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Research article

Resveratrol induces apoptosis, autophagy and endoplasmic reticulum stress in colon cancer cells

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Abstract

Objective: Resveratrol exerts various biologic effects. This study was designed to investigate its anti-cancer effect and its impact on cell apoptosis, autophagy, and endoplasmic reticulum stress (ER-stress) in colon cancer.

Methods: Colon cancer cells were treated with resveratrol. Cell viability, apoptosis, and autophagy were assessed by MTT, flow cytometry, and green fluorescence protein (GFP)-tagged LC3B analysis, respectively. Gene expression was detected by qRT-PCR and western blot. Xenograft model was subjected to verify the effects of resveratrol on tumorigenesis of colon cancer cells in vivo.

Results: The viability of colon cancer cells was reduced by resveratrol. The cell apoptosis and the protein levels of apoptotic markers (cleaved-PARP and cleaved-Caspase3) were increased by resveratrol. The protein level of autophagic marker (MAP1LC3B) and the LC3B dots accumulation were elevated by resveratrol. Autophagy inhibitor (3-MA) partially reversed the effect of resveratrol on cell apoptosis. The protein and mRNA levels of ER-stress markers (IRE-1 and ATF6) and pro-apoptotic signals (GRP-78, GADD153) were up-regulated by resveratrol. The tumor growth of colon cancer cells was suppressed by resveratrol in vivo.

Conclusion: Resveratrol exerts anti-cancer function of colon cancer, which is associated with its induction effect of cell apoptosis, autophagy and ER-stress.

Keywords: resveratrol; colon cancer; apoptosis; autophagy; endoplasmic reticulum stress

INTRODUCTION

Colon cancer is one of the most common malignant tumors throughout the world and has high morbidity and mortality[1]. Thus, it is important to develop efficient drugs for the treatment of colon cancer. Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a natural polyphenolic compound and extensively exists in wine and multiple plants, including peanuts, grapes, as well as herbs[2,3] . It is widely recognized for its diverse pharmacological properties, such as anti-oxidative, anti-inflammatory and anti-aging. For instance, resveratrol can act as a scavenger of reactive oxygen species such as hydroxyl- and superoxide- radicals to exert its anti-oxidative activity[4,5] . Resveratrol can also exert anti-inflammatory function via blocking NF-κB and p38MAPK pathways [6]. As well, the anti-aging effect of resveratrol has been found in lower organisms, such as zebrafish and yeast[7,8] . Apart from the anti-oxidative, anti-inflammatory, and anti-aging activities, resveratrol has been proved to exert strong anti-cancer property of many cancers, including breast cancer, pancreatic cancer, and ovarian cancer[9-11]. In addition, resveratrol has anti-tumor function of colon cancer [12,13].

Apoptosis, autophagy, and endoplasmic reticulum stress (ER-stress) are closely related to cancer development and progression. Apoptosis can hinder cancer progression by removing harmful cancer cells, while the inhibition of apoptosis can result in infinite proliferation of cancer cells and thus promotes cancer development and progression[14,15] . Autophagy is also an adaptive response of cancer cells under environmental stress[19] . During cancer progression, autophagy can protect cancer cells from the many adverse factors, such as hypoxia and nutrition deficiency[20]. Nevertheless, anti-cancer agents-induced autophagy can mediate apoptosis of cancer cells in certain cancers[21,22]. ER-stress is mainly a survival-promoting response of cancer cells under various adverse conditions, which is mediated by unfolded protein response (UPR) [16]. Whereas when ER stress is severe and prolonged, IRE-1, ATF-6, and PERK, the signaling sensors of UPR, can activate pro-apoptotic signals, such as GADD153 and GRP-78, thereby promoting cell apoptosis[17]. Previous studies indicated that resveratrol can induce apoptosis, autophagy, and ER-stress of many

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types of cancer cells[18,19]. However, whether resveratrol can induce cell apoptosis, autophagy, and ER-stress in colon cancer is still unknown. In this study, we aimed to verify its anti-cancer effect and its impact on cell apoptosis, autophagy, and ER-stress in colon cancer

MATERIALS AND METHODS

Cell lines and cell culture

Human colon cancer cell lines HCT116 and HT29 were purchased from ATCC. Cells were cultured in the RPMI 1640 medium containing 10% foetal bovine serum and incubated at 37°C, 5% CO_2.

MTT assay

Cell viability of HCT116 and HT29 was detected by MTT Kit (Beyotime, China). In brief, HCT116 and HT29 cells were pretreated with different concentrations of resveratrol for 24 h or pretreated with 300 μM resveratrol for different times. Then cells (5×10^3-1×10^4) were seeded onto 96-well plate and incubated for 24 h. MTT solution and formanzan were added into each well. The absorbance at 570 nm was measured by a microplate reader (Biorad, USA) to quantify the cell viability. Each experiment was performed at least in triplicate.

Flow cytometry assay

Cell apoptosis of HCT116 and HT29 was detected using Annexin V-FITC/PI Apoptosis Detection Kit (YEASEN, China). Briefly, cells were washed with cooled PBS buffer and resuspended in the binding buffer. Annexin V-FICT and PI staining solution were added into cell suspension. After 15 min incubation in the dark, cells were analyzed by a flow cytometer (BD Bioscience, USA).

GFP-LC3B analysis

SHCT116 and HT29 cells were transfected with the 0.5 μg green fluorescence protein (GFP)-tagged LC3B expression vector (Cell Biolabs, USA) using Lipofectamine 2000 reagent (Invitrogen, USA). 24 h after transfetcion, HT29 cells and HCT116 cells were treated with 300 μM resveratrol and 5μM autophagy inhibitor 3-MA. Then cells were fixed with formaldehyde for 10 min and washed with PBS buffer for 3 times. Last, cells were observed under a fluorescence microscope (Olympus, Japan).

Total RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from HCT116 cells using Trizol reagent (Takara, Japan). After the quality and concentration of RNA were determined, 1000 ng RNA was used to synthesize cDNA using Reverse transcription Kit (Takara, Japan). cDNA and SYBR Green Master Mix (CWBIO, China) were sujected to perform qRT-PCR. The relative expression of target genes (GRP-78, GADD153, IRE-A, and ATF6) was calculated by $2^{-\Delta\Delta CT}$ method. GAPDH was used to normalize the expressions of traget genes.

Western blot

Protein was isolated from HCT116 and HT29 cells by RIPA lysis buffer (CWBIO, China) containing protease inhibitor. After measured the concentration by BCA Kits (CWBIO, China), 30 μg protein was separated by SDS-PAGE. Then protein was transferred onto PVDE membranes and blocked for 1 h. The PVDE membranes were incubated with primary antibodies: anti-Caspase3 (Abcam, 1:5000), anti-PARP (GeneTex, 1:2000), anti-MAP1LC3B (AmyJet Scientific, 1:1500), anti-GADD153 (Abcam, 1:1000), anti-IRE-1 (Abcam, 1:1000), anti-TAF-6 (GeneTex, 1:2000), anti-Tubulin (Abcam, 1:5000) at 4 °C overnight, followed by incubation with corresponding secondary antibodies at room temperature for 2 h. The specific protein bands were visualized using chemiluminescence detection system (Biorad, USA).

Xenograft models

BALB/c-nude mice (5 weeks old, 20.0±0.5 g) were purchased from the Beijing Weitong Lihua Laboratory Animal Technology Co., Ltd. (China). All mice were housed in the SPF condiction and provided with enough food and water. After a week of acclimatization, they were subcutaneously inoculated with HTC116 cells (1×10^6) and randomly divided in four groups: Control group (without resveratrol and 3-MA treatment), (30 mg/kg) group, 3-MA (1 mg/kg) group, resveratrol +3-MA group (30 mg/kg +1 mg/kg 3-MA treatment). Resveratrol or 3-MA treatments were performed once per 3 days for 18 consecutive days. Tumor volume and animal weight were measured once per 3 days. All animal experimental producers were approved by the First Affiliated Hospital of Zhengzhou University.

Statistical analysis

All dats were analyzed by SPSS 20.0 and expressed as mean ± standard deviation. The group differences were compared using student's t-test. P-value <0.05 indicated statistically significant.

RESULTS

Resveratrol inhibited cell viability of colon cancer cells

The chemical structure of resveratrol was shown in Fig. 1A. To evaluate the effect of resveratrol on cell viability of colon cancer cells, HCT116 and HT29 cells were treated with different concentrations of resveratrol for 24 h or treated with 300 μM resveratrol for different times, and then cell viability was detected. The results showed that resveratrol inhibited the cell viability of HCT116 and HT29 cells in a dose-dependent manner and signifi-

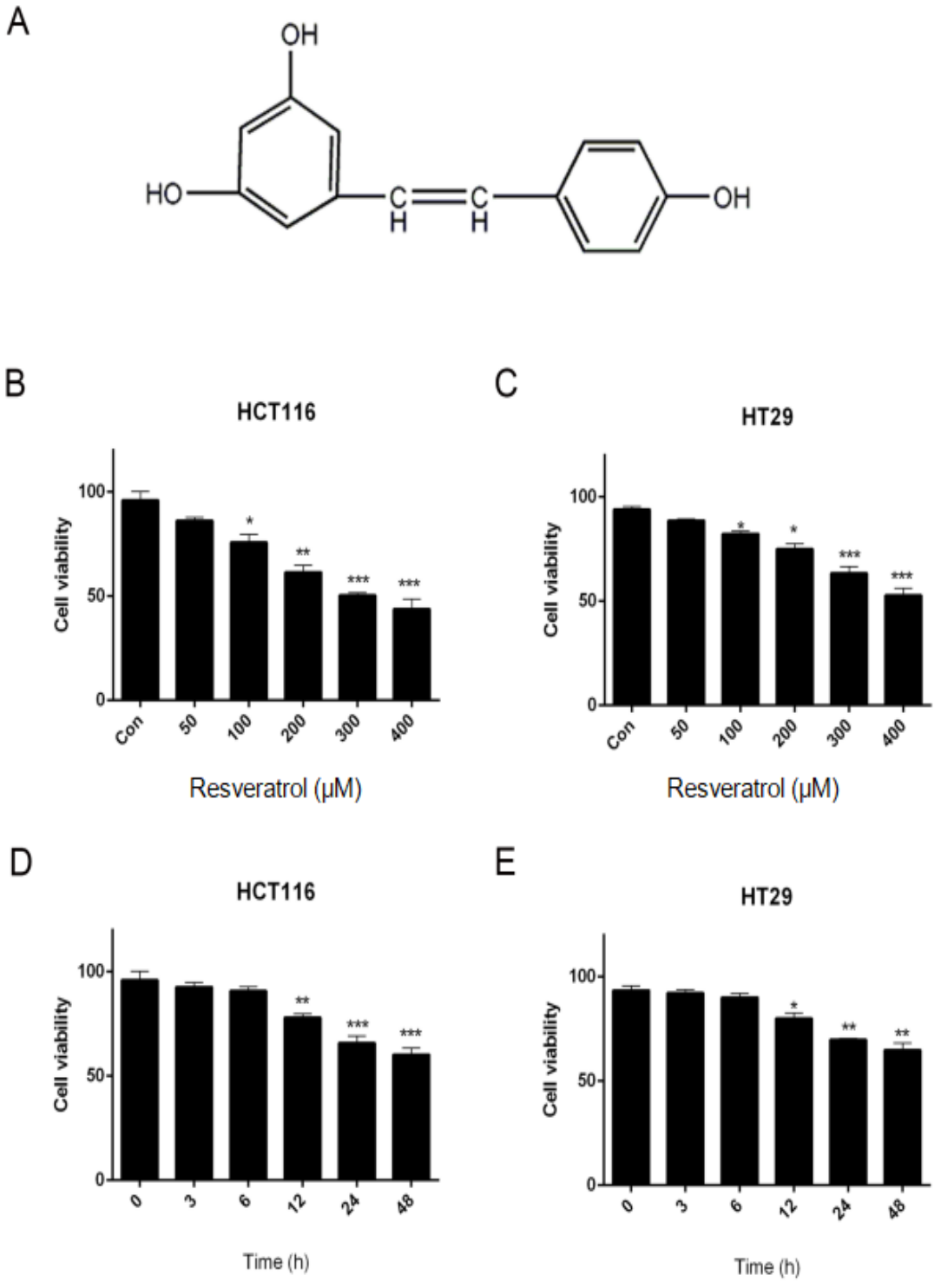

Figure 1. Effect of resveratrol on the growth of colon cancer cells. (A) Chemical stucture of resveratrol. (B&C) HCT116 and HT29 cells were treated with different doses of resveratrol (50, 100, 200, 300, and 400 μM) for 24 h. Cell viability of HCT116 (B) and HT29 (C) was detected by MTT assay. (D&E) HCT116 and HT29 cells were treated with 300 μM resveratrol for different times (0, 3, 6, 12, 24, and 48 h). Cell viability of HCT116 (D) and HT29 (E) was detected by MTT assay. *p< 0.05, **p< 0.01, ***p< 0.001.

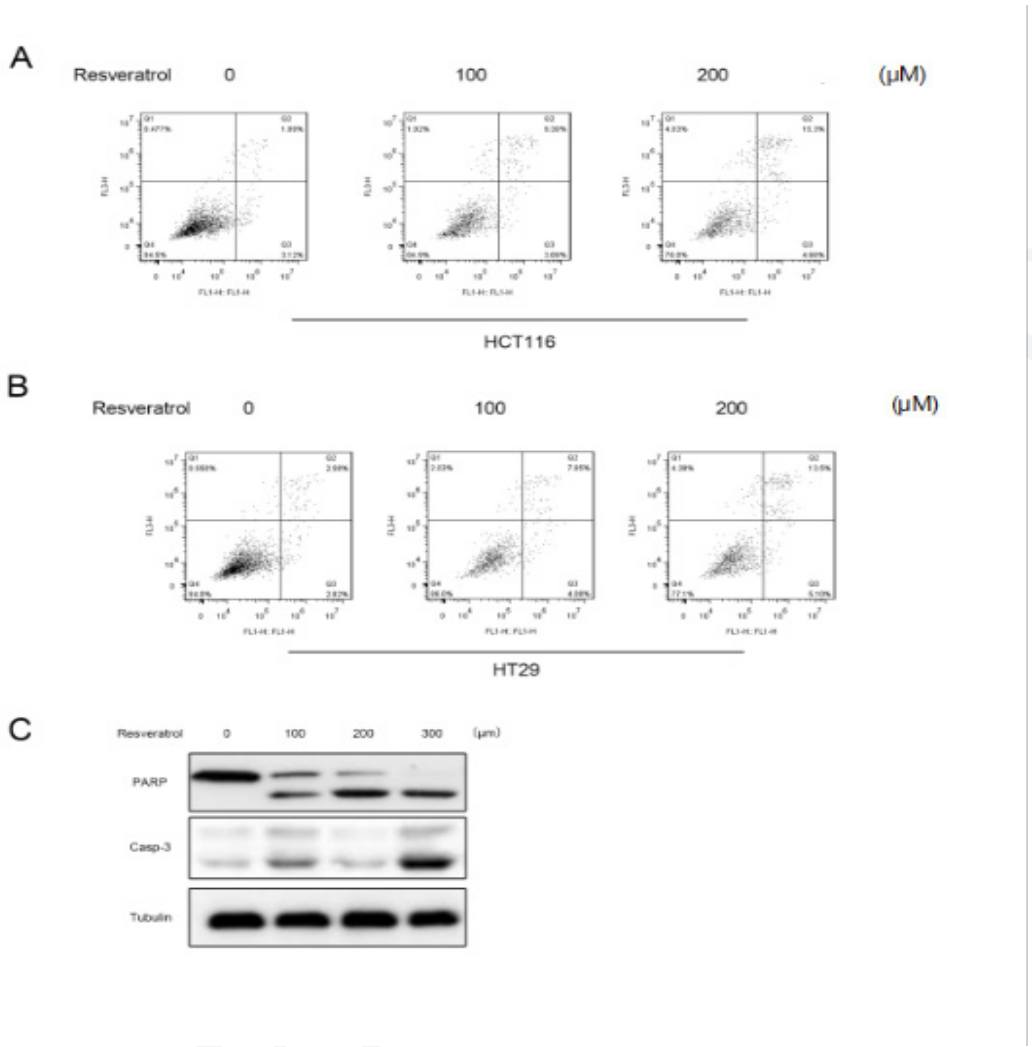

Figure 2. Effect of resveratrol on the apoptosis of colon cancer cells. (A&B) HCT116 and HT29 cells were treated with different doses (0, 100, and 200 μM) of resveratrol for 24 h. Cell apoptosis of HCT116 (A) and HT29 (B) was detected by flow cytometry assay. (C) HCT116 cells were treated with different doses (0, 100, 200, 300 μM) of resveratrolfor 24 h. The protein levels of PARP and Caspase3 were detected by western blot. Tubulin was used as a reference.

cantly inhibited the cell viability when the concentration reached 100 μM (Fig. 1B&C). The results also revealed that 300 μM resveratrol decreased the cell viability of HCT116 and HT29 cells in a time-dependent manner and evidently decreased the cell viability when the treatment time reached 12 h (Fig. 1D&E). These results indicated that resveratrol inhibited cell viability of colon cancer cells in a dose- and time-dependent manner.

Resveratrol induced apoptosis of colon cancer cells

In order to assess the impact of resveratrol on the apoptosis of colon cancer cells, HCT116 and HT29 cells were treated with different concentrations of resveratrol for 24 h. Then the apoptotic cells were detected by flow cytometry assay and the expression of apoptotic markers (PARP and Caspase3) was determined by western blot. The flow cytometry result showed that 100 and 200 μM resveratrol promoted apoptosis of HCT116 and HT29 cells (Fig. 2A&B). The western blot result revealed that resveratrol up-regulated the cleaved-PARP and cleaved-Caspase3 protein level in HCT116 cells (Fig. 2C). Those results suggested resveratrol induced apoptosis of colon cancer cells in a dose-dependent manner.

Resveratrol induced autophagy of colon cancer cells

Next, we explore the influence of resveratrol on the autophagy of colon cancer cells. The western blot results showed that resveratrol up-regulated the protein level of MAP1LC3B in a dose-dependent manner in HCT116 and HT29 cells (Fig. 3A&B). The immunofluorescence

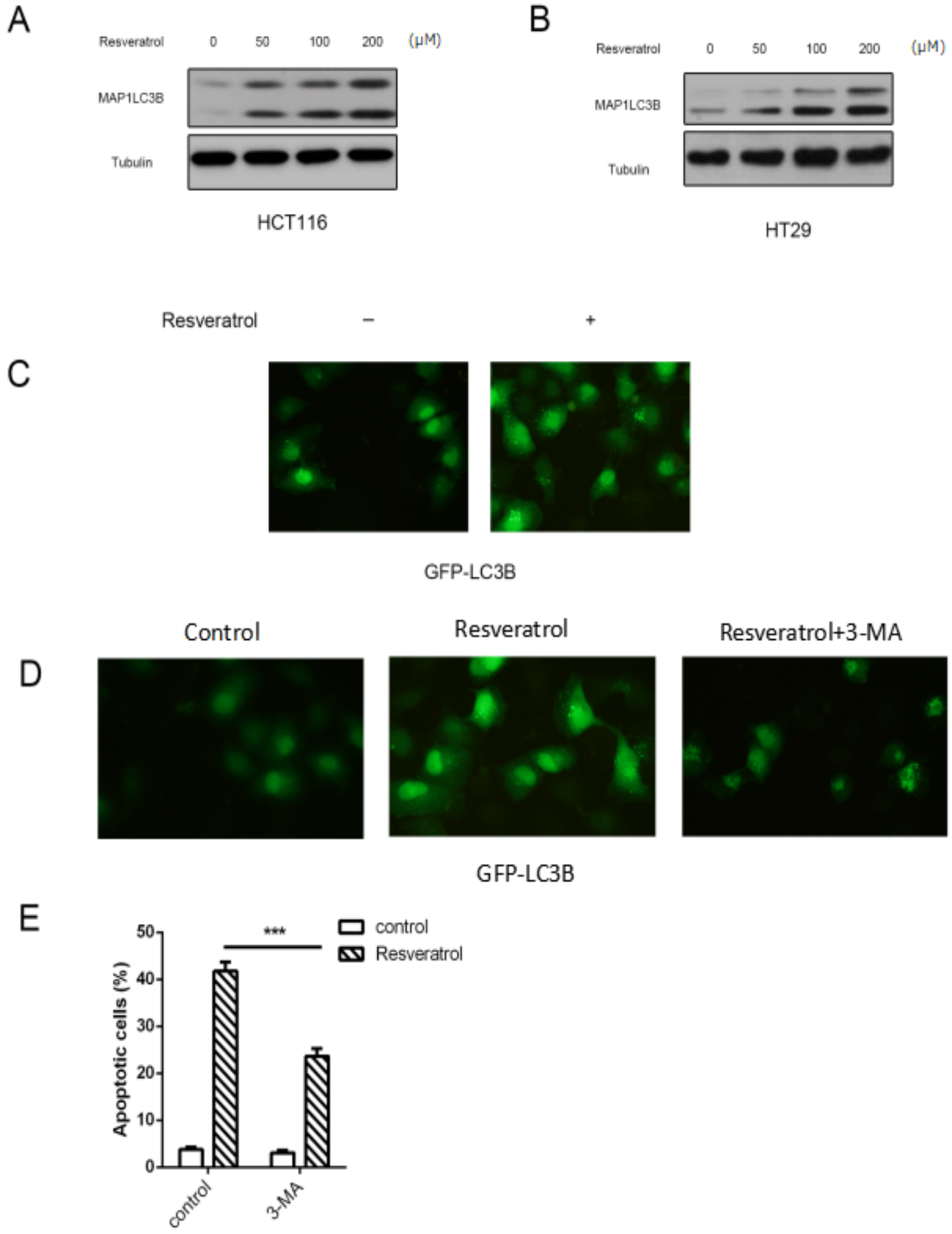

Figure 3. Effect of resveratrol on the autophagy of colon cancer cells. (A&B) HCT116 and HT29 cells were treated with different concentrations (0, 50, 100, and 200 μM) of resveratrol for 24 h. The protein level of MAP1LC3B in HCT116 (A) and HT29 cells (B) was determined by western blot. (C) HT29 cells were transfected with GFP-LC3B plasmid for 24 hours before and then exposed to 300 μM resveratrol for 8 hours. The accumulation of LC3B was deteced by immunofluorescent assay. (D) HCT116 cells were transfected with GFP-LC3B plasmid for 24 hours. Then cells were retreated with autophagy inhibitor 3-MA (5 μM) for 30 min and exposed to 300 μM resveratrol for 8 h. The accumulation of LC3B was deteced by immunofluorescent assay. (E) HCT116 cells were pretreated with 5 μM 3-MA for 30 min, then cells were treated with control or 300 μM resveratrol for 24 hours. Cell apoptosis was detected by flow cytometry assay. ***p< 0.001.

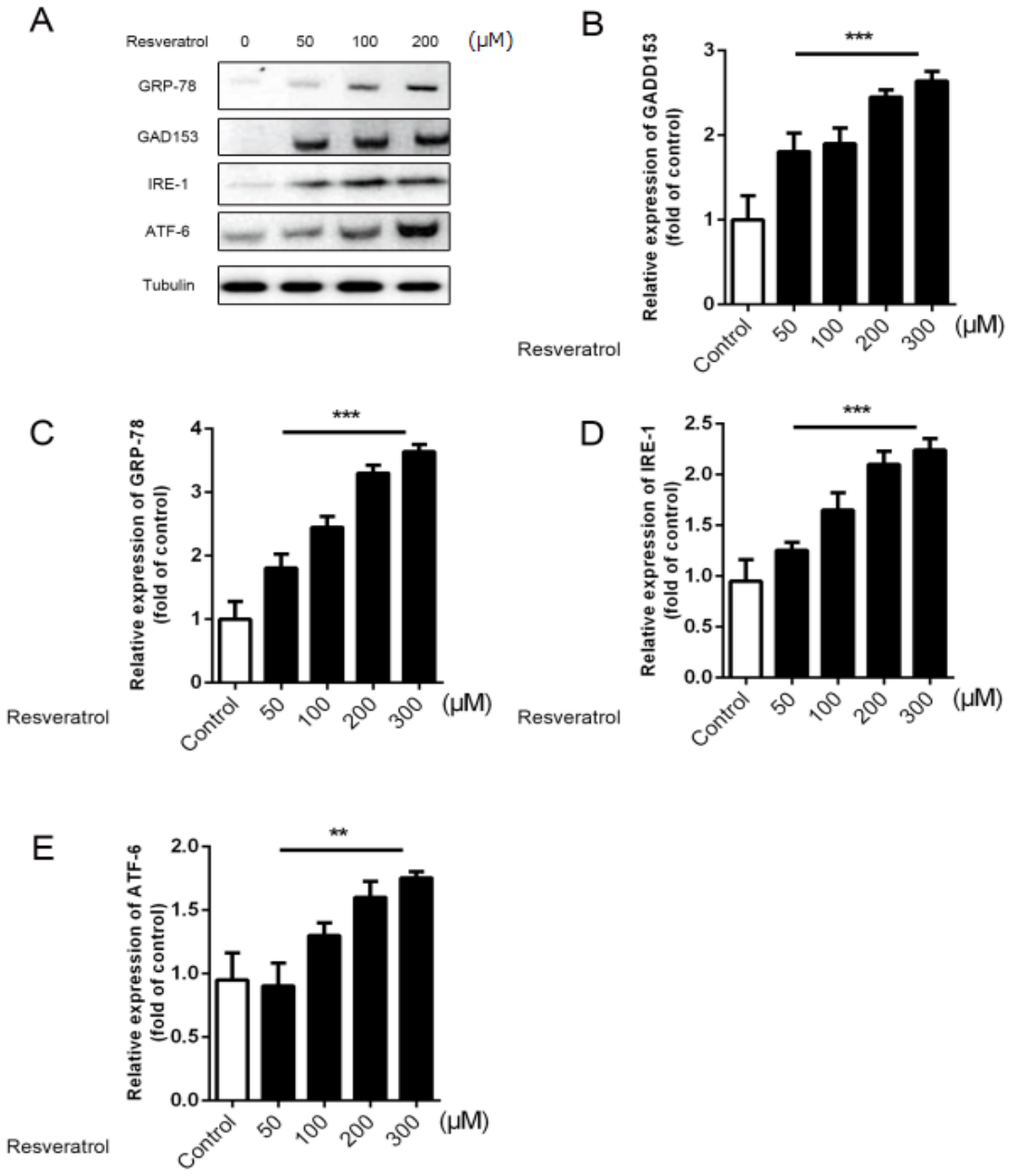

Figure 4. Effetc of resveratrol on ER-stress of colon cancer cells. (A) HCT116 cells were treated with different concentrations (0, 50, 100, and 200 μM) of resveratrol for 24 h. The protein levels of ER-stress related proteins (GRP-78, GADD153, IRE-1, and ATF6) were detected by western blot. Tubulin was used as a reference. (B-E) HCT116 cells were treated with different doses (0, 100, 200, 300 μM) of resveratrolfor 24 h. The mRNA levels of GADD153 (B), GRP-78 (C), IRE-1 (D), and TAF-6 (E) were determined by qRT-PCR. **p< 0.01, ***p< 0.001.

staining result revealed that resveratrol increased the LC3B dots accumulation in the HT29 cells (Fig. 3C). Similarly, resveratrol also augmented the LC3B dots accumulation in the HCT116 cell, while autophapy inhibitor 3-MA eliminated the effect (Fig. 3D). These results suggested that resveratrol stimulated the autophagy of colon cancer cells.

To verify whether autophagy mediates the resveratrol-induced apoptosis of colon cancer cells, HCT116 cells were treated with resveratrol or/ and 3-MA, and then cell apoptosis was detected by flow cytometry assay. The result showed that the effect of resveratrol on the apoptosis of HCT116 cells was partially reversed by 3-MA treatment (Fig. 3E), implying that autophagy me-

diates the apoptosis induced by resveratrol.

Resveratrol induced ER-stress of colon cancer cells

Aiming to investigate the effect of resveratrol on ER-stress of colon cancer cells, HCT116 cells were treated with different concentrations of resveratrol, and then the expressions GRP-78, GADD153, IRE-1, and ATF6 were determined by western blot and qRT-PCR. As shown in Fig. 4A, resveratrol elevated the protein levels of GRP-78, GADD153, IRE-1, and ATF6 in a dose-dependent manner. As well, resveratrol increased the mRNA levels of GADD153, GRP-78, IRE-1, and ATF6 in a dose-dependent manner (Fig. 4B-E). These results demonstrated that resveratrol induced ER-stress of colon cancer cells.

Resveratrol attenuated tumorigenesis of colon cancer cells *in vivo*

To verify the effect of resveratrol on tumorigenesis of colon cancer cells *in vivo*, HCT116 cells were inoculated into nude mice to produce xenografts model. Mice were treated with resveratrol or/ and 3-MA and tumor volume and animal weight were measured once per 3 days. As shown in Fig. 5A, resveratrol suppressed the tumorigenesis of HCT116 cells *in vivo*, while 3-MA treatment reversed the effect. The result also revealed that resveratrol or /and 3-MA had no obvious influence on the body weight of mice (Fig. 5B). Those data confirmed that resveratrol attenuated tumorigenesis of colon cancer cells *in vivo*.

DISCUSSION

Resveratrol exerts beneficial effects in many diseases. For example, resveratrol plays a neuroprotective role in neonatal hypoxic-ischemic brain injury[20]; resveratrol exerts prevention and treatment function of Alzheimer's disease[21]; it also has cartilage protection effect in experimental osteoarthritis[22]. In addition, it has been confirmed that resveratrol has strong anti-cancer activity of various cancers in both cell culture and animal carcinogenesis models, including colon cancer[23]. In this study, our result showed that resveratrol inhibited the viability of colon cancer cells in a dose- and time-dependent manner in vitro. The result also revealed that resveratrol inhibited tumor growth of colon cancer cells in vivo. Therefore, our results demonstrated that resveratrol induced growth inhibition of colon cancer in vitro and in vivo.

It has been widely reported the induction effect of resveratrol on apoptosis, autophagy, and ER-stress of various types cells. In uterine sarcoma cells, resveratrol triggers apoptosis via inhibiting Wnt signaling pathway [24]. In leukemia cells, resveratrol contributes to cell autophagy and apoptosis through inhibiting Akt/mTOR pathway and activating p38-MAPK pathway[25]. In hepatoma cells, resveratrol promotes cisplatin-induced apoptosis and enhances palmitate-induced ER stress[26,27]. Since apoptosis, autophagy, and ER-stress play vital roles in the development and progression of cancers, we investigated the effects of resveratrol on them in the present study. We found that resveratrol facilitated apoptosis of colon cancer lines HCT116 and HT29 in a dose-dependent manner. As well, resveratrol up-regulated the protein levels of cleaved-PARP and cleaved-caspase3 in HCT116 cells. Our results were in accordance with previous study which indicated that resveratrol induces apoptosis of colon cancer line SW480[23]. The present data also showed that resveratrol up-regulated the autophagic marker MAP1LC3B expression and increased the LC3B dots accumulation in colon cells. Moreover, resveratrol elevated the protein levels of ER-stress markers IRE-1 and ATF6 in colon cells. Hence, these results showed that resveratrol induced apoptosis, autophagy, and ER-stress of colon cells and that might be associated with its anti-cancer function of colon cancer.

Increasing studies demonstrated that autophagy and ER stress, the important adaptive response mechanisms, take part in the response of cancer cells to anti-cancer agents. According to previous study, penfluridol attenuates pancreatic tumor growth through promoting autophagy–mediated apoptosis [28]. Cyptotanshinone exerts anti-cancer effects of liver and breast cancer cells via inducing ER-stress-mediated apoptosis[29].In addition, recent studies confirmed the ability of resveratrol in inducing ER-stress mediated apoptosis in many types of cancer cells. In malignant melanoma cell line, resveratrol promotes cell apoptosis and blocks cell cycle via enhancing ER-stress and oxidative stress[30]. In human nasopharyngeal carcinoma cell lines, resveratrol induce ER-stress-mediated apoptosis through up-regulating expression of IRE-1, ATF6, p-PERK, and CHOP[31]. In the current study, our result showed that resveratrol treatment promoted apoptosis of colon cells, while autophagy inhibitor 3-MA treatment partially reversed the induction effect of resveratrol on apoptosis. This result indicated that autophagy mediates resveratrol-induced apoptosis of colon cancer cells. In addition, the results showed that resveratrol up-regulated the expressions of ER-stress-related pro-apoptotic signals GADD153 and GRP-78, suggesting ER-stress involves in the resveratrol-induced apoptosis of colon cancer cells. Therefore, those results indicated that autophagy and ER-stress mediated resveratrol-induced apoptosis of colon cancer cells.

In conclusion, the present study suggested that resveratrol exerts anti-cancer function of colon cancer in vitro and in vivo, which is associated with its induction effects on apoptosis, autophagy, and ER-stress.

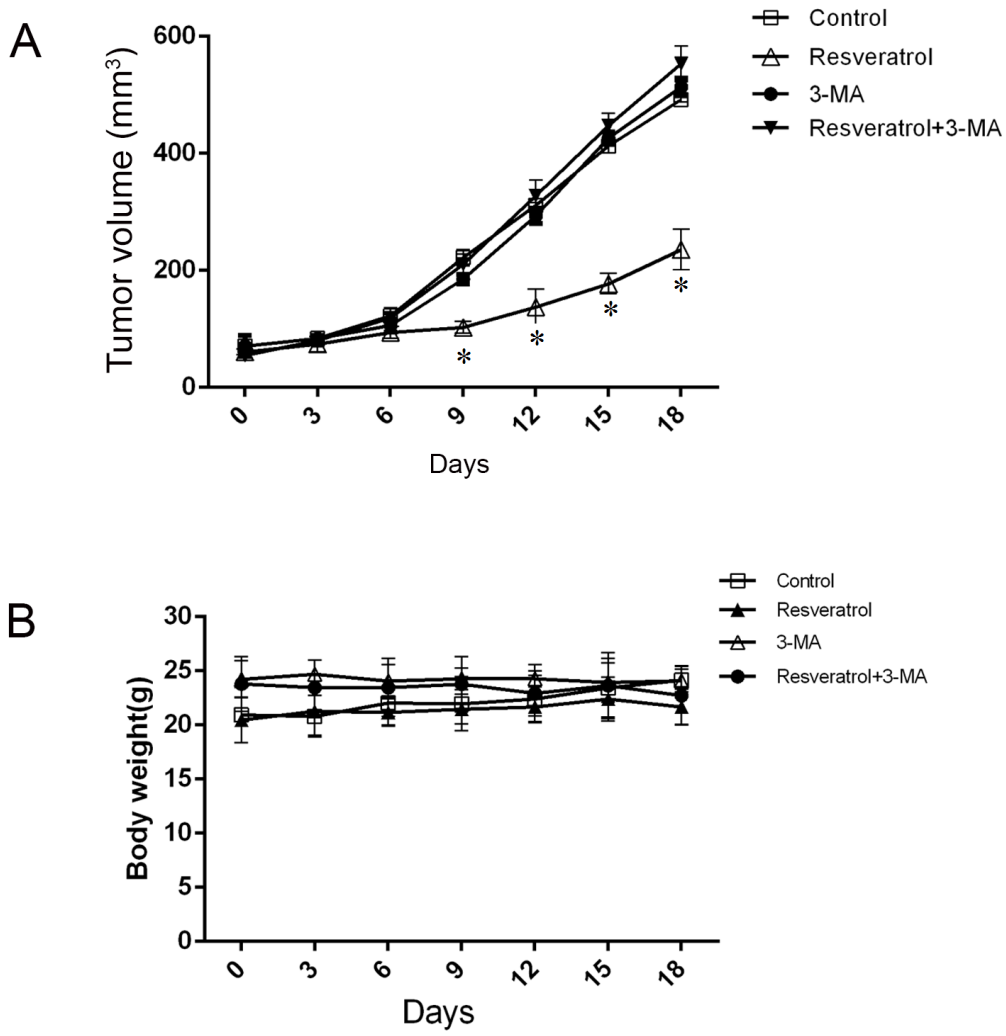

Figure 5. Effect of resveratrol on tumorigenesis of colon cancer cells in vivo. HCT116 cells were inoculated into nude mice to produce xenografts model. Mice were divided into control group (without resveratrol and 3-MA treatment), resveratrol (30 mg/kg) group, 3-MA (1 mg/kg) group, resveratrol +3-MA group (30 mg/kg +1 mg/kg 3-MA treatment). Resveratrol or 3-MA treatments were performed once per 3 days for 18 consecutive days. Tumor volume (A) and body weight (B) were measured once per 3 days. n=6 *p<0.05.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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