Life Sciences 218 (2019) 16–24

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Endoplasmic reticulum stress triggered by Soyasapogenol B promotes apoptosis and autophagy in colorectal cancer

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ARTICLE INFO

Keywords:
Soy B
Colorectal cancer
Endoplasmic reticulum stress
Apoptosis
Autophagy

ABSTRACT

Aim: Colorectal cancer (CRC) is a common human malignancy which accounts for 600,000 deaths annually at the global level. Soyasapogenol B (Soy B), an ingredient of soybean, has been found to exert anti-proliferative activities in vitro in human breast cancer cells. The current study aimed to evaluate the efficacy of Soy B in suppressing CRC.

Methods and materials: The effect of Soy B on cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay. The effect of Soy B on cell proliferation was determined using colony formation assay. The percentage of apoptotic cells was determined by the TUNEL assay and flow cytometry following Annexin V-FITC/Propidium Iodide (PI) double staining. JC-1 staining was performed to examine the change in mitochondrial membrane potential. Autophagy was examined by acridine orange staining and mRFP-GFP-LC3 adenovirus transfection. Caspase-12 activities were determined by ELISA kit. Western blotting was used to determine the expression of relevant proteins. To investigate the role of autophagy in the pro-death and pro-apoptotic activities of Soy B, autophagy inhibitors Bafilomycin A1 (Baf-A1) and Atg5 siRNA were utilized. TUDCA and CHOP shRNA were utilized to block ER stress. Moreover, a CRC xenograft murine model was used to analyze the therapeutic efficacy of Soy B in vivo.

Key findings: Soy B treatment decreased the number of viable cells and colonies formed in CRC cell lines. Moreover, Soy B treatment promoted the apoptotic cell death via the intrinsic pathway and autophagy which positively contributed to cell death and apoptosis. In addition, our results showed that ER stress, triggered by Soy B, mediated apoptosis and autophagy. In vivo results revealed that Soy B could suppress tumor growth, which was associated with increased ER stress, accompanied with apoptosis and autophagy induction.

Significance: Soy B was able to promote cell death in vitro and in vivo. Our findings highlight the possibility of utilizing Soy B as a chemotherapeutic agent to prevent and treat CRC.

1. Introduction

Colorectal cancer (CRC) is a common human malignancy which accounts for 600,000 deaths annually worldwide [1]. Recent statistics indicate that the incidence rate of CRC has been steadily increasing in China, ranking behind lung cancer and gastric cancer [2]. Currently, surgical resection remains the mainstream therapeutic approach for CRC therapy [3]. Despite the introduction of novel therapeutic regimens including systemic chemotherapy, radiotherapy, immunotherapy and targeted therapy, the clinical outcomes for patients undergoing surgery are far from being satisfactory [4]. Therefore, the discovery of novel chemotherapeutic agents is highly desired.

Upon external or internal stimuli such as calcium homeostasis, disruption or accumulation of unfolded or misfolded proteins, cells adopt a self-defense response known as endoplasmic reticulum (ER) stress to handle the change in the surrounding environment and restore homeostasis. This cell equilibrium of proteins is achieved by activating the unfolded protein response (UPR). UPR can be triggered by three distinct signaling, which involves IRE1/XBP1, PERK-eIF2α-ATF4, and ATF6, respectively [5]. The involvement of ER stress in a variety of human disorders has been well documented. With respect to human malignancies, it is proposed that ER stress plays a crucial role in the development and progression of cancer under stressful growth conditions such as hypoxia [6]. Thus, targeting of ER stress should be an effective strategy in cancer prevention and treatment [7].

Soybeans have been reported to promote various health functions [8]. They include many functional food components, such as soy protein, β-conglycinin, isoflavones, soy peptides, lectin, trypsin inhibitor,

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https://doi.org/10.1016/j.lfs.2018.12.023
Received 8 November 2018; Received in revised form 6 December 2018; Accepted 13 December 2018
Available online 14 December 2018
0024-3205/

lecithin, tocopherol, and saponins [8]. In particular, soyasaponins are triterpene glycosides that possess an oleanane-type aglycone with one or two polysaccharide chains [9]. Several studies have established that soyasaponins can exert several functions, such as antioxidative [10], cholesterol-lowing [11], anti-kidney disease progression [12], anti-inflammatory [13], renin-inhibiting [14], hepatoprotective [15], and anti-obesity [11]. Interestingly, soyasaponins have also been found to exhibit antineoplastic activities [16]. Particularly, Soyasapogenol B (Soy B) was found to exert anti-proliferative activities in human breast cancer cells in vitro [17]. The pro-apoptotic activities of Soy B have also been evidenced in hepatocellular carcinoma cell lines [18]. However, whether Soy B could exhibit anti-cancer activities in CRC still remains unclear. In this study, our results revealed the Soy B could promote apoptosis and autophagy in CRC cells via the intrinsic pathway. Our findings also showed that ER stress induced by Soy B mediated its effect on apoptosis and autophagy. Furthermore, our results showed that Soy B can suppress tumor growth in xenograft murine models.

2. Materials and methods

2.1. Cell culture

HCT116 and SW480 cells (Human CRC cell lines) obtained from ATCC (Shanghai, China) were cultured in DMEM medium (Hyclone, Logan, UT) containing 10% FBS (Hyclone, Logan, UT) in a CO_2 humidified incubator at 37 °C.

2.2. Cell proliferation assay

A CCK-8 kit obtained from Beyotime (Shanghai, China) was applied to determine the cell viability in accordance with the manufacturer's protocols. Then the absorbance of the viable cells was measured by the spectrophotometer (Tecan Group Ltd., Männedorf, Switzerland).

2.3. Colony formation assay

CRC cells (3×10^4 cells) were suspended in 1.6 mL RPMI agarose medium (10% FBS and 0.33% agarose) supplemented with DMSO or Soy B. The cells were then plated in 6-well plates covered with a layer of solidified DMEM agarose medium. The cells were maintained for two weeks in a humidified atmosphere of 5% CO2 at 37 °C. Colonies with > 0.1 mm diameter were counted and photographed by Nikon Eclipse TE2000-U microscope.

2.4. TUNEL assay

After 48 h incubation with Soy B, CRC cells were stained with the TUNEL dye (Beyotime, Wuxi, China). The ratio of TUNEL positive cells to the total number of cells was calculated after counting at least 300 cells per field in five random fields under 100× magnification.

2.5. Apoptosis assay

Apoptosis kit (BD Pharmingen, Franklin Lakes, NJ) was applied to assess cell apoptosis according to the manufacture's protocols and using flow cytometer (Beckman Coulter Inc., Miami, FL) measured the apoptotic population of cells. Cells that presented Annexin V-FITC positive/PI negative were considered as early apoptotic cells. Cells that presented Annexin V-FITC positive/PI positive were considered as late apoptotic cells. The apoptosis was calculated as the sum of early and late apoptosis.

2.6. Mitochondrial depolarization assay

Following incubation with Soy B for 48 h, the change in MMP was evaluated by JC-1 staining as previously described [19]. Following treatment, cells were stained with JC-1 for 20 min. Cells were then harvested and rinsed with JC-1 staining Buffer (1×) twice. The stained cells were imaged immediately at 200× using a fluorescence microscope (OLYMPUS, Japan).

2.7. Western blot

Western blots were conducted in accordance with standard protocols. A total of 40 μg protein samples were electrophoresed on SDS-PAGE before transferring to a PVDF membrane. The membranes were then blocked with 5% (w/v) non-fat milk and washed with Tris-buffered saline-Tween solution (TBST) and the membranes were incubated overnight at 4 °C with primary antibodies according to manufacturer's instructions. The primary antibodies against LC3B, p62, CHOP and GRP78 was purchased from Santa Cruz (Santa Cruz, CA). The primary antibodies against Beclin 1, Atg5, cleaved caspase-3, cleaved PARP, cleaved caspase-9, cleaved caspase-12, cyto C, *p*-PERK, ATF4, *p*-eIF2a, Bcl-2, Bax were purchased from Abcam (Shanghai, China), GAPDH and COX-IV were used as internal control and purchased from Beyotime (Shanghai, China). Goat anti-rabbit IgG-HRP (Beyotime, Shanghai, China) served as the second antibodies and β-actin was applied as internal control. Protein levels were measured using chemiluminescent substrate (KPL, Guildford, UK) and visualized using BandScan software (Glyko, Novato, CA).

2.8. Acridine orange staining

CRC cells were treated with different concentrations of Soy B for 48 h and washed with 1 × PBS for three times. Then, cells were stained with 0.01% acridine orange (Solarbio, China) for 5 min and observed under a red filter fluorescence microscope (BX53, OLYMPUS, Tokyo, Japan).

2.9. mRFP-GFP-LC3 adenovirus transfection

CRC cells were transfected with mRFP-GFP-LC3 adenoviruses (Hanbio, China) for 48 h, and then treated in the presence and absence of indicated concentration of Soy B for 48 h. The formation of autolysosome was detected and analyzed using a confocal microscope, and photographed cells under 400× magnification. Yellow puncta and red puncta represented autophagosome and autolysosome, respectively.

2.10. Caspase-12 activity assay

The activity of caspase-12 was measured using an ELISA kit following the manufacturer's instructions (Abcam, Shanghai, China). The activity of caspase-12 was assessed by measuring the fluorescence intensity.

2.11. Transfection with Atg5 siRNA or CHOP shRNA

The shRNA oligos targeting CHOP was synthesized according to previously published sequences [20] and the Atg5 siRNA was purchased from Cell Signaling Technology (Boston, MA), with the nonsense sequence as the control. Cells were transfected with 30 nM shRNA using Lipofectamine 3000. Forty-eight hours after transfection, cells were harvested for further analysis.

2.12. In vivo CRC tumor model

All animal experiments were approved by the Institutional Animal Care and Use Committee at Qingdao University. Eight-week-old male athymic BALB/c nu/nu mice were injected with 10^7 HCT116 cells in their left flanks. Twenty-one days after implantation, the mice were randomized into 3 groups (6 mice/group) to receive a daily i.p. injection with the following: (1) vehicle (0.9% sodium chloride plus 1%

DMSO), (2) 25 mg/kg Soy B dissolved in vehicle, and (3) 50 mg/kg Soy B dissolved in vehicle. The tumor volume and body weight of mice were measured twice every week until the 30th day, and tumor tissue samples were collected for histopathological evaluation. The tumor tissue sections were stained with H&E and IHC according to standard protocols. Briefly, 4-μm consecutive sections were deparaffinized in xylene, rehydrated in a graded ethanol series and submerged in EDTA antigen retrieval buffer for 15 min in a microwave oven. Endogenous peroxidase activity was blocked with a 20 min incubation with 3% hydrogen peroxide in absolute methanol, followed by 5% BSA for 15 min to block non-specific binding. The sections were incubated with specific primary antibodies and the secondary antibody, and stained with the 3,30-diaminobenzidine tetrachloride (DAB) chromogen to visualize immunoreactive cells.

2.13. Statistical analysis

The data are presented as mean ± SD (Standard Deviation) and represent the results of three separate experiments unless otherwise stated. All statistical analyses were conducted using SPSS software 16.0 (SPSS Inc., Chicago, IL). Groups were compared by One-way ANOVA followed by Dunnett's *t*-test, and $P < 0.01$ was considered statistically significant.

3. Results

3.1. Soy B decreases the viability of CRC cells

The chemical structure of Soy B was presented in Fig. 1A. Based on our results from preliminary experiments, cell viability was tested following incubation with Soy B ranging from 5, 10 and 20 μM for 24 or 48 h. When HCT116 cells were incubated with Soy B for 24 h, only a dosage of 20 μM produced a considerable loss in cell viability (Fig. 1B). When the incubation lasted for 48 h, Soy B at 5, 10 and 20 μM significantly decreased the cell viability. As for CRC cell line SW480, Soy B at 10 and 20 μM for 24 h was able to greatly reduce the cell viability (Fig. 1B). When the treatment duration was increased to 48 h, more profound decrease in the number of viable cells was observed (Fig. 1B). Soy B treatment for 48 h obviously affected the shape and reduced adhesive force of both HCT116 and SW480 cells, in comparison to the control group (Fig. 1C). The effect of Soy B on cell proliferation was assessed by colony formation assay. In the present study here, Soy B was able to impressively promote cell death (Fig. 1D). In contrast, Soy B did not present any cytotoxic activities on normal human colon mucosal epithelial cell line NCM460 (Supplementary Fig. 1). Present findings here evidenced that Soy B can selectively promote cell death in tumor cells.

3.2. Soy B promotes intrinsic apoptosis in CRC cells

Given the crucial role of apoptosis induction in eradicating tumor cells, apoptotic cell death was detected by TUNEL assay and flow cytometry following treatment with Soy B for 48 h. The number of TUNEL positive cells increased after Soy B treatment (Fig. 2A). Meanwhile, flow cytometry assay also showed Soy B at 10 and 20 μM was able to increase the apoptotic percentage in HCT by > 4 folds and 9 folds respectively compared to control cells (Fig. 2B). In SW480 cells, the apoptotic percentage was elevated to over 10% and 20% respectively by Soy B at 10 and 20 μM (Fig. 2B). The cleavage of caspase-3 and PARP were important hallmarks for cell apoptosis. Our results exhibited that Soy B treatment for 48 h produced a dose-dependent increase in the expression level of cleavage of caspase-3 and PARP (Fig. 2C). Cleavage of caspase-8 and caspase-9 indicated the activation of extrinsic and intrinsic apoptotic signaling pathway, respectively. Moreover, we found that Soy B activated capase-9 in a dose-dependent manner while it did not alter the intracellular level of cleaved caspase-8 (Fig. 2C), suggesting that Soy B mainly triggered apoptotic cell death via intrinsic pathway. Soy B treatment produced loss of mitochondrial membrane potential as detected by JC-1 staining (Fig. 2D). At the same time, Soy B promoted the release of cytochrome C from mitochondria to cytosol, decrease in Bcl-2 expression, and an increase in Bax expression (Fig. 2E). This evidence validated the involvement of intrinsic apoptotic pathway in the pro-apoptotic activities of Soy B.

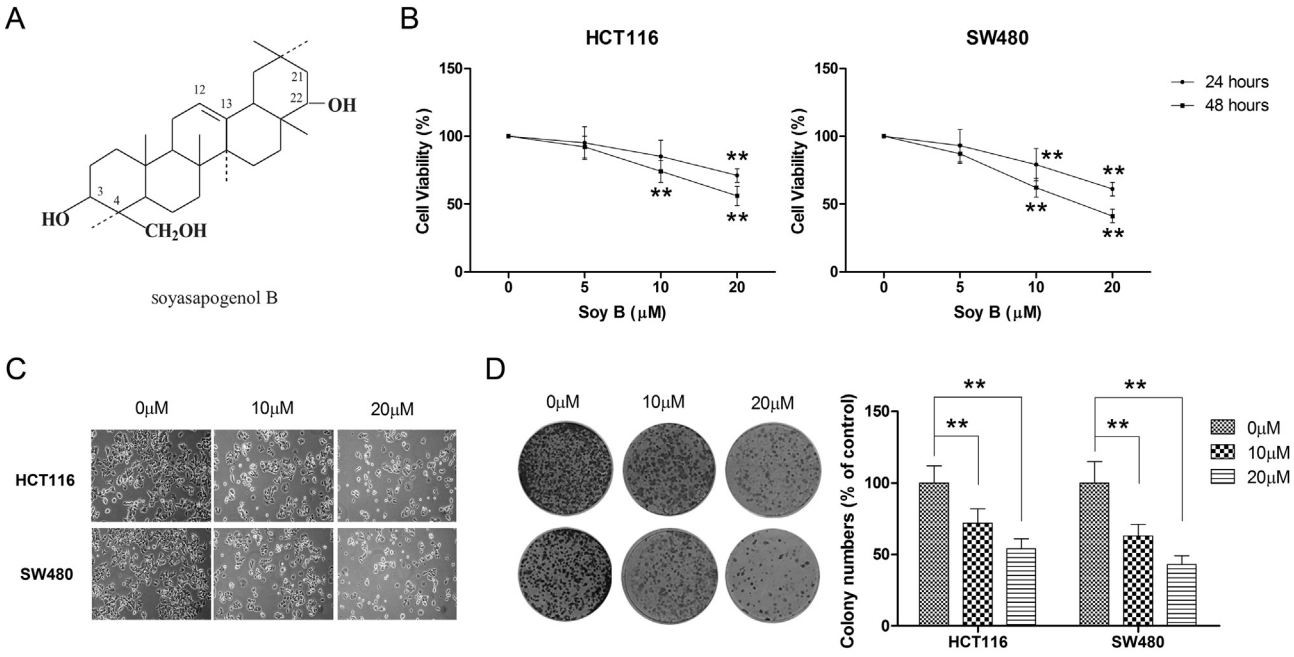

Fig. 1. Soy B suppresses cell proliferation in CRC cells. A. Chemical structure of Soy B. B. CRC cells were treated with Soy B at the indicated concentration for 24 or 48 h. Soy B dose-dependently reduced cell viability of HCT116 and SW480 cells. C. CRC cells were treated with Soy B at the indicated concentration for 48 h before the morphological change was examined by microscopy. D. Soy B suppressed the colony-formation of CRC cells. $^{**}P < 0.01$.

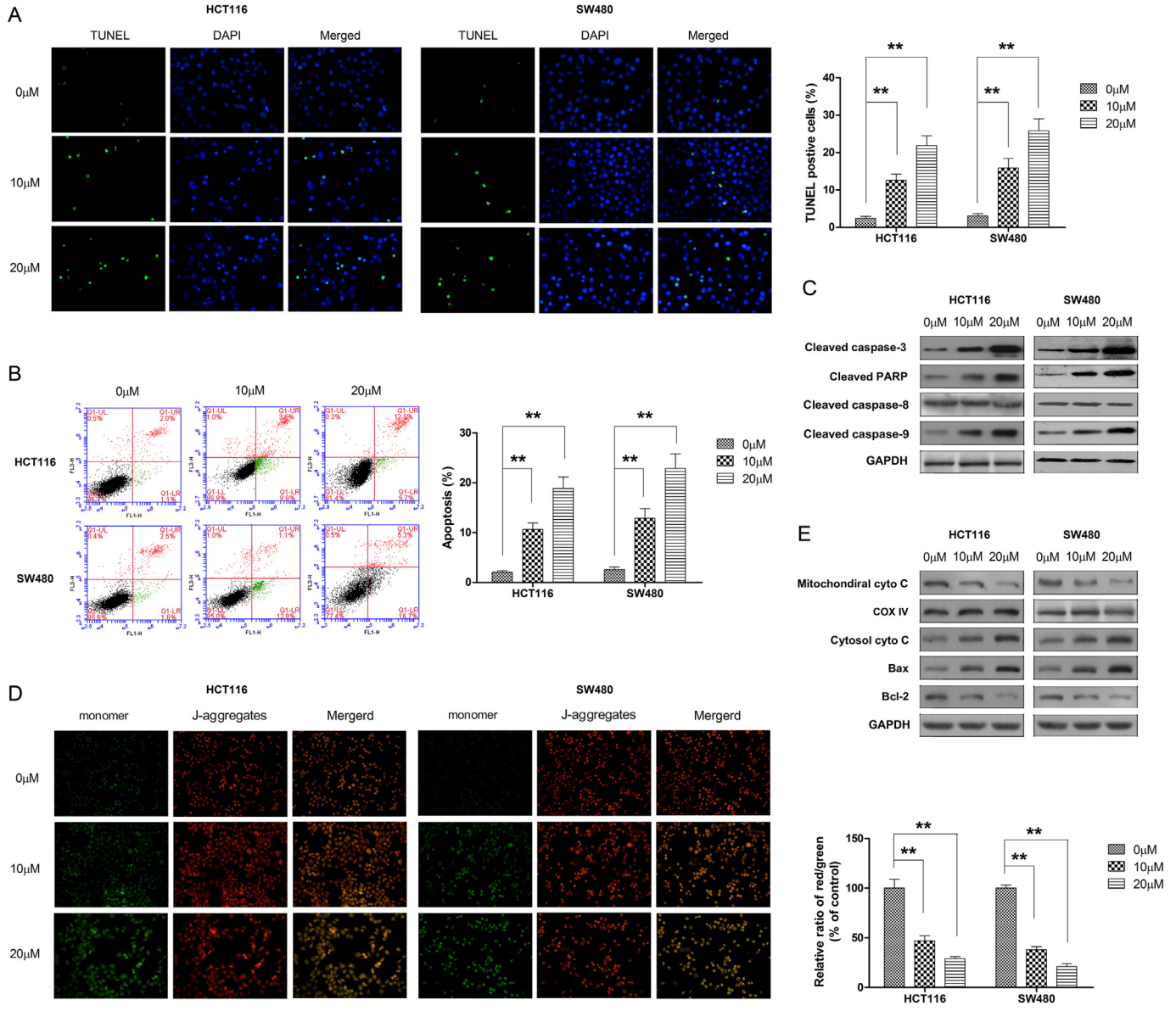

Fig. 2. Soy B promotes apoptotic cell death in CRC cells. CRC cells were treated with Soy B at the indicated concentration for 48 h before the assay was performed. A. Apoptotic cell death was determined by TUNEL assay. B. Cell apoptosis was determined by flow cytometry. C. Cleavage of caspase-3, caspase-8, caspase-9 and PARP was determined by Western blot. D. Change of mitochondrial membrane potential was examined by JC-1 staining. E. The release of cytochrome C from mitochondria to cytosol, and expression of Bcl-2 and Bax were determined by Western blot. $^{**}P < 0.01$.

3.3. Autophagy induction by Soy B positively contributes to its effect on cell death and apoptosis in CRC cells

Upon exposure to 10 and 20 μM Soy B for 48 h, Soy B effectively increased the development of AVO in a dose-dependent manner compared to the control group as detected by AO staining (Fig. 3A). We also confirmed the autophagy induction effect of Soy B by detecting the development of autolysosome and the expression of autophagy-associated markers. Western blotting results also supported the above conclusion, as Soy B treatment significantly increased the expression of LC3-II/LC3-I, Beclin 1 and Atg5, while decreased the expression of p62 (Fig. 3B). Further, a mRFP-GFP-LC3 adenovirus was used to monitor the autophagy flux in cells, and the decrease of GFP implies the fusion of autophagosome with lysosome. Thus, after merging, yellow puncta and red puncta refer to autophagosome and autolysosome, respectively. Soy B notably increased the number of red puncta, and decreased the number of yellow puncta, indicating that Soy B effectively enhanced autophagy flux (Fig. 3C). These results indicate that Soy B induces autophagy in CRC cells.

Next, experiments were conducted to examine the contribution of autophagy in Soy B-induced cell death and apoptosis. Bafilomycin A1 (Baf A1), which is an autophagy inhibitor that inhibits lysosomal degradation of autophagosomes, was used to block the autophagic flux. A pretreatment with Baf A1 for 4 h before incubation with Soy B markedly rescued HCT116 and SW480 cells from Soy B-induced cell death (Fig. 4A). Besides, the pro-apoptotic effects of Soy B were significantly compromised when autophagy was inhibited (Fig. 4B). Correspondingly, Baf A1 also attenuated Soy B-induced elevation in the expression level of cleavage caspase-3, PARP and Bax, as well as decrease in the expression level of Bcl-2 (Fig. 4C). To further investigate the role of autophagy in Soy B-induced cell death and apoptosis, CRC cells were transfected with siRNA targeting Atg5 to block autophagy. Although Atg5 knockdown did not produce any apparent change in cell viability, Atg5 knockdown partly dampened Soy B-induced cell death in both CRC cell lines (Fig. 4D). Results from flow cytometry and Western blot assay also showed that Atg5 knockdown reduced Soy B-induced

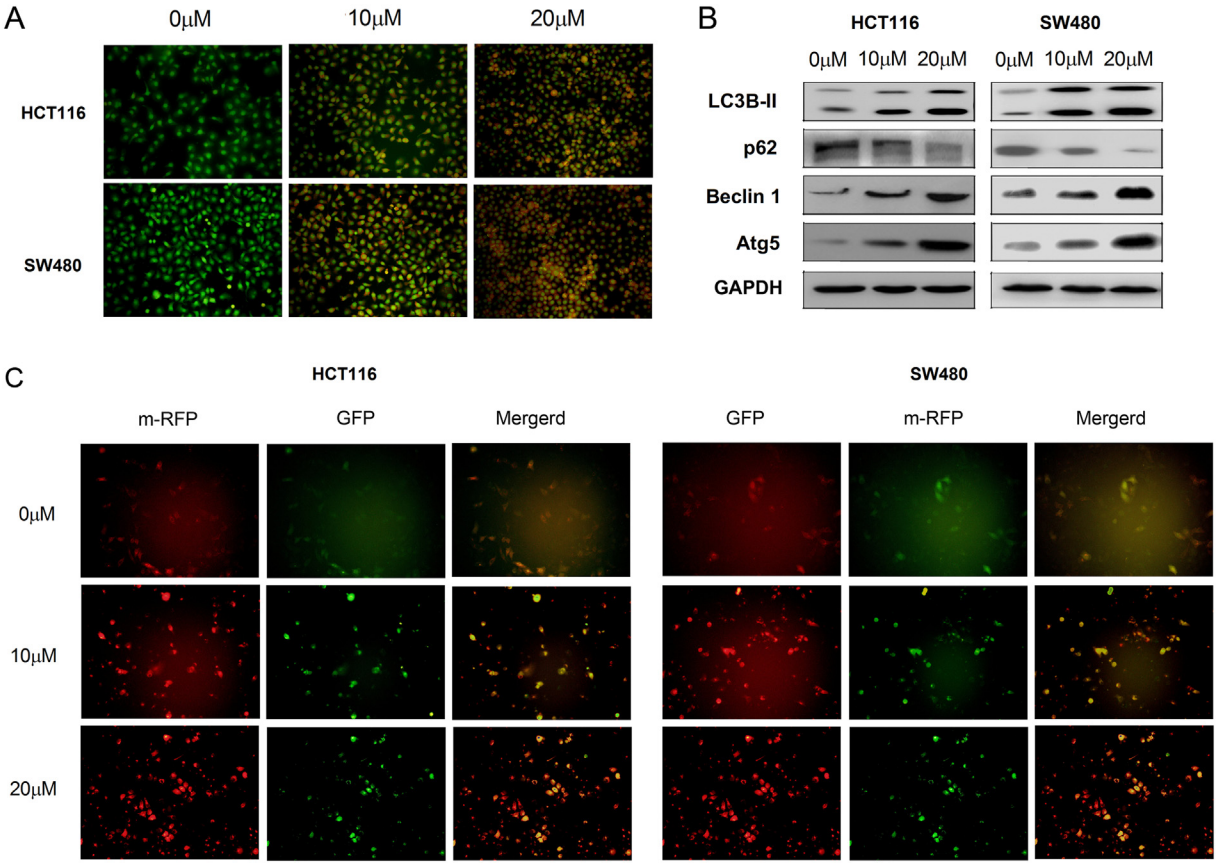

Fig. 3. Soy B promotes autophagy in CRC cells. CRC cells were treated with Soy B at indicated concentration for 48 h before assay was performed. A. Formation of AVO was visualized by AO staining. B. Protein levels of autophagic markers LC3BII, p62, Beclin1 and Atg5 were determined by Western blot. C. Autophagic flux was visualized by transfection with mRFP-GFP-LC3 adenovirus. $^{**}P < 0.01$.

apoptotic cell death (Fig. 4E and F). Thus, this evidence suggests that autophagy induced by Soy B positively contributes to its effect on cell death and apoptosis.

3.4. Soy B triggers ER stress to promote apoptosis and autophagy

ER stress has been found to play a role in the interplay of apoptosis and autophagy in cancerous cells [21,22]. Thus, we examined whether ERS was involved in the Soy B-induced apoptosis and autophagy. Since caspase-12 activation is an important indicator of ERS, the change in caspase-12 activities was examined by ELISA. Soy B treatment notably increased the activities of caspase-12 in both HCT116 and SW480 cells (Fig. 5A). Afterwards, the protein markers for ERS were examined. Interestingly, Soy B treatment also up-regulated ERS-associated proteins in a dose-dependent manner, suggesting that Soy B could trigger ER stress in both HCT116 and SW480 cells (Fig. 5B). Then the role of ER stress in autophagy and apoptosis induction by Soy B was first examined by utilizing ER stress inhibitor, TUDCA. The pretreatment with TUDCA for 4 h before incubation with Soy B remarkably rescued HCT116 and SW480 cells (Fig. 5C). In addition, apoptotic cell death produced by Soy B was also significantly reduced by pretreatment with TUDCA (Fig. 5D). Western blot assay also showed that TUDCA could attenuate Soy B-induced caspase-3 activation, Bcl-2 suppression and Bax elevation (Fig. 5E). In addition, our results showed that ER stress inhibition also compromised the capability of Soy B to promote autophagy, as demonstrated by the decrease in the expression of LC3BII, Beclin 1 and Atg5 by TUDCA plus Soy B compared with Soy B only (Fig. 5E). To confirm the role of ER stress in the occurrence of apoptosis and autophagy following Soy B treatment, we showed that silencing CHOP blocked ER stress. Similar to the results obtained with TUDCA pretreatment, CHOP knockdown reduced Soy B-induced cell death and apoptosis (Fig. 5F, G and H). Altogether, these results showed that ER stress mediated the effects of Soy B on apoptosis and autophagy.

3.5. Soy suppresses tumor growth and promotes apoptosis and autophagy in vivo

Based on the aforementioned results from in vitro experiments, xenograft murine model was developed to evaluate the efficacy of Soy B in vivo. Soy B at both dosages (25 mg/kg/day and 50 mg/kg/day) was able to suppress the tumor growth (Fig. 6A). Subsequently, the apoptosis of cell in tumor tissue was determined by TUNEL assay, which showed that Soy B dose-dependently increased the apoptotic cell death in vivo (Fig. 6B). IHC and Western blot assay also showed that Soy B treatment correlated with increased the expression of cleaved caspase-3, Bax, CHOP, GRP78 and LC3BII while it decreased the expression of Bcl-2 and Ki-67 in tumor tissue (Fig. 6C and D), which implied that Soy B triggered ER stress and promoted apoptosis and autophagy in vivo. In addition, these results demonstrated that Soy B treatment at 20 mg/kg/day and 40 mg/kg/day did not cause any huge change in the body weight of model animals. Further, histological examination showed that Soy B did not cause any apparent change in the histology in heart, liver, spleen, lung and kidney (Fig. 6F). From these results, we can ascertain that Soy B had no adverse effects in vivo.

4. Discussion

Owing to their good safety profile, natural compounds have been applied as novel anti-cancer chemotherapeutic agents. Soy B is a soyasaponin compound found in soybean. During the past two decades,

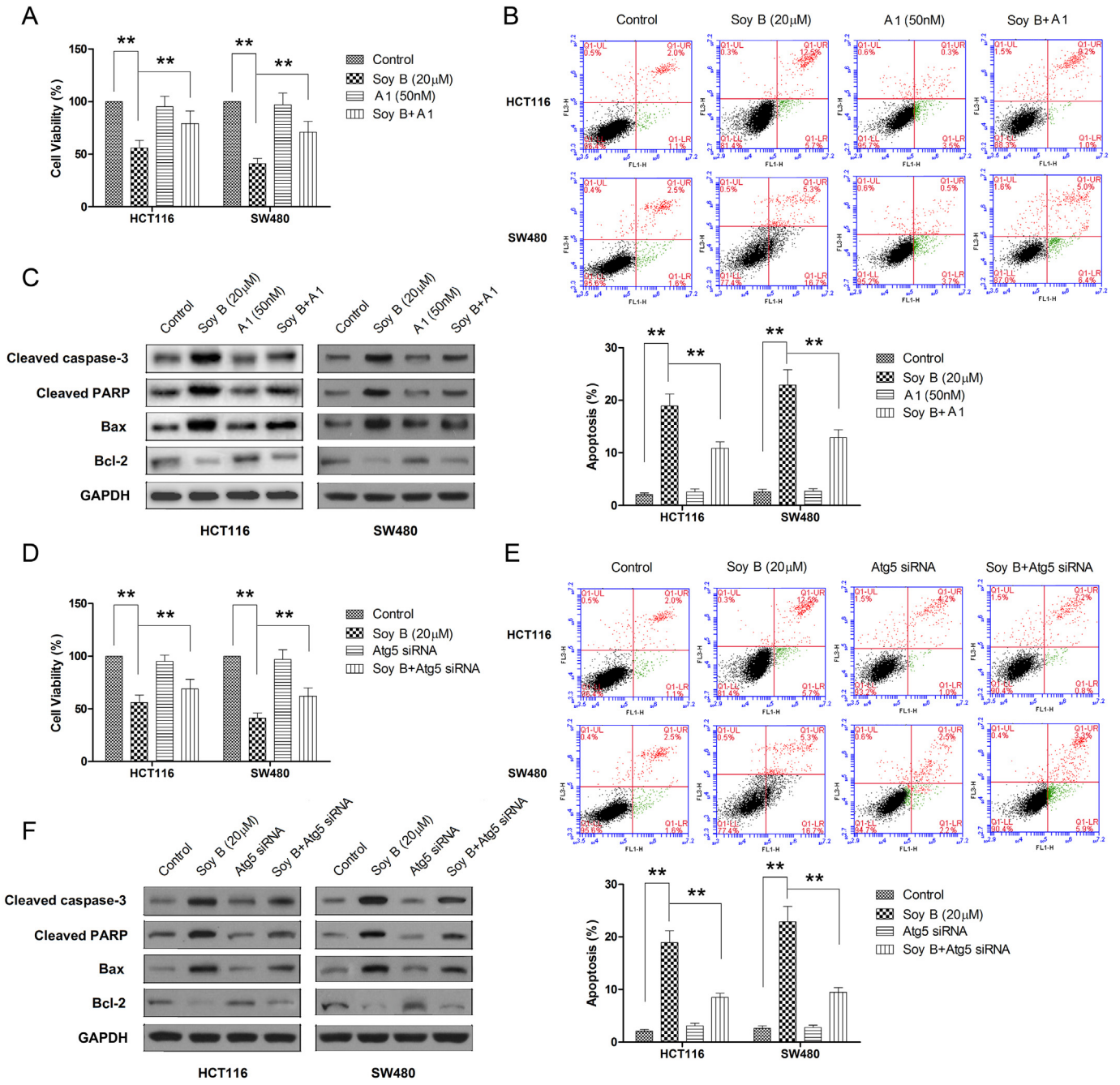

Fig. 4. Inhibition of autophagy compromises Soy-B induced cell death and apoptosis. A. Baf A1 pretreatment reduced Soy B-induced loss of cell viability. B. Baf A1 pretreatment reduced Soy B-induced cell apoptosis. C. Baf A1 decreased Soy B-induced cleavage of caspase-3 and PARP, increase in Bax expression, and decrease in Bcl-2 expression. D. Atg5 knockdown reduced Soy B-induced loss of cell viability. E. Atg5 knockdown reduced Soy B-induced cell apoptosis. F. Atg5 knockdown decreased Soy B-induced cleavage of caspase-3 and PARP, increase in Bax expression, and decrease in Bcl-2 expression. $^{**}P < 0.01$.

Soy B has been reported to possess various therapeutic activities against many disorders including platelet aggregation, dyslipidemia immune diseases, and inflammatory conditions [23–25]. The anti-proliferative activities of Soy B on breast cancer cells were firstly documented in 2002 [17]. Since then, the anti-cancer activities of Soy B have been evidenced in hepatocellular carcinoma cells and glioma cells [18,26]. However, no further study has been conducted to examine its role in CRC and the associated molecular mechanisms. In our case, we found that Soy B promoted apoptosis and autophagy in CRC cells. Additionally, our findings indicate that ER stress triggered by Soy B positively contributing to its inducing effect on apoptosis and autophagy. In vivo studies also showed that Soy B suppressed tumor growth, which was associated with apoptosis and autophagy induction.

Apoptosis induction is a main strategy in chemotherapy when eradicating cancerous cell [27]. In this work, our findings revealed that Soy B was able to promote apoptosis in CRC cells. Triggering of caspase cascade has a major function in apoptosis. The effect of Soy B on activation of caspase-3, caspase-8, caspase-9 and PARP was examined in the present study. Our findings demonstrated that casaspe-9 and caspase-3 activation mediated the pro-apoptotic effect of Soy B in CRC cells. However, no activation of caspase-8 was observed at the tested dosage. Since cleavage of caspase-8 is the hallmark for activation of death receptor apoptotic pathway, our results suggested the pro-apoptotic activities of Soy B in CRC cells were not involved in the extrinsic

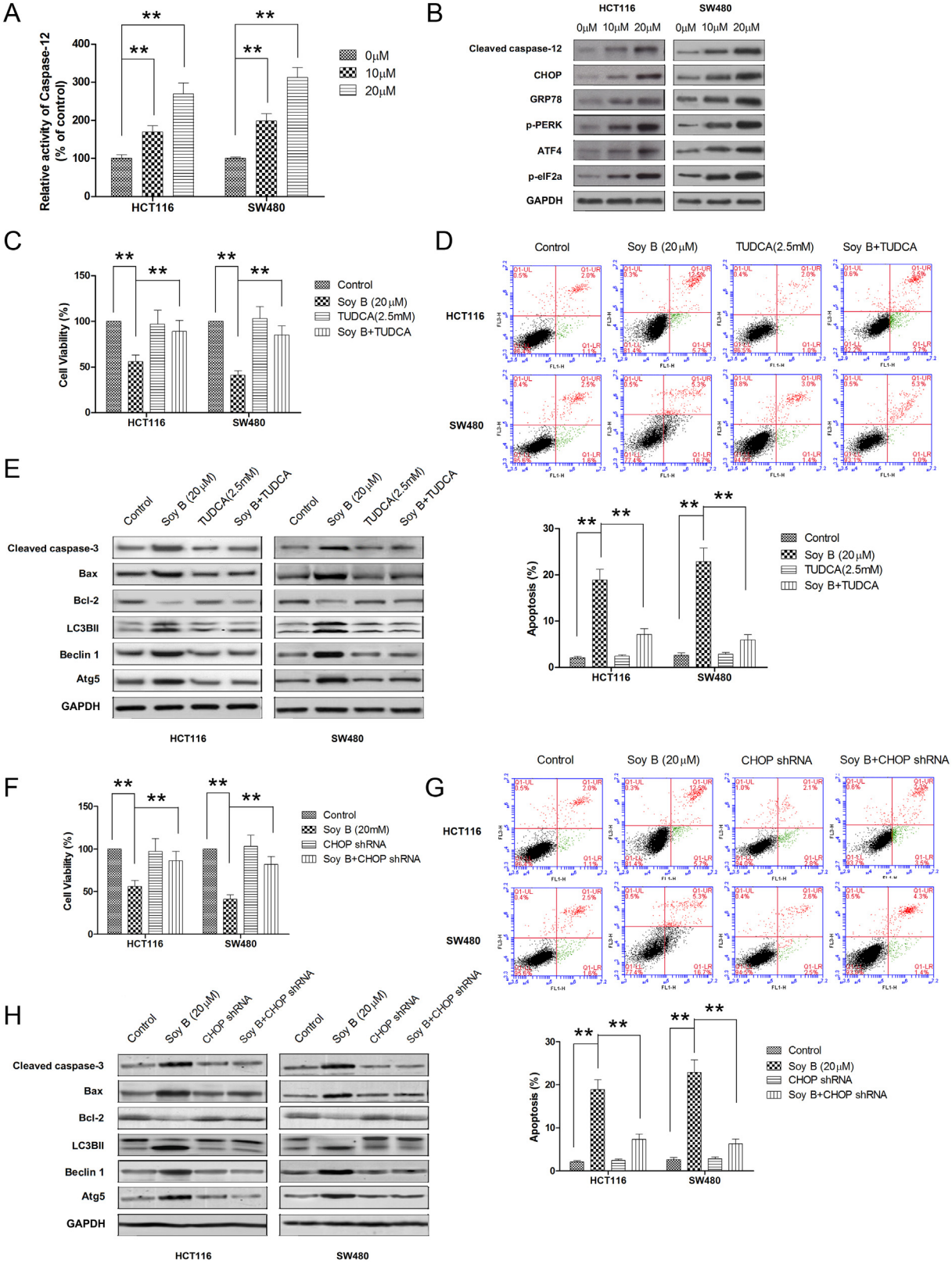

Fig. 5. ER stress mediated Soy B-induced autophagy and apoptosis. A. Soy B increased the activities of caspase-12. B. Soy B dose-dependently elevated the expression of marker proteins for ER stress. C. TUDCA pretreatment reduced Soy B-induced loss of cell viability. D. TUDCA pretreatment reduced Soy B-induced cell apoptosis. E. TUDCA decreased Soy B-induced cleavage of caspase-3 and PARP, increase in Bax, LC3BII, Beclin1 and Atg5 expression, and decrease in Bcl-2 expression. F. CHOP knockdown reduced Soy B-induced loss of cell viability. G. CHOP knockdown reduced Soy B-induced cell apoptosis. H. CHOP knockdown decreased Soy B-induced cleavage of caspase-3 and PARP, increase in Bax, LC3BII, Beclin1 and Atg5 expression, and decrease in Bcl-2 expression. $^{**}P < 0.01$.

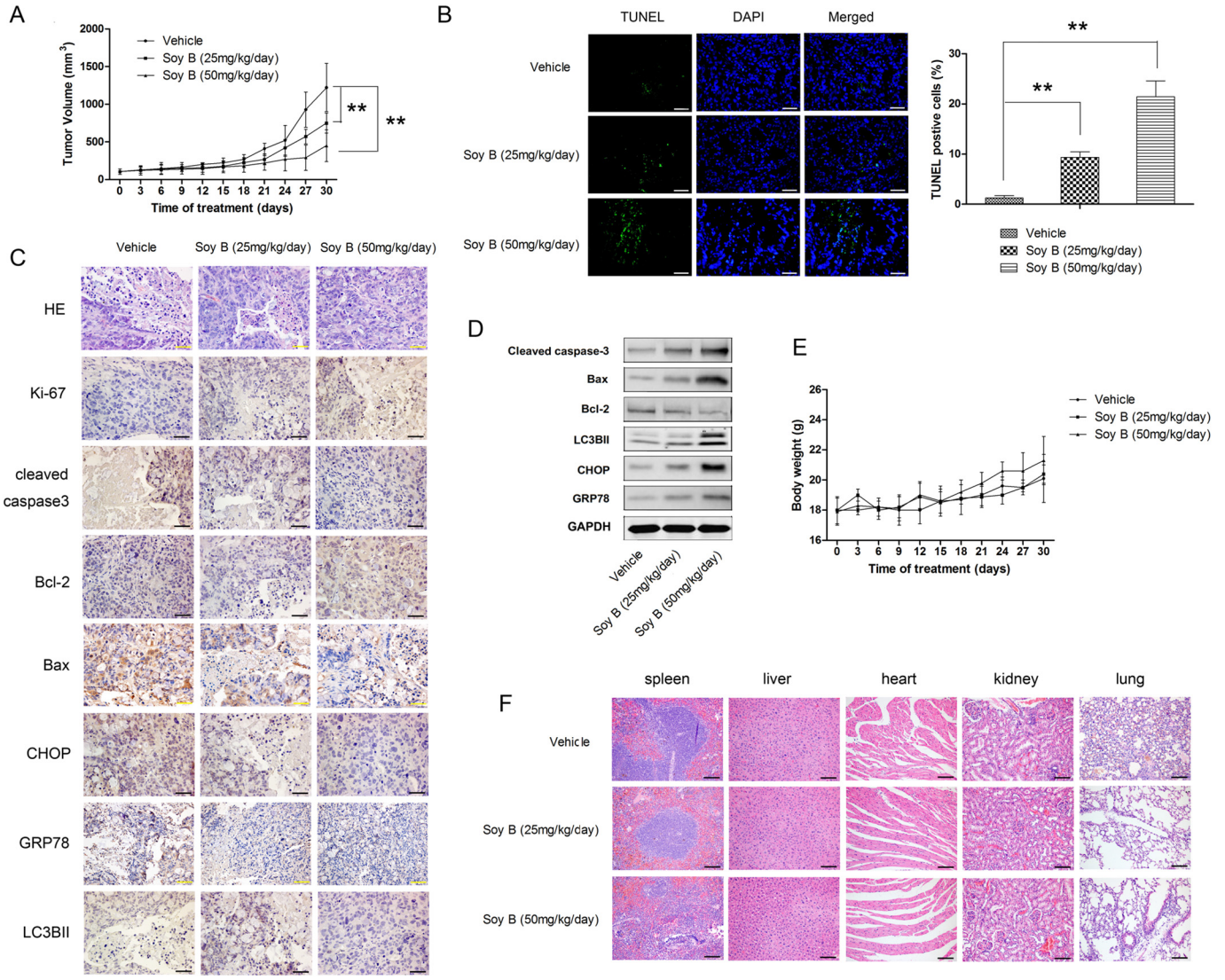

Fig. 6. Soy B suppresses tumor growth in xenograft model ($n = 6$). A. Soy B treatment inhibited tumor growth in a dose-dependent manner. B. Soy B treatment enhanced cell apoptosis in tumor tissue. C. IHC results for Ki-67, cleaved caspase-3, Bcl-2, Bax, CHOP, GRP78 and LC3BII in tumor tissue. D. Western blot results for cleaved caspase-3, Bcl-2, Bax, CHOP, GRP78 and LC3BII in tumor tissue. E. Soy B did not markedly affect the body weight of animal model. F. Soy B did not cause marked histological damage in spleen, liver, heart, kidney and lung tissue. $^{**}P < 0.01$.

apoptosis. We further examined the role of mitochondria in the pro-apoptotic activities of Soy B. We found that Soy B treatment led to loss of mitochondrial membrane potential, release of cytochrome C from mitochondria to cytosol, upregulation of anti-apoptotic molecule Bax and downregulation of pro-apoptotic molecule Bcl-2, supporting the contribution of mitochondrial pathway in Soy B-induced apoptosis. Our results were consistent with Yanamandra's findings in glioma cells, which showed that Soy B promoted cell apoptosis in caspase-dependent way and correlated with release of cytochrome C and activation of capasse-9 and caspase-3 [26].

Autophagy, also termed as second cell programmed death, has been a focus of cancer research in the past decade [28]. The contradictory role of autophagy has been well established in studies. Unlike apoptosis, autophagy has been found to promote or suppress cancer progression depending on the microenvironment or the agents that trigger autophagy [28]. In this study, Soy B promoted autophagy in HCT116 and SW480 cells, as evidenced by an increase of the expression levels of LC3-I to LC3-II, Atg5, and Beclin-1, in addition to the decrease of the expression level of p62. XAG treatment also dramatically increased the number of AVO and autolysosome. Further, the inhibition of autophagy by a specific inhibitor (Baf A) or Atg5 siRNA dampened the anti-growth effect of Soy B on CRC cells, suggesting that autophagy induced by Soy B suppressed cell growth. Mounting evidence has shown that apoptosis and autophagy are correlated [29]. Autophagy could enhance or abrogate apoptotic effect induced by anti-cancer drugs in cancer cells, which has been widely reported. For instance, Sheng et al. have showed that isovitexin induced cytotoxic autophagy in liver cancer cells, and blocking autophagy abrogated the pro-apoptotic effect of isovitexin [30]. Similarly, autophagy induced by Quercetin and Prodigiosin enhanced apoptotic cell death in ovarian cancer and glioblastoma cells, respectively [21,22]. Contrary to previous studies, our findings also showed that blocking autophagy could dramatically comprise the pro-apoptotic activities of Soy B in CRC cells. In fact, the protective role of autophagy against apoptosis has also been previously reported. Zhang et al. demonstrated that Bufalin conferred protection against pro-apoptotic activities of autophagy in human gastric cancer cells by showing that suppressing autophagy augmented the pro-apoptotic activities of Bufalin. [31]. These seemingly contradictory results imply that autophagy exerts a context-dependent role in the apoptosis of tumor cells.

ER stress is a response of cells when handling changes in the surrounding environment and restore homeostasis. Recently, the link

between ER stress and programmed cell death including autophagy and apoptosis has been established in a variety of cancerous cells, including melanoma cells [32], sarcoma cells [30], glioblastoma cells [21], gastric cancer cells [31], and hepatocellular carcinoma cells [33]. In the present study, we also found that the blocking of ER stress by a specific inhibitor or CHOP shRNA abrogated Soy B-induced cell death. Furthermore, Soy B promoted autophagy and apoptosis by triggering ER stress. Hence, our results support the idea that triggering ER stress can function as an anti-cancer mechanism.

In summary, our findings demonstrate that Soy B can suppress CRC growth in vitro and in vivo via apoptosis induction. What's more, our results show that ER stress can trigger apoptosis following Soy B treatment. Soy B has been reported to have a better bioavailability than that of corresponding soyasaponins [7]. Moreover, Soy B has a good safety profile in human. Therefore, our findings in present study provide the basis for further investigation on the use of Soy B in the prevention and treatment of CRC.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2018.12.023.

Declare of conflict

None.

Funding

None.

Author contribution

Hui Zhang designed the research; Luping Wang, Lu Yun, Xiaojun Wang and Liying Sha performed experiments; Luning Wang and Yingying Sui analyzed the data; Luping Wang wrote the paper.

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