Neoplasma 2021; 68(6): 1236–1244

doi:10.4149/neo_2021_210422N552

Inhibiting endoplasmic reticulum stress mediated-autophagy enhances the pro-apoptotic effects of resveratrol derivative in colon cancer cells

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Received April 22, 2021 / Accepted July 7, 2021

Previous studies have demonstrated that endoplasmic reticulum stress (ERS) might play a major role in inducing cellular autophagy and apoptosis in multiple types of cancer. Herein, we observed that trans-3,5,4'-trimethoxystilbene (TMS) exposure facilitated apoptotic cell death and ERS-mediated autophagy in colon cancer SW480 and HCT116 cells. Interestingly, our data demonstrated that ERS was not involved in TMS-induced apoptosis. However, ERS notably induced protective autophagy in SW480 and HCT116 cells. In addition, inhibiting cellular ERS significantly improved the pro-apoptotic effects of TMS. Thus, our results indicated that TMS-mediated autophagy was dependent on ERS, while apoptotic cell death might be induced in the ERS-independent pathway after TMS treatment. Generally, inhibiting ERS-mediated autophagy can enhance the pro-apoptotic effects of TMS. TMS might be a potential therapeutic agent for colon cancer treatment.

Key words: endoplasmic reticulum stress, trans-3,5,4'-trimethoxystilbene, apoptosis, autophagy, colon cancer

Colon cancer is a common human malignancy, which accounts for more than 650,000 deaths annually worldwide. Surgical resection and chemotherapy are normally offered to patients with colon cancer, however, both of them have limited therapeutic efficacy and significant adverse effects [1, 2]. Thus, the development of novel therapeutic agents to improve colon cancer patients' prognoses is urgently needed. Resveratrol, a natural polyphenolic compound that is abundant in blueberries, grapes, and peanuts, possesses numerous pharmacological activities. Previous researches have demonstrated that resveratrol can induce apoptosis in various human tumor cells via ROS-dependent endoplasmic reticulum stress (ERS) [3]. Although resveratrol has shown remarkable anti-cancer effects and protective effects of cardiovascular diseases in numerous pre-clinical studies, its poor pharmacokinetic parameters have restricted its clinical application [4]. Adding methoxy or hydroxyl groups to the stilbene backbone of resveratrol generates modified resveratrol derivatives, such as the trans-3,5,4'-trimethoxystilbene (TMS), which possess improved bioavailability and stability [5]. Unlike resveratrol, TMS can bind membrane proteins with high binding affinity. Furthermore, upon uptake, TMS can enter cells unaltered, which increases its stability within cells.

Recently, several studies have shown that TMS can exert anti-tumor effects in several human malignant tumors, including lung malignancies, cholangiocarcinoma, prostate adenocarcinoma, and osteosarcoma [6, 7]. Annick et al. have demonstrated that TMS exhibits stronger anti-cancer activity than that of resveratrol and could induce apoptosis in malignant MCF-7 cells [8]. In addition, 20 μM TMS increases intracellular ROS levels and induces apoptosis by phosphorylating JNK, p38, and MAPK in breast cancer cells [9]. Another study has shown that 10 μM TSM sensitizes osteosarcoma cells to apoptosis by activating Bax, p53, and caspase-3 [10]. Furthermore, TMS could modulate both autophagic and apoptotic cell death in human lung cancer cell line A549 via the p53 dependent signaling [11]. However, only a few studies have been conducted to confirm the potential inhibit effect of TMS on colon cancer. Therefore, herein we explored the potential pharmacological effects of TMS on colon cancer cell lines SW480 and HCT116 and discussed the underlying molecular mechanisms by which TMS modulates the progressing of colon cancer.

Autophagy is a conserved catabolic process in eukaryotic cells, which can catabolize cytoplasmic organelles and proteins. Recent studies have shown that autophagy might function as a "double edges sword" in tumorigenesis. The

roles of autophagy in tumor development are complicated, promotion or inhibition of autophagy-induced malignancy mainly depends on different cancer microenvironments [12]. Autophagy can inhibit tumorigenesis by decreasing the toxic accumulation of impaired organelles or proteins. Abnormal high expressions of p62/SQTSM1 in cancer cells may contribute to tumor progression by activating NF-κB signaling and inhibiting autophagy [13]. Previous studies have shown that the reduced p62/SQTSM1 expressions in cancer via inducing autophagy might inhibit cancer development [14]. In addition, inducing autophagy via upregulating Beclin-1 could significantly suppress breast cancer progression [15]. In contrast, other studies found that autophagy could also promote cancer progression under certain conditions. For example, autophagy could promote pancreatic cancer development both *in vivo* and *in vitro*, while inhibition of autophagy could significantly suppress tumor progression [12]. In summary, developing novel agents by targeting autophagy may provide potential benefits for a subset of cancer patients. Recently, some studies have confirmed the critical roles of endoplasmic reticulum stress (ERS) in apoptotic cell death and autophagy in cancers. For example, several natural plant chemicals can trigger autophagy and apoptotic cell death in human cancers via induction of ERS, which is mediated through the Bcl-2/p-STAT3 signaling activation [15]. Another study showed that stimulation of ERS in lung cancer cells with mutant p53 gene might induce apoptotic cell death and autophagy by inhibiting the mTOR/Akt/PI3K pathway [16]. Therefore, stimulation of ERS in cancer cells might be a promising antitumor strategy by inducing apoptosis and autophagy. Herein, we explored the potential effects of TMS on colon cancer cells and discovered its underlying molecular mechanisms for providing new insights into the biological activity of TMS.

Materials and methods

Cell lines and chemicals. The colon cell lines SW480 and HCT116 were purchased from the American Type Culture Collection (ATCC). All the cell lines were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. All the chemical agents were purchased from Sinopharm Chemical Reagent Company (Beijing, PRC) unless otherwise indicated. TMS (CAS Number 22255-22-7) was provided by Wako Pure Chemical Industries (Osaka, Japan) (purity >98%) and dissolved in 0.1% DMSO. For the control group, 0.1% DMSO was used as a vehicle. Tauroursodeoxycholic acid (TUDCA), Bafilomycin A1 (Baf A1), and 3-Methyladenine (3-MA) were proved by Sigma-Aldrich (MO, USA).

Cell proliferation assay. Colon cancer cells were seeded into 96-well plates at a density of 2.0×10^3/well for 24–48 h incubation. Then, the cells were treated with 15 μl Cell Counting Kit-8 (CCK-8, Sigma-Aldrich) and incubated for 3 h. Next, a microplate reader (Bio-Rad, USA) was used to detect the absorbance at optical density (OD) 450 nm to analyze the viability of colon cancer cells.

Apoptosis analysis. To evaluate cell apoptotic death, terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate-biotin nick end labeling (TUNEL) assay was applied using a commercial kit (Beijing Biotech, China) as previously described [16]. Each sample was tested three times by two independent researchers.

Mitochondrial membrane potential. Colon cancer cells were stained with JC-1 following the instructions of the manufacturer (Invitrogen, USA) as previously described [17]. Briefly, cells were incubated with JC-1 for 20 min. 2×10^4 JC-1 stained cells were quantified by FCM assay. The changes of mitochondrial membrane potential were analyzed according to the formation of JC-1 aggregates (red) and monomers (green), respectively.

Western blotting. Total proteins were extracted in RIPA cell lysis buffer containing protease and phosphatase inhibitors. For isolating mitochondrial proteins, cells were incubated with mitochondrial proteins isolation reagent (Biomed, PRC) following the instruction manual. The mitochondrial and cytosolic proteins were prepared as previously reported [18]. Cleaved caspase-8 (ab 20421), cleaved caspase-3 (ab13847) at dilutions 1 : 500; cleaved caspase-9 (ab2154) at dilutions 1 : 500; Bcl-2 (ab13167) at dilutions 1 : 1000; Bak (ab14148) at dilutions 1 : 200; Bax (ab 51212) at dilutions 1 : 200 and PARP-1 (ab12623) at dilutions 1 : 1000 were purchased from Abcam (Cambridge, USA). The primary antibodies used for Beclin-1 (CS-8760), p62/SQSTM1 (CS-3129), Atg5 (CS-8104), and LC3B-II (CS-3510) at dilutions 1 : 200 were purchased from Cell Signaling Technology (Trask Lane Danvers, USA). The concentration of proteins was detected by BCA protein assay kit (Life Technologies, USA) for preparing samples. Western blot analysis was performed using a method described previously [16]. Membranes were analyzed with ImageJ software.

Quantifying autophagy using acridine orange. Acidic vesicular organelle (AVO) staining was applied to quantify the volume of the acidic components in the cells. In acridine orange-stained cells, the nucleolus and cytoplasm fluoresce dim red and bright green, however acidic compartments fluoresce bright red. For AVO staining, cells were incubated at 37 °C for 25 min in culture media with 2 μg/ml acridine orange (Biotopped Life Sciences, USA). After trypsinization and washing with PBS, cells were suspended with PBS at 4 °C and kept in a dark place. The fluorescence signal was observed and assessed by fluorescence microscopy (Olympus DP80, Japan).

mRFP-GFP-LC3 puncta assay. Cells were plated in 12-well plates, transfected with mRFP-GFP-LC3 adenovirus (Takara, PRC) for 3.5 h. After incubation with TMS for 48 h, the samples were examined by a confocal microscope (Olympus DP80, Japan). After microscopic imaging, the yellow puncta indicated autophagosomes (RFP+GFP+), while the red puncta indicated autophagolysosomes (RFP+GFP−). The intensity of autophagy flux was analyzed according to the number of red and yellow puncta.

shRNA or siRNA transfection. Colon cancer cells were incubated in 12-well plates, and then the cells were transfected with Atg5 siRNA, scramble siRNA, or CHOP shRNA, using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, USA) for 72 h as previously described [16].

Statistical analysis. Statistical analyses were performed with SPSS 22.0 software (Chicago, USA). The data were expressed as the means ± standard deviation (SD). A two-tailed independent Student's t-test was applied for the comparison of two experimental groups. Statistical differences among more than two groups were assessed by one-way ANOVA with Tukey's correction for multiple comparisons. Statistical significance was defined as $p<0.01$.

Results

TMS suppresses cells viability and induces apoptosis in colon cancer cells. CCK-8 assay was applied for examining the effects of TMS on SW480 and HCT116 cells' viability. Our results showed that treatment with TMS (5, 10 μM) for 24 and 48 h remarkably changed the cell shape of SW480 and HCT116 cells (Figure 1A). In addition, the CCK-8 assay results indicated that TMS might dose- and time-dependently suppress colon cancer cells growth (Figure 1B). These results demonstrated that TMS could inhibit colon cancer cell viability *in vitro*. To determine whether the suppressive effects of TMS on colon cancer cells growth was induced

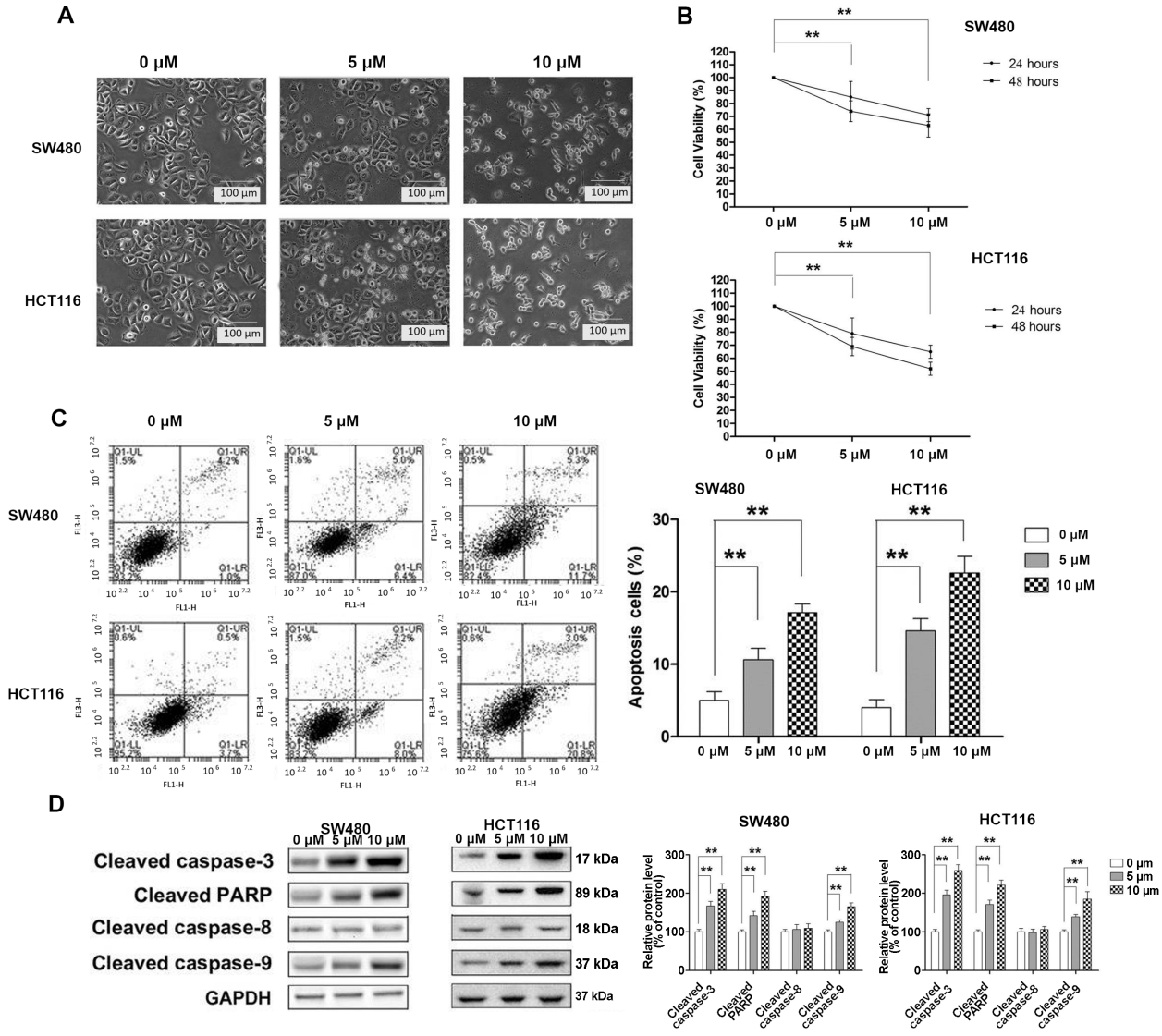

Figure 1. TMS suppresses colon cancer viability by inducing cell apoptosis. A) SW480 and HCT116 cells were pre-treated with 5 and 10 μM of TMS, the morphologic changes of colon cancer cells were detected by a microscope (100× magnification). B) SW480 and HCT116 cells were exposed to 5 and 10 μM of TMS for different time points, cells viability was examined by the CCK-8 assay. C) Cell apoptosis was examined by FCM assay after TMS treatment. D) The expression of several apoptosis-related proteins was examined by western blot assay after TMS exposure in colon cancer cells. Data are expressed as mean ± SD, **$p<0.01$.

by apoptotic cell death, cellular apoptosis was examined by the flow cytometry (FCM) assay. Interestingly, TMS (5, 10 μM) exposure dose-dependently upregulated the ratio of apoptotic cell ($p<0.01$, Figure 1C). Furthermore, western blot results indicated that the expression of several apoptotic-related proteins was significantly increased after TMS exposure in colon cancer cells except for the expression of cleaved caspase-8 (Figure 1D).

TMS triggers intrinsic mitochondrial apoptosis signaling. The above studies have shown that TMS remarkably increased the cleavage of caspase-9/3 and PARP ($p<0.01$), while the expression of cleaved caspase-8 was not changed. These results indicated that TMS might induce colon cancer cells' apoptosis via intrinsic mitochondrial signaling. To verify this hypothesis, we conducted JC-1 staining to detect whether TMS treatment could regulate mitochondrial membrane potential (MMP). Our results showed that after TMS treatment the monomer in both SW480 and HCT116 cell lines was significantly increased while J-aggregate was decreased (Figure 2A). These data indicated that TMS might decrease the MMP of the colon cancer cells. Next, we further examined protein expressions of Bcl-2, Bak, Bax, mitochondrial and cytosolic cytochrome C in mitochondrial apoptosis signaling in SW480 and HCT116 cells. Our results suggested that TMS significantly increased the expressions of cytosol cytochrome C and upregulated Bak and Bax expressions while Bcl-2 was downregulated in SW480 and HCT116 cells ($p<0.01$, Figure 2B). In conclusion, these data indicated that TMS might induce apoptotic cell death via intrinsic mitochondrial signaling. Recent studies have demonstrated that autophagy might stimulate or inhibit cancer cells apoptosis, which depends on different cancer microenvironments [12]. Therefore, we explored the potential roles of autophagy in apoptosis mediated by TMS in colon cancer cells. Firstly, we detected the effects of TMS on acidic vesicle organelle (AVO, an indicator for autophagy) formation by acridine orange staining. AVO staining can quantify the volume of the acidic components in the cells. In acridine orange-stained cells, the nucleolus and cytoplasm fluoresce dim red and bright green, however acidic compartments fluoresce bright red. Our results indicated that TMS significantly promoted AVO formation in colon cancer cells (Figure 2C). Then, we further examined the pro-autophagy effects of TMS via analyzing the expressions of autophagy-related proteins such as Beclin-1, p62/SQSTM1, Atg5, and LC3B-II. Our results showed that treatment with TMS upregulated the expressions of Atg5, Beclin-1, and LC3B-II while reducing the expressions of p62/SQTSM1 ($p<0.01$, Figure 2D). Furthermore, LC3/GFP/mRFP adenovirus was applied to examine intracellular autophagy flux, the reduced GFP indicates the fusion of lysosome with autophagosome. Therefore, red puncta and yellow puncta represent autolysosome and autophagosome after merging, respectively. In our study, TMS significantly decreased the proportion of yellow puncta while the proportion of red puncta was improved, which indicated that TMS could enhance autophagy flux in SW480 and HCT116 cells (Figure 2E).

TMS induced autophagy and partially reversed apoptosis in colon cancer cells. To further explore the relationship between apoptosis and autophagy in colon cancer cells after TMS treatment, siRNA targeting Atg5 or specific autophagy inhibitors (25 nM and 5 mM 3-MA) were applied to suppress the autophagy in SW480 and HCT116 cells [19, 20], and then apoptotic cell death was analyzed by FCM assay and western blot. siRNA assay removes the target mRNA (and hence the protein) from the cell, whereas specific ERS inhibitors only block the function of a protein. In this study, we performed both assays to provide stronger evidence on whether blocking autophagy could effectively enhance apoptosis. As expected, Atg5 siRNA effectively suppressed target gene expression (Supplementary Figure S1). Our results indicated that cells were more sensitive to apoptosis induced by TMS after transfecting with Atg5 siRNA or pre-treating with Baf A1 or 3-MA for 4 h ($p<0.01$, Figures 3A, 3B, 3D). Furthermore, western blot results also demonstrated that blocking autophagy could further improve the cleavage of caspase-3 and PARP as well as the expressions of Bax/Bcl-2 after TMS treatment ($p<0.01$, Figures 3C, 3E). The above data suggested that the autophagy might exhibit protective effects in TMS-induced apoptosis in colon cancer cells.

Apoptosis was partially reversed by autophagy via activating ERS signaling after TMS treatment in colon cancer cells. Previous studies have shown that ERS can induce autophagy in malignant tumors [3]. Herein, we explored whether ERS was involved in TMS-mediated autophagy. Western blotting was performed to examine the expressions of ERS markers, such as GRP78, p-eIF2α, IRE1α, and ATF6. The results showed that TMS treatment significantly increased ERS-related proteins dose-dependently ($p<0.01$). Furthermore, TMS also upregulated the cleavage of caspase-12, a critical protein that can induce stress apoptotic cell death (Figure 4A). The above data suggested that TMS might induce ERS in SW480 and HCT116 cells. Then, we used an ERS inhibitor (TUDCA) to block ERS, ER stress-related genes were detected by qRT-PCR after treatment (Supplementary Figure S2). Interestingly, TUDCA could significantly reverse the increased expressions of Beclin1, LC3B-II, and Atg5 and the decreased expressions of p62/SQTSM1 by TMS treatment ($p<0.01$, Figure 4B). Moreover, we used shRNA-targeted CHOP to further suppress ERS in colon cancer cells (Supplementary Figure S2). Western blot (Supplementary Figure S3) analyses revealed that compared with the negative control shRNA group, knockdown of CHOP via specific shRNA significantly decreased its protein expression. Consistent with the above studies, TMS-mediated autophagy was significantly attenuated by CHOP shRNA transfection in SW480 and HCT116 cells ($p<0.01$, Figure 4C). In conclusion, our results suggested that TMS might induce autophagy in colon cancer cells via activating ERS. Previous studies have demonstrated

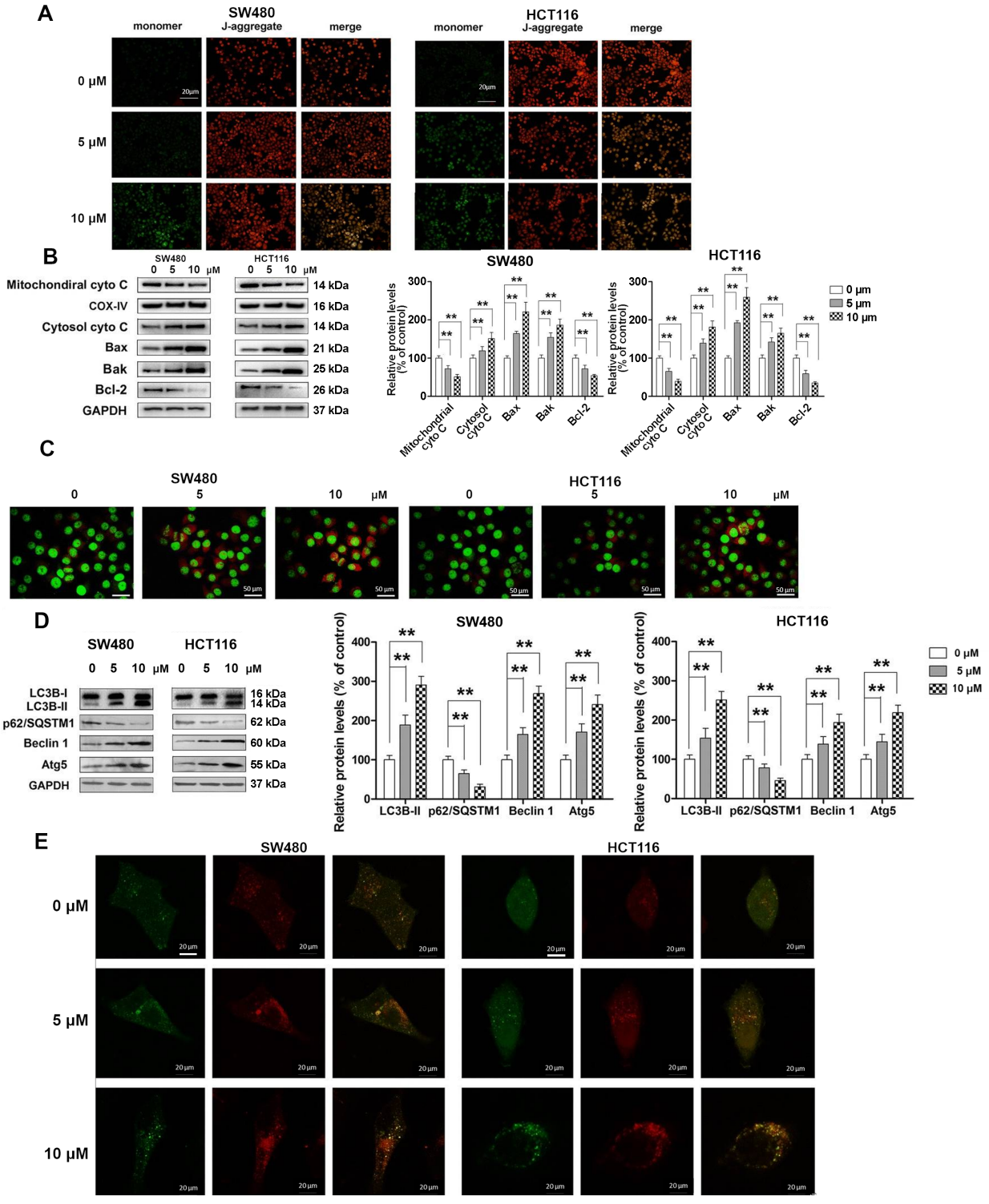

Figure 2. TMS induces colon cancer cells' intrinsic apoptotic cell death and stimulates cell autophagy. A) Fluorescence microscopy was applied to detect the effects of TMS on mitochondrial membrane potential (MMP) by examining JC-1 staining. B) Western blot assay was performed to examine the expressions of intrinsic apoptosis-related proteins. C) Acridine orange dye was applied for detecting the effects of TMS on acidic vesicle organelles development in SW480 and HCT116 cells. Red plots indicate acidic vesicle organelles. D) The expressions of autophagy-related proteins were examined by western blot assay. E) Recombinant adenovirus with mRFP-GFP-LC3 was transfected into SW480 and HCT116 cells. Fluorescence microscopy was applied to examine the effects of TMS on autolysosome formation (200× magnification). Red puncta and yellow puncta imply autolysosome and autophagosome, respectively. Scale bar = 50 μm. Data are expressed as mean ± SD, **p<0.01.

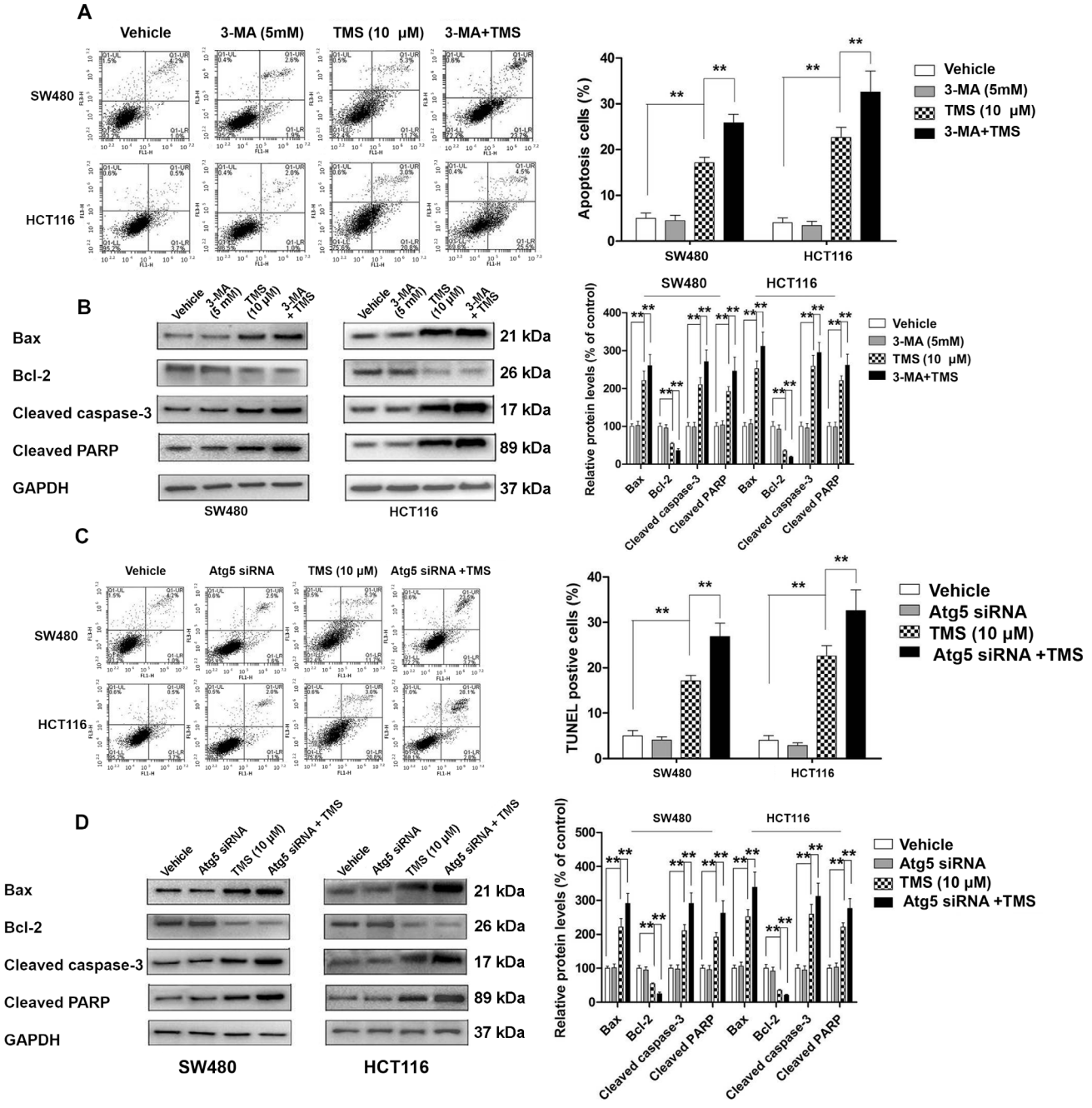

Figure 3. TMS induced autophagy and partially reversed apoptosis in colon cancer cells. A) SW480 and HCT116 cells were treated with 3-MA (5 mM) for 8 h and 10 μM TMS or vehicle were added to cells after 3-MA treatment. FCM was applied to detect cell apoptosis. B) Colon cancer cells were pre-treated with Baf A1 (25 nM) for 4 h in the presence of TMS. Cell apoptosis after TMS treatment was detected by flow cytometry. C) The expressions of apoptosis-related proteins were examined in colon cancer cells by western blot assay. D, E) siRNA was used to inhibit Atg5 transcription in colon cancer cells, then, cells were treated with TMS (10 μM), cell apoptosis was determined by the TUNEL method and western blot. The data were presented as mean ± SD from three independent experiments. **p<0.01

that ERS might induce apoptotic cell death in cancers [8]. However, in this study, we found that blocking ERS by TUDCA notably promoted the pro-apoptotic effects of TMS in colon cancer cells. The raise of apoptotic cells proportion, caspase-3 cleavage, and the decrease of Bcl-2/Bax ratio suggested that ERS might not be involved in TMS-mediated apoptotic cell death. However, ERS might mediate protective autophagy in colon cancer cells (Figures 4D, 4E). The above studies indicated that inhibiting ERS mediated-autophagy may enhance the pro-apoptotic effects of TMS.

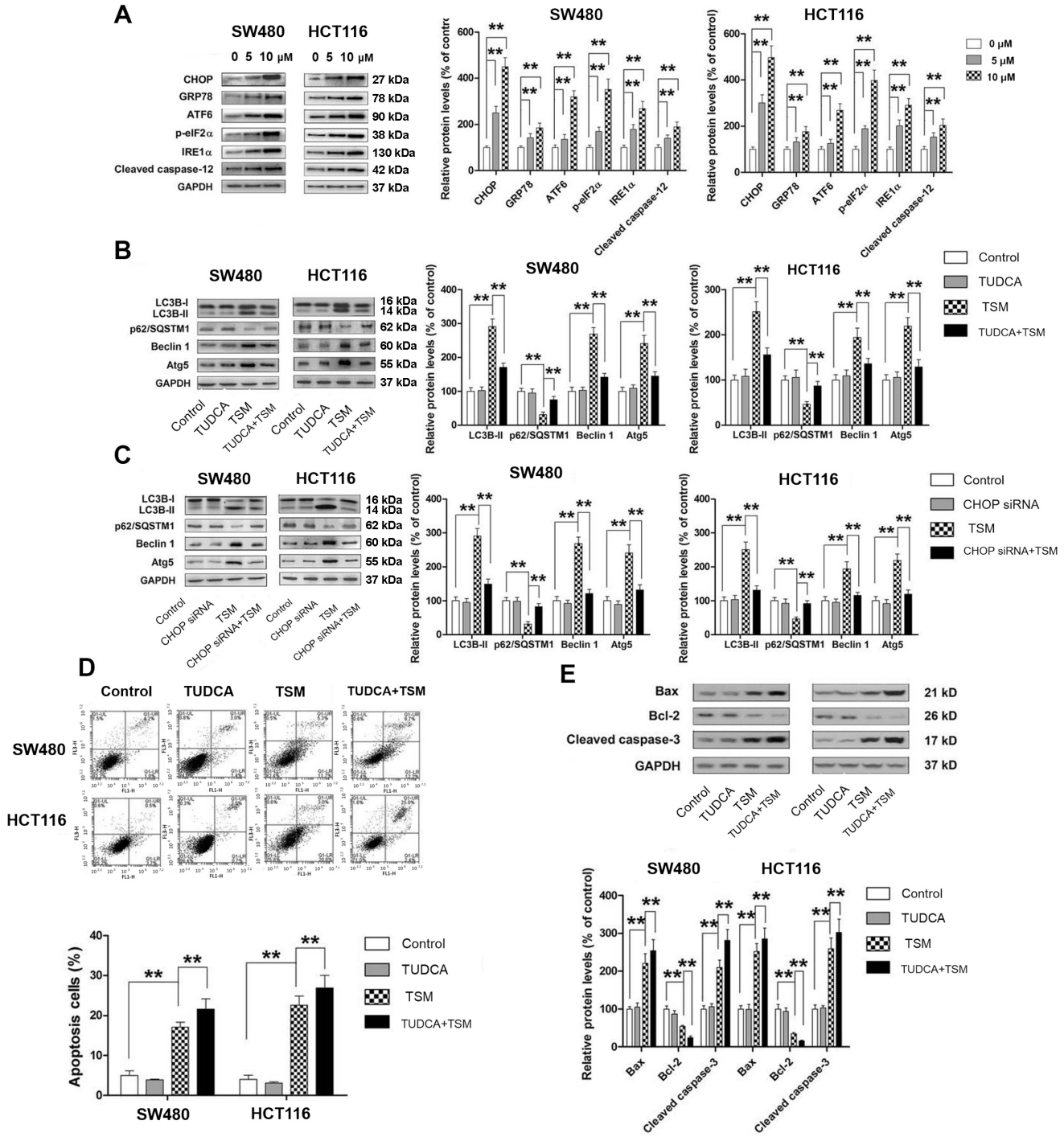

Figure 4. Cell apoptotic death was partially reversed by autophagy via activating ERS signaling pathway after TMS treatment on colon cancer cells. A) SW480 and HCT116 cells were pre-treated with TMS (5, 10 μM). ERS-related proteins expressions were determined by western blot assay. B) After co-treatment with ERS inhibitor (TUDCA) and TMS (10 μM) on colon cancer cells, the protein expressions of Beclin 1, LC3B-II, Atg5, and p62/SQTSM were analyzed by western blot assay. C) After CHOP shRNA transfection, cells were exposed to TMS (10 μM) for 72 h. The expressions of autophagy-related proteins were examined by western blot assay. (D) Cells were exposed to TUDCA and incubated with TMS (10 μM) for 24 h Then, cell apoptosis was analyzed by FCM assay. E) Cells were exposed to TUDCA and incubated with TMS (10 μM) for 24 h, the cleavage of caspase-3 and the protein expressions of Bcl-2, Bax were analyzed by western blot assay. **p<0.01

Discussion

Recent studies for designing new analogs of resveratrol with better pharmacological activities have yielded several novel stilbenes with diverse aryl substituents, of which TMS is a major promising derivative of resveratrol. Previous studies have reported that TMS is more active than resveratrol as an anti-tumor agent in various cancer cell lines [21]. In the current study, we found that TMS significantly suppressed colon cancer growth by inducing apoptotic cell death. In addition, TMS could induce protective autophagy by stimulating ERS in colon cancer. Inhibiting ERS or autophagy could enhance the pro-apoptotic effects of TMS in colon cancer cells.

Cell apoptosis plays a pivotal role in the pathogenesis of human cancers. Apoptotic cell death can be induced by endogenous mitochondrial signaling or exogenous apoptosis signaling [6]. Dysregulation of these apoptosis signaling may lead to the resistance of cell death, which is a hallmark of cancer [22]. In the current research, we found that TMS induced apoptosis in SW480 and HCT116 cells by activating mitochondrial apoptosis signaling, according to the increased expression of the cleaved caspase-3/9, cytosol cytochrome C, Bak, and Bax proteins, as well as the decreased expression of Bcl-2 in colon cancer cells. Different from apoptotic cell death, autophagy can either suppress or facilitate tumor development, which mainly depends on diverse cancer types or microenvironments [23]. In the current research, our results showed that TMS mediated autophagy in SW480 and HCT116 cells, as indicated by the increased expressions of Atg5, LC3, and Beclin-1, and the decreased expression of p62/SQTSM1. In addition, inhibiting TMS-mediated autophagy significantly facilitated apoptotic cell death in colon cancer cells.

The relationship between apoptosis and autophagy is complex in different cancer cells. Both processes play important roles in regulating cell survival and death. Apoptosis and autophagy may be stimulated by the same stresses. Previous studies have suggested that ERS might play a pivotal role in inducing autophagy and apoptosis in different cancers [12]. Herein, we observed that TMS exposure facilitated apoptotic cell death and ERS-mediated autophagy in SW480 and HCT116 cells. Interestingly, our data demonstrated that ERS was not involved in TMS-induced apoptosis, however, ERS notably induced protective autophagy in colon cancer cells. Furthermore, inhibiting cellular ERS significantly improved the pro-apoptotic effects of TMS. Thus, our study suggested that TMS-mediated autophagy was dependent on ERS, while apoptotic cell death might be induced in the ERS-independent pathway after TMS treatment. In consistent with this work, a recent study by Qomaladewi et al. has also shown that stimulation of ERS only induced autophagy, while apoptosis was not affected in cancer cells [24]. Our study indicated that exploring novel agent which could inhibit ERS mediated-autophagy should be combined with TMS to achieve more effectiveness in cancer treatment. In conclusion, inhibiting ERS mediated-autophagy may enhance the pro-apoptotic effects of TMS. TMS might be a potential therapeutic agent for colon cancer treatment. However, further *in vivo* studies should be performed to better elucidate the complex linkage between autophagy and apoptosis mediated by TMS.

Supplementary iformation is available in the online version of the paper.

Acknowledgments: This research was supported by the projects of science and technology development plan of Jilin province (20190201203JC, 20190201214JC) and the projects of Guangdong Basic and Applied Basic Research Foundation (2019A1515110167).

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Inhibiting endoplasmic reticulum stress mediated-autophagy enhances the pro-apoptotic effects of resveratrol derivative in colon cancer cells

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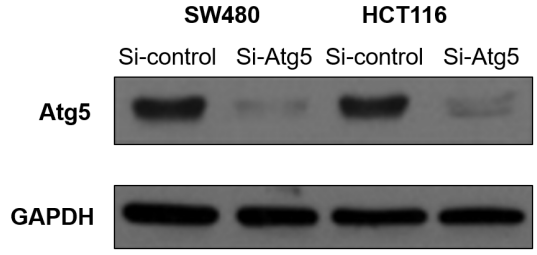
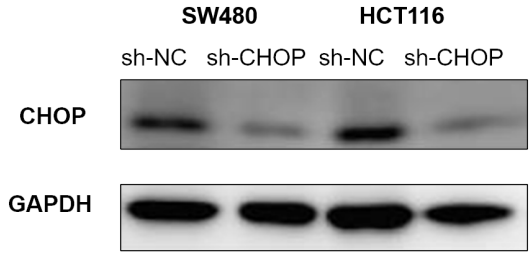
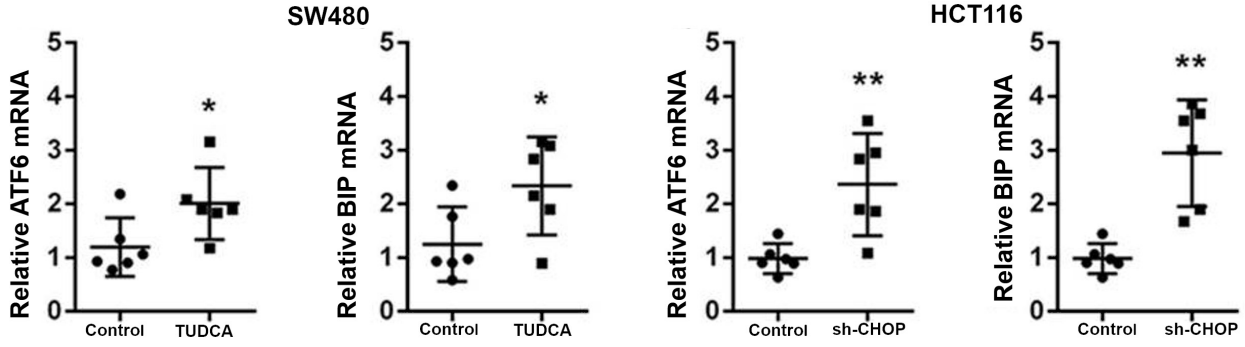
Supplementary Information



Supplementary Figure 1. Western blot results showed that Atg5 siRNA effectively suppressed target gene expression.



Supplementary Figure 2. shRNA targeted CHOP was used to further suppress ERS in colon cancer cells.



Supplementary Figure 3. Knockdown of CHOP via specific shRNA significantly decreased its protein expression.