Y-P partially rescued the GA-induced dysregulation of these apoptosis- and mitochondria-associated proteins, and such rescue effects were largely blocked by FOXO1 deficiency (Figure 5I-J).

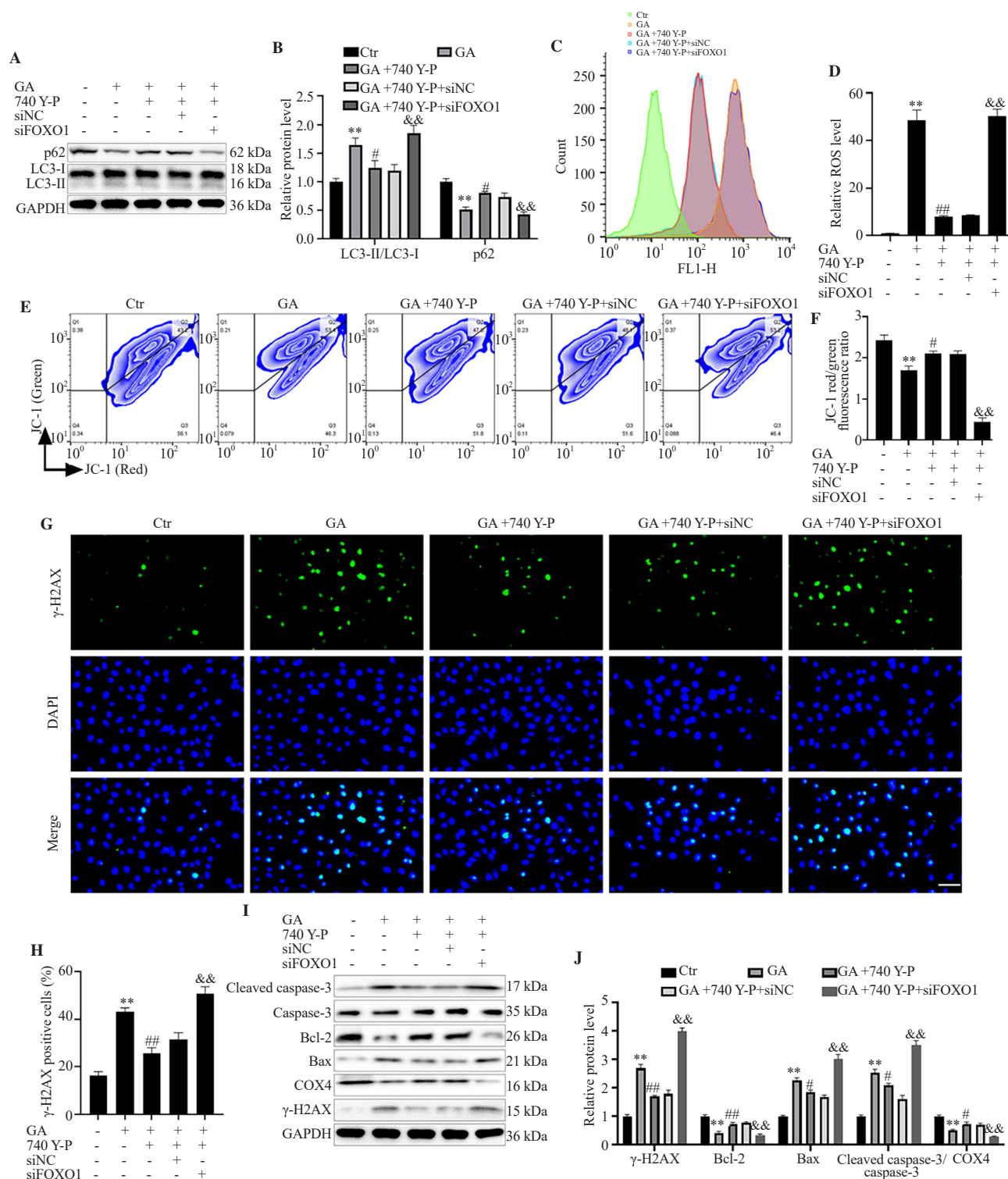


Figure 5. GA induces autophagy-related changes, and apoptosis in gastric cancer cells *via* the PI3K/Akt/FOXO1 axis. A-B: Immunoblotting analysis of LC3-II/I and p62 in MKN-45 cells. C-D: Intracellular ROS levels. E-F: Flow cytometric evaluation of MMP and the JC-1 aggregate/monomer ratio. G-H: Nuclear distribution of γ -H2AX foci visualized by immunofluorescence (scale bar = 50 μ m) and the percentage of γ -H2AX-positive MKN-45 cells. I-J: Protein levels of γ -H2AX, cleaved caspase-3, Bcl-2, Bax, and COX4 detected by Western blot. Data are presented as mean $\pm$ SD from 3 independent experiments ($n = 3$). ** $P < 0.001$ vs. the control group; # $P < 0.05$, ### $P < 0.001$ vs. the GA group; && $P < 0.001$ vs. the GA+740 Y-P + siNC group.

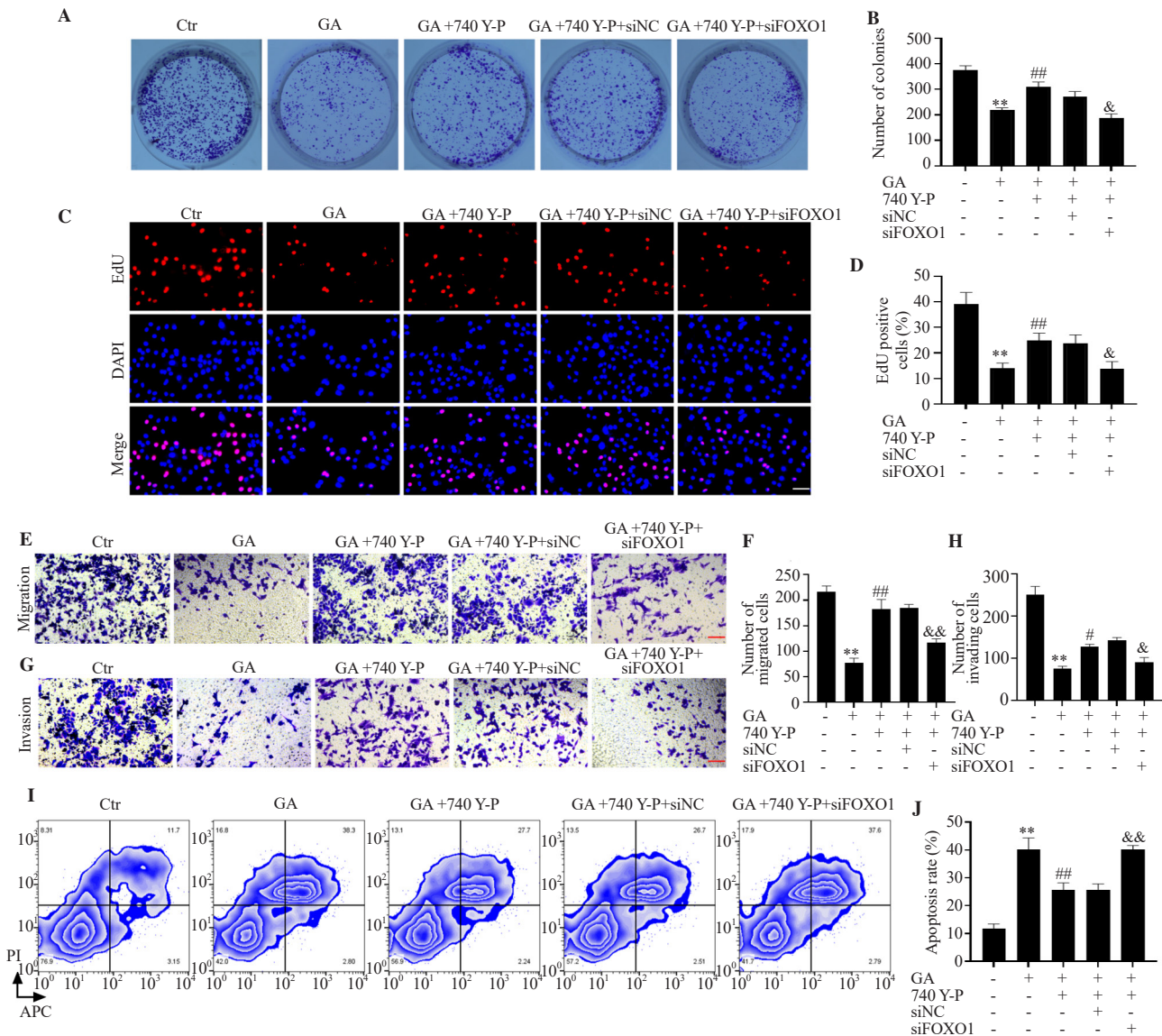


Figure 6. GA suppresses the malignant progression of gastric cancer cells *via* the PI3K/Akt/FOXO1 axis. A-B: Evaluation of MKN-45 cell clonogenicity and colony numbers. C-D: Proliferation of MKN-45 cells assessed by EdU incorporation assays (scale bar = 50 μ m) and EdU-positive cell fractions. E-F: Cellular motility assessed by Transwell migration assay (scale bar = 100 μ m) and migrated cell counts. G-H: Invasiveness of MKN-45 cells determined through Transwell invasion assay (scale bar = 100 μ m) and invaded cell numbers. I-J: Flow cytometric detection of apoptosis and apoptotic rates. Data are presented as mean $\pm$ SD from 3 independent experiments ($n = 3$). ** $P < 0.001$ vs. the control group; # $P < 0.05$, ## $P < 0.001$ vs. the GA group; & $P < 0.05$, && $P < 0.001$ vs. the GA+740 Y-P + siNC group.

3.6. Suppressive effects of GA on gastric cancer cell malignant phenotypes are associated with the PI3K/Akt/FOXO1 axis

The involvement of the PI3K/Akt/FOXO1 axis in the antitumor effects of GA in gastric cancer cells was further examined using pharmacological and genetic rescue experiments. The results from colony formation and EdU staining demonstrated that GA significantly reduced proliferation capacity and clonogenic potential of MKN-45 cell ($P < 0.05$), whereas 740 Y-P partially restored clonogenic capacity and DNA synthesis activity. FOXO1 knockdown in the GA + 740 Y-P group weakened this restorative effect, as reflected by a renewed decrease in colony numbers and a

further reduction in the proportion of EdU-positive cells (Figure 6A-D).

The migratory and invasive properties of gastric cancer cells were significantly reduced by GA, as evidenced by Transwell chamber assays ($P < 0.05$). PI3K activation partially restored the movement and invasiveness of the cells, yet this compensatory effect was compromised by siFOXO1 transfection, which further reduced the populations of migrated and invasive cells (Figure 6E-H). Additionally, quantitative assessment by flow cytometry showed that GA treatment significantly increased the apoptotic index of MKN-45 cells ($P < 0.05$), while 740 Y-P partially weakened this effect. FOXO1 knockdown in the GA + 740 Y-P group diminished the effect of 740

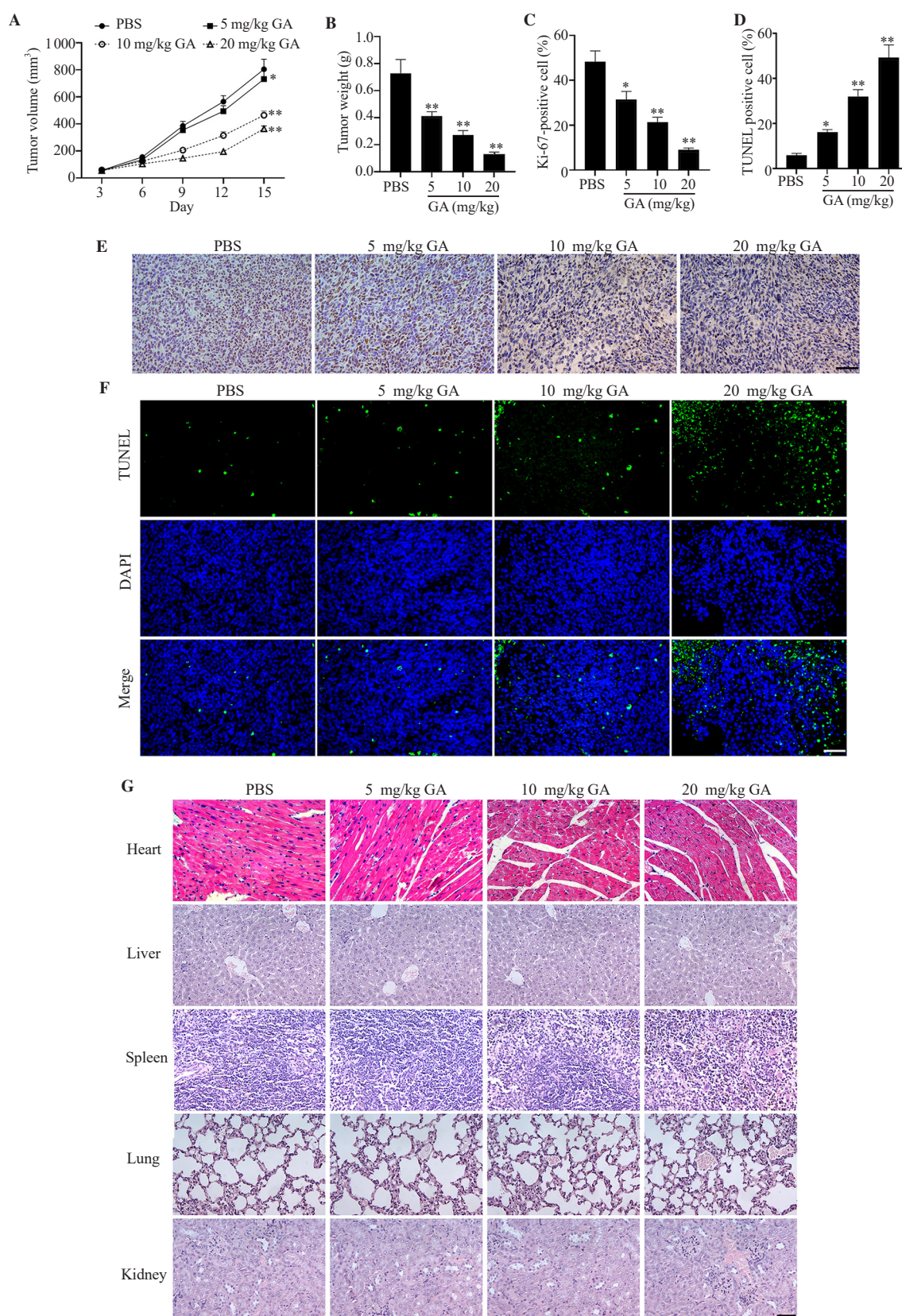


Figure 7. GA suppresses the growth of gastric cancer xenografts *in vivo* without obvious histopathological abnormalities in major organs. A: Tumor growth of xenografts in nude mice. B: Tumor weight. C&E: Immunohistochemical assessment of the proliferative marker Ki-67 (scale bar = 50 μ m) and Ki-67-positive cell fractions. D&F: Apoptosis in tumor tissues determined by TUNEL fluorescence staining (scale bar = 50 μ m) and percentage of TUNEL-labeled cell. G: Histopathological examination (H&E staining) of vital organs (heart, liver, spleen, lung, and kidney) (scale bar = 50 μ m). Data are presented as mean $\pm$ SD (n = 6 mice per group). All histological quantifications were derived from 5 random fields per section. * P <0.05, ** P <0.001 *vs.* the PBS group.

Y-P, resulting in a renewed increase in apoptosis (Figure 6I-J).

3.7. GA suppresses gastric tumor xenograft growth *in vivo* without obvious histopathological abnormalities in major organs

To evaluate the *in vivo* antitumor effect of GA, a subcutaneous xenograft model was established in nude mice using MKN-45 cells, followed by intragastric administration of GA at different doses. The excised tumors from each group are shown in Supplementary Figure

2. Compared with the PBS control group, GA significantly inhibited xenograft growth, as evidenced by decreased tumor volume, reduced tumor size, and lower tumor weight, with a dose-dependent trend ($P < 0.05$) (Figure 7A-B).

Immunohistochemical staining showed that GA treatment significantly reduced Ki-67-positive expression in tumor tissues ($P < 0.05$) (Figure 7C&E), indicating suppressed *in vivo* tumor cell proliferation. TUNEL fluorescence staining showed that GA significantly increased the number of TUNEL-positive cells in tumor tissues, suggesting increased tumor cell apoptosis ($P < 0.05$) (Figure

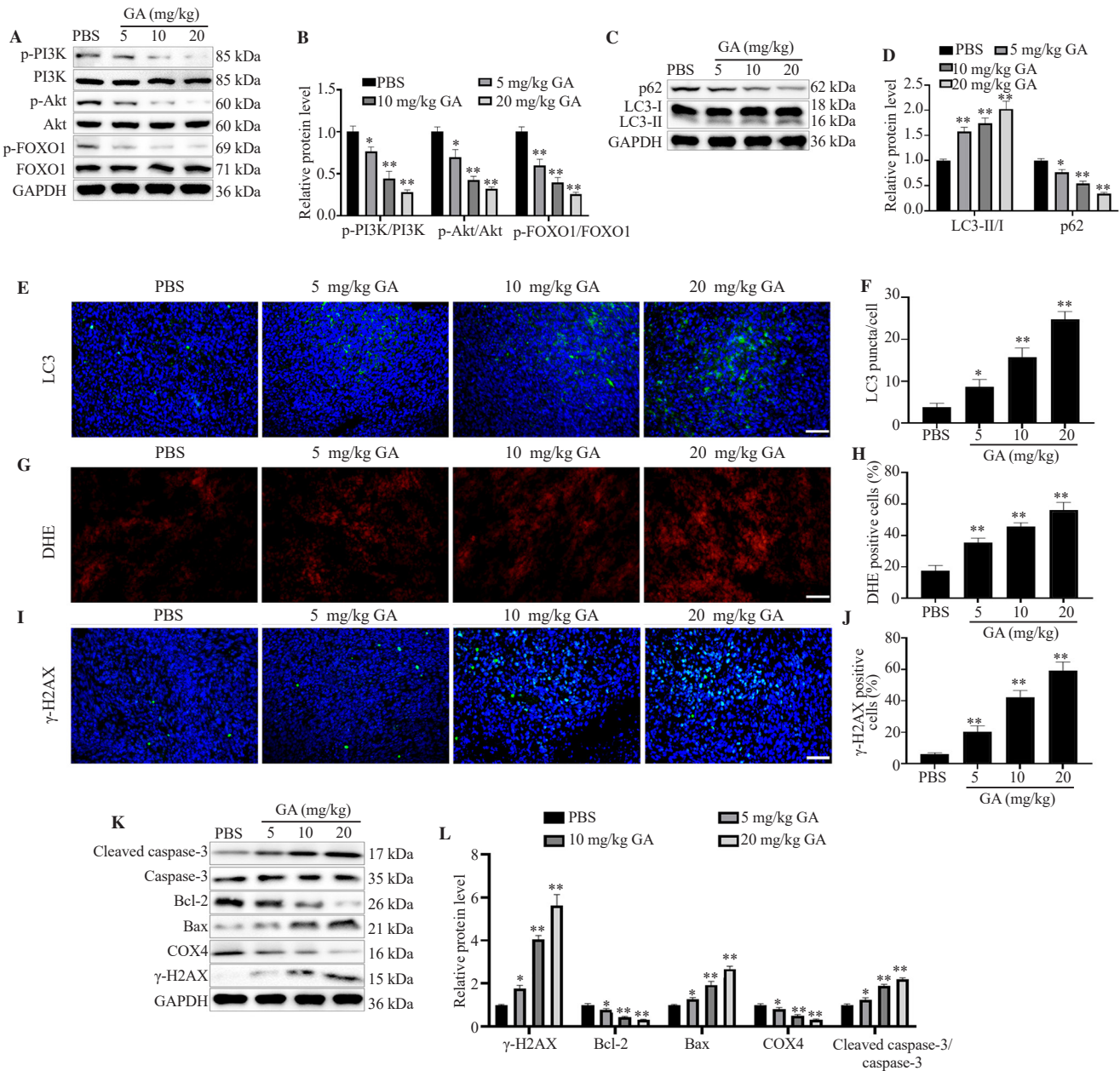


Figure 8. GA inhibits gastric cancer progression *in vivo* via the PI3K/Akt/FOXO1 axis. A-B: Immunoblotting analysis of the phosphorylation and total expression of PI3K, Akt, and FOXO1. C-D: Protein expression of LC3-I/II and p62. E-F: Immunofluorescence visualization of LC3B-positive foci (scale bar = 50 μ m), and the number of LC3 puncta per cell. G-H: Intratumoral ROS production detected by DHE staining (scale bar = 50 μ m) and the DHE-positive cell proportion. I-J: Distribution of γ -H2AX-labeled DNA damage foci (scale bar = 50 μ m) and γ -H2AX-positive cell fractions. K-L: Western blot analysis of γ -H2AX, cleaved caspase-3/caspase-3, Bcl-2, Bax, and COX4. Data are presented as mean $\pm$ SD. For histological and immunofluorescence analyses, 5 random fields per section were quantified. For Western blot analysis of tumor tissues, 3 biological replicates per group were analyzed. * $P < 0.05$, ** $P < 0.001$ vs. the PBS group.

7D&F). After GA intervention, H&E staining of the major organs, including the heart, liver, spleen, lung, and kidney, showed no significant histopathological abnormalities, suggesting no obvious toxic effects on major organs within the tested dose range (Figure 7G).

3.8. GA suppresses gastric cancer progression *in vivo*, accompanied by PI3K/Akt/FOXO1 modulation, autophagy-related changes, and apoptosis-related changes

To further investigate the *in vivo* molecular mechanism, markers involved in the PI3K/Akt/FOXO1 axis, autophagy, and mitochondria-associated apoptosis were examined in xenograft tumor tissues. *In vivo* results demonstrated that GA treatment significantly reduced protein levels of p-PI3K and p-Akt, as well as p-FOXO1 (Ser256)/FOXO1 ratio ($P < 0.05$), indicating that GA inhibits the PI3K/Akt/FOXO1 signaling axis in tumor tissues (Figure 8A-B). Assessment of autophagy showed that GA significantly increased the LC3-II/LC3-I ratio and decreased p62 protein expression ($P < 0.05$). Consistently, immunofluorescence staining of LC3B revealed an increased number of LC3 fluorescent puncta in tumor tissues, suggesting autophagy-related changes (Figure 8C-F). DHE staining showed that GA significantly increased the proportion of DHE-positive cells in tumor tissues, suggesting elevated ROS levels ($P < 0.05$) (Figure 8G-H). Both immunofluorescence and Western blot analyses demonstrated that GA significantly increased both the proportion of γ -H2AX-positive cells and the protein expression of γ -H2AX ($P < 0.05$), indicating severe DNA damage in xenograft tissues (Figure 8I-L). GA also significantly increased Bax expression and the cleaved caspase-3/caspase-3 ratio, while it significantly decreased Bcl-2 and COX4 protein levels ($P < 0.05$), suggesting mitochondrial dysfunction and apoptosis-related changes.

4. Discussion

Globally, gastric cancer is one of the most frequently diagnosed gastrointestinal malignancies and remains a major cause of cancer-related mortality. Because of its invasiveness, metastatic potential, and poor prognosis, gastric cancer continues to pose a major challenge in cancer research and clinical treatment[28]. Although comprehensive therapeutic strategies for gastric cancer have continued to evolve in recent years, treatment resistance, recurrence, and distant metastasis remain the major obstacles limiting survival benefits in patients with advanced-stage disease[29]. Therefore, there is an urgent need to identify natural small molecules with well-defined molecular targets, satisfactory safety profiles, and potent antitumor activity. In this study, we investigated the effects of GA, a natural bioactive compound derived from ginger, on the malignant

progression of gastric cancer and further explored its underlying molecular mechanisms. GA treatment suppressed gastric cancer cell growth, migration and invasion while promoting cell death *in vitro* and inhibiting tumor growth *in vivo*. Mechanistic investigations suggested that the PI3K/Akt/FOXO1 axis is involved in the effects of GA, and that modulation of this axis is associated with autophagy-related changes and apoptosis-related responses.

Aberrant proliferation and evasion of apoptosis are fundamental biological features underlying the sustained progression of gastric cancer[30]. In the present study, initial screening across multiple cell lines showed that GA exerted inhibitory effects on several gastric cancer cell lines, with MKN-45 cells being the most sensitive, whereas normal gastric mucosal epithelial GES-1 cells showed relatively low sensitivity to GA. These findings suggest that GA may possess a certain degree of tumor selectivity. Further functional experiments showed that GA reduced the viability of MKN-45 cells, suppressed colony formation and EdU incorporation, impaired migratory and invasive capacities, and increased the proportion of apoptotic cells. These results indicate that GA not only inhibits the short-term proliferative activity of gastric cancer cells, but also affects their long-term clonogenic potential and migratory and invasive behaviors. Overall, our results support the anti-gastric cancer potential of GA and suggest that it may merit further investigation.

Mitochondria are important regulators of cellular metabolism and programmed cell death and play a critical role in cancer cell survival[31]. The dissipation of MMP, coupled with an intracellular surge of ROS and the dysregulation of Bcl-2 family proteins, represents a cardinal signature of mitochondrial-mediated apoptotic initiation[32,33]. In the present study, GA treatment reduced MMP in gastric cancer cells and increased both total intracellular ROS and mitochondria-derived ROS, together with decreased COX4 and Bcl-2 levels, increased Bax expression, and enhanced caspase-3 cleavage. These changes suggest that GA is associated with mitochondrial dysfunction and apoptosis-related changes. In addition, GA enhanced γ -H2AX expression, indicating a DNA damage response. Previous studies have shown that excessive ROS can not only directly impair mitochondrial structure and function, but also trigger nuclear DNA damage, thereby amplifying pro-apoptotic signaling and promoting tumor cell death[34]. Therefore, ROS accumulation, γ -H2AX upregulation, and changes in mitochondrial apoptosis-related proteins observed in this study collectively support the conclusion that GA is associated with oxidative stress, mitochondrial dysfunction, and apoptosis-related changes.

Autophagy has dual roles in cancer, and its function depends on tumor type, disease stage, and the cellular stress context[35]. On the one hand, autophagy can help tumor cells eliminate damaged organelles and maintain metabolic adaptation[36]. On the other hand, excessive or sustained autophagy activation may also cooperate with

cell death programs, thereby suppressing tumor progression[37]. In the present study, GA increased the LC3-II/LC3-I ratio, reduced p62 protein abundance, and increased the formation of LC3B-positive puncta, suggesting autophagy-related changes. Pharmacological inhibition of autophagy *via* 3-MA partially abrogated the impact of GA on gastric cancer cell proliferation, migration, invasion, and apoptosis, suggesting that autophagy may contribute, at least in part, to these effects. These findings suggest that, under the conditions of this study, autophagy may function more as a pro-death-associated process contributing to GA-induced antitumor activity rather than serving solely as a cytoprotective response. Together with the dissipation of MMP, the increase in ROS levels, and the changes in apoptosis-related proteins, these findings suggest that the autophagy-related changes induced by GA may be functionally coupled to mitochondrial damage and apoptosis, ultimately being associated with suppression of the malignant progression of gastric cancer cells.

The PI3K/Akt pathway is a well-defined prosurvival signaling axis in oncology, and its persistent activation promotes cell proliferation, metabolic adaptation, migration, invasion, and resistance to apoptosis[13]. As a key downstream transcription factor of this pathway, FOXO1 is frequently phosphorylated and inactivated in response to PI3K/Akt hyperactivation, thereby weakening its tumor-suppressive function. Previous studies have shown that activated FOXO1 can directly regulate the transcription of autophagy- and apoptosis-related genes such as *LC3*, *Bnip3*, and *Bim*, and is therefore broadly involved in cellular stress responses[16]. In the present study, GA reduced the levels of p-PI3K and p-Akt and was associated with reduced inhibitory phosphorylation of FOXO1. These findings suggest that the PI3K/Akt/FOXO1 axis may be involved in the observed autophagy-related changes and apoptosis-related responses. The PI3K activator 740Y-P partially attenuated the GA-induced effects on autophagy, mitochondrial/DNA damage, and malignant phenotypes, whereas FOXO1 knockdown partially restored these GA-induced effects. Collectively, these findings suggest that FOXO1 may serve as a link between suppression of upstream survival signaling and downstream autophagy-related and apoptosis-related responses, and that the PI3K/Akt/FOXO1 axis may represent an important molecular basis for the anti-gastric cancer effect of GA. Although FOXO1 nuclear translocation and its direct transcriptional target genes were not further examined in this study, the current evidence supports the involvement of this signaling axis in the antitumor mechanism of GA.

The xenograft model further supported the present findings. In the xenograft model, GA administration reduced tumor growth and tumor weight, accompanied by decreased Ki-67 positivity and increased TUNEL-positive cells. These observations support the *in vivo* antitumor and pro-apoptotic effects of GA. More importantly, the changes observed in the PI3K/Akt/FOXO1 axis, autophagy-related markers, ROS, γ -H2AX, and apoptosis-related proteins

in tumor tissues were largely consistent with the *in vitro* findings, suggesting general consistency between the *in vivo* and *in vitro* findings. In addition, H&E staining revealed no obvious pathological abnormalities in the major organs, suggesting that GA did not cause obvious histopathological damage to major organs within the tested dose range. These findings provide experimental support for the further investigation of GA as a potential therapeutic candidate for gastric cancer.

However, several limitations of the present study should be acknowledged. Regarding mechanistic depth, although autophagy-related changes and FOXO1 modulation were identified as important events, the precise relationship between autophagy-related changes and mitochondrial dysfunction was not further dissected. Similarly, the evidence for mitochondria-associated apoptosis remains partly indirect without the measurement of cytochrome C release or caspase-9 activation. In addition, the precise transcriptional network of FOXO1 requires further validation through ChIP-qPCR or nuclear translocation assays. Furthermore, the reliance on a single cell line, MKN-45, may not fully capture the inherent heterogeneity of gastric cancer, requiring broader validation in diverse molecular subtypes. Finally, while our *in vivo* results demonstrate the antitumor efficacy and lack of obvious histopathological abnormalities in major organs at the tested doses, its full pharmacokinetic profile and long-term toxicity remain to be elucidated. Future studies integrating electron microscopy, mitochondrial isolation, and systematic pharmacodynamic evaluation will be important to fully assess the clinical translational potential of GA.

Overall, this study showed the inhibitory effect of GA on gastric cancer and explored the associated mechanisms in both *in vitro* and *in vivo* models. GA may, at least in part, suppress the malignant progression of gastric cancer and inhibit xenograft growth in association with inhibition of PI3K/Akt signaling, reduced inhibitory phosphorylation of FOXO1, autophagy-related changes, and apoptosis-related responses. These findings not only expand the current evidence supporting the antitumor activity of GA in gastric cancer, but also provide an experimental basis for future mechanistic studies focusing on autophagy and mitochondrial homeostasis.

The present findings suggest that GA suppresses gastric cancer progression. In cellular models, GA restricted the proliferation, motility, and invasive capacity of gastric cancer cells while concurrently promoting apoptosis. Mechanistically, these antitumor activities appear to be associated, at least in part, with suppression of the PI3K/Akt/FOXO1 signaling cascade and reduced inhibitory phosphorylation of FOXO1, together with autophagy-related changes, ROS accumulation, DNA damage, and apoptosis-related changes. Furthermore, GA administration in xenograft models inhibited tumor growth and reduced proliferative markers without causing obvious histopathological abnormalities in major organs at the tested doses. Collectively, GA may inhibit gastric cancer

progression *via* modulation of the PI3K/Akt/FOXO1 axis, along with autophagy-related and apoptosis-related changes, supporting its further investigation as a natural bioactive compound in gastric cancer.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

Authors' contributions

YZ and RM conceived and designed the research, conducted experiments, analyzed data, and drafted and revised the manuscript critically for important intellectual content. TW, XM, and HW contributed to the acquisition, analysis, and interpretation of data, and provided substantial intellectual input during the drafting and revision of the manuscript. LZ participated in the conception and design of the study, and played a key role in data interpretation and manuscript preparation. All authors have read and approved the final version of the manuscript.

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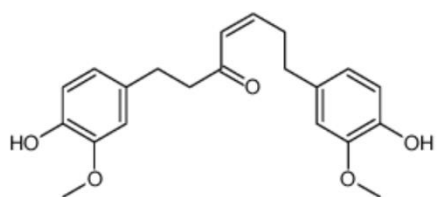
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Supplementary Figure 1. Chemical structure of gingerenone A (GA).



Supplementary Figure 2. Macroscopic appearances of harvested tumors from each treatment group.