

Inhibition of FOXO1-Mediated Autophagy Promotes Paclitaxel-Induced Apoptosis in MDA-MB-231 Cell Lines

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Research Article

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Abstract

Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancers and often produces resistance to paclitaxel (PTX) therapy. Autophagy plays an important cytoprotective role in PTX-induced tumor cell death and targeting autophagy is promising to improve the efficacy of tumor chemotherapy in recent years. Here, we reported that PTX induced both apoptosis and autophagy of MDA-MB-231 cells, and inhibition of autophagy enable to promote apoptotic cell death. Furthermore, we found that FOXO1 enhanced PTX-induced autophagy by a transcriptional activation pattern in MDA-MB-231 cells, which was associated with its downstream target genes *ATG5*, *VPS34*, *BECN1* and *MAP1LC3B*. The knockdown of FOXO1 attenuated the survival of MDA-MB-231 cells under the PTX treatment. These findings will be beneficial to improve the treatment efficacy of PTX and to develop the autophagic target therapy of TNBC.

Introduction

Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancers with poor clinical outcomes and early recurrence, which make it become a new hot spot of research in recent year [1]. Presently, chemotherapy remains standard treatment for TNBC, such as the use of single-agent taxanes including paclitaxel [2, 3]. However, tumor cells often escape apoptosis through some ways leading to lower therapeutic effect. It has been reported that autophagy could protect tumor cells from damage to escape apoptosis during paclitaxel treatment [4]. Therefore, the treatment approaches targeted autophagy may provide a novel therapeutic value for TNBC.

Autophagy is a classical self-digestive process, which could maintain cell homeostasis and ensure cell survival in stress conditions through degrading and recycling damaged large organelles and protein aggregates. It also plays a pivotal role in tumor progression, which can prevent tumor initiation in early developing cancer but protect tumor cells from various stress damages in fully developed tumors [5–7]. Many chemotherapy drugs can induce autophagic occurrence by activating different signaling pathway. For example, SKF-96365, a store-operated calcium entry inhibitor, can induce cytoprotective autophagy by preventing the release of cytochrome C in colorectal cancer cells, which is mechanistically involved in AKT-related signaling [8]. In addition, apatinib-induced autophagy in anaplastic thyroid carcinoma cells is related to downregulation of p-AKT and p-mTOR signals [9]. PTX treatment often also activates different autophagy-related pathways depending on different tumor types. In A549 lung cancer cells, PTX induced autophagy by regulating autophagy-related genes *ATG5* and *BECN1* [10]. Whereas, PTX induced autophagy by upregulating *TXNDC17* gene in ovarian cancer, which shortened survival of a number of patients [11]. However, the key molecule or mechanism associated with PTX-induced autophagy in TNBC cells remains unclear.

Apoptosis is one of terminal paths of cell death, which is closely related to morphogenesis during elimination of aged or harmful cells to maintain adult tissue homeostasis [12]. There are some cross-talks between autophagy and apoptosis have been reported in some tumors: autophagy may be an

adaptive stress response prior to apoptotic cell death or enable apoptosis or could antagonize apoptosis [7]. Therefore, the relationship between apoptosis and autophagy is critical to tumor targeted-therapy.

In this study, we investigated the molecular mechanism of PTX chemotherapy in vitro with MDA-MB-231 cells. We found that PTX induced both apoptosis and autophagy, and inhibition of autophagy contributed to enhance apoptotic cell death. Furthermore, FOXO1 played a critical role in PTX-induced autophagy by a transcriptional activation pattern, and knockdown of FOXO1 was able to attenuate the survival of MDA-MB-231 cells. The finding will be beneficial to improve the efficacy of PTX and the target therapy of TNBC.

Materials And Methods

Reagents and antibodies

All the experimental reagents and antibodies are purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated in this study. Bafilomycin A1 (Baf A1) was purchased from Sangon Biotech, China. The antibodies used in this study are anti-FOXO1 antibody (Cat# 2880, Cell signaling Technology, USA), anti-phospho-FOXO1 antibody (Cat# WL03634, Wanlaibio, China) and anti-Lamin AC antibody (Cat# 10298-1-AP, Proteintech, USA).

Cell culture

The MDA-MB-231 triple-negative breast cancer cell lines was purchased from Chinese Cells Bank. Cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Cat# C11995500BT, Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Cat# 10099-141, Gibco, USA), penicillin (100 units/ml) and streptomycin (100 µg/ml) (Invitrogen, Carlsbad, CA) in a humidified atmosphere of 5 % CO₂ at 37 °C.

Cell morphological observation

Exponentially growing MDA-MB-231 cells were transferred to 12-well plates and cultured at 37 °C in a 5% CO₂ atmosphere. Cells were treated with different concentration of PTX for 24 h. When the cells were at 60 to 70% confluence, they were rinsed twice with PBS, and the supernatant was discarded. Then, images were taken using an OLYMPUS IX 71 microscope (OLYMPUS, Tokyo, Japan).

Annexin V-FITC/PI staining for apoptosis analysis

The cell apoptosis assay was performed according to manufacturer's instructions using Annexin V-FITC/PI test kit (Cat# FXP018-100, 4A Biotech Co., Ltd, China). The cell flow cytometry analysis was performed by using a Beckman CytoFLEX flow cytometer (Beckman, California, USA).

Immunofluorescence

The immunofluorescence analysis of LC3 and FOXO1 proteins was performed according to our previous study [13]. In brief, the treated cells were fixed in methanol for 10 min and blocked with a buffer containing 1% BSA and 0.1% Triton X-100 for 1 h. Then the fixed cells were incubated with primary antibodies against LC3B and FOXO1, respectively, at 4 °C overnight. After that, cells were incubated with secondary fluorescence-conjugated antibodies for 1 h to visualize via laser confocal microscopy (OLYMPUS FV 1000, Tokyo, Japan).

Colony formation assay

The assay was performed according to our previous study [14]. Briefly, the adherent cells were treated with or without 3-MA (5nM) and/or PTX for 72 h and then cultured for 15 days. Thereafter, the cells were fixed and stained with 10% Giemsa (Solarbio, Beijing, China). The colonies were washed, air dried, imaged and counted. Finally, colonies formation ratio was calculated according to the formula, Colony formation ratio = No. of colonies/No. of seeded cells × 100%.

Small interfering RNA (siRNA) and transient transfection

MDA-MB-231 cells were seeded into 96 or 6-well plates. Then the control random small interference RNA (siRNA) or targeting FOXO1-targeted siRNA (Santa Cruz Biotechnology, California, USA, 100 pmol/well) were transfected into MDA-MB-231 cells using the siRNA transfection reagent (Santa Cruz Biotechnology, California, USA) according to the manufacturer's protocol. After 7 h transfection, cells were treated with paclitaxel for an additional 24 h. Then, the cells were collected and cell lysates were prepared for q-PCR and western blotting. Cells were also used for cell viability and apoptosis analysis.

Reverse transcription and q-PCR

Total RNA was extracted by Trizol agent (Invitrogen, California, USA). cDNA was synthesized from total RNA using a Prime-Script RT reagent kit (TaKaRa, Japan). The obtained cDNA was used as a template in SYBR green-based q-PCR (CFX-96, Bio-Rad, California, USA). The mRNA expression levels of the ATG genes were assessed with quantitative polymerase chain reaction (q-PCR). GAPDH was used for normalization. The primers are shown in supplementary Table 1.

Western blotting

At the end of the designed treated time, cells were washed twice with PBS and collected. Then, total protein concentrations of cell lysates were determined with a BCA Protein Assay kit (Beyotime, Shanghai, China). Protein samples (total protein loading of 100 µg) were separated by 12% SDS-PAGE and transferred onto PVDF membranes. These membranes were incubated for 30 min in 5% BSA buffer (Solarbio, Beijing, China) with gentle shaking to block non-specific binding before incubation with the diluted primary antibody (LC3B: 1:2000, p62: 1:2000, FOXO1: 1:1000, p-FOXO1: 1:1000, β-actin: 1:5000) overnight at 4 °C. Subsequently, membranes were incubated with 5000-fold diluted secondary antibody (BD, California, USA) for 90 min at room temperature. The membrane was washed three times in PBS, for

10 min each time. Then, the membrane was treated for 3 min in the dark with reagent from an Easysee Western Blot Kit (Transgene, Alsace, France).

Statistical analysis

All western blotting and image data presented are representatives from at least 3 independent experiments. The numeric data are presented as means \pm standard deviation (SD) from 3 independent experiments and analyzed using Student's *t*-test.

Results

PTX induced cytotoxicity and apoptosis in MDA-MB-231 cells

PTX is known as the chemotherapeutic agent to promote the polymerization of tubulin, which disrupts normal microtubule dynamics, thereby leading to cell death [15]. To determine the effect of PTX on the MDA-MB-231 cell viability, we firstly performed morphological observation and CCK-8 assay after PTX treatment. The morphological changes revealed that the higher dose of PTX, the more MDA-MB-231 cell death (Fig. 1A). Furthermore, the CCK-8 assay showed cell survival rate was also decreased with the increase of PTX dose (Fig. 1B). These results indicated that PTX induced MDA-MB-231 cell death in a dose-dependent manner.

To further determine whether the observed cell death was caused through PTX-induced apoptosis pathway, we performed PI and Annexin V staining coupled with flow cytometry. As shown in Fig. 1C, PTX exposure resulted in a dose-dependent increase of apoptosis rate. In addition, the expression level of apoptosis markers, Bcl-2 and Bax, were also analyzed by western blot after PTX treatment. As shown in Fig. 1D, the expression level of Bcl-2 was decreased in a dose-dependent manner, while Bax was increased correspondingly. These results indicated that PTX induced apoptosis in MDA-MB-231 cells.

PTX induced autophagy in MDA-MB-231 cells

Under the PTX inducing MDA-MB-231 cell apoptosis, we investigated the effect of PTX on cell autophagy. We found that there was an increase of LC3-II and decrease of p62 in a dose-dependent manner in MDA-MB-231 cells (Fig. 2A), indicating the increased autophagy level. In addition, exposure to PTX resulted in a further increase of LC3 puncta in the presence of Baf-A1 (Fig. 2B and C), suggesting that PTX increased autophagy flux level. Therefore, PTX also induced autophagy at the same time of inducing apoptosis in MDA-MB-231 cells.

Inhibition of autophagy increase PTX-induced apoptosis cell death in MDA-MB-231 cells

Given PTX enabled to promote apoptosis simultaneously, to enhance autophagy in MDA-MB-231 cells, we investigated whether inhibition of autophagy contributed to improve PTX-induced apoptotic cell death. After treatment with 3-MA, inhibition of autophagy was clearly observed, including significant decrease of LC3-II/LC3-I ratio, and increase of p62 expression and LC3 puncta (Fig. 3A-D). Furthermore,

the formation of cell colonies was effectively inhibited when the dose of PTX was 10 nM and 30 nM (Fig. 3E and F). These results indicated that inhibition of autophagy with 3-MA promoted PTX-induced apoptosis in MDA-MB-231 cells.

PTX increase the FOXO1 expression in MDA-MB-231 cells

Autophagy is regulated by autophagy-regulated genes, such as *FOXO1*, *PTEN*, *LKB1*, *mTOR*, *SESN1*, *EPG5*, *TSC1*, *AKT*, *LMNA*, *AMBRA1* and *DRAM1* genes, which are essential for autophagy signaling pathways [16-18]. We measured mRNA level of these genes by real-time PCR and found that 20 nM PTX induced a 2.7-fold increase of FOXO1 mRNA in MDA-MB-231 cells (Fig. 4A). Therefore, we focused on the role of FOXO1 in PTX-treated MDA-MB-231 cells at the following assays. Firstly, we detected that FOXO1 protein expression after PTX treatment. Indeed, PTX induced the significant elevated expression of FOXO1, but not change phosphorylated FOXO1 which was translocated into cytoplasm to regulate autophagy (Fig. 4B and C). In addition, the upstream inhibitor of FOXO1, phosphorylated AKT, showed a markedly down-regulated pattern (Fig. 4D and E). Thus, we speculated that the elevated FOXO1 expression was related to the declined p-AKT level. Since FOXO1 as a transcript factor, we asked whether FOXO1 monitoring autophagy in the PTX-treat MDA-MB-231 cells was in a transcriptional activation pattern. Therefore, we further detected the location of FOXO1 by WB and immunofluorescence. The result showed that FOXO1 was mainly distributed in nucleus, which further confirmed that FOXO1 exerting its autophagy-regulated function was in a transcriptional activation pattern (Fig. 5A and B).

FOXO1 Is required for PTX-induced autophagy in MDA-MB-231 cells

To investigate the function of FOXO1 in PTX-induced autophagy in MDA-MB-231 cells, we knocked down FOXO1 expression via small interfering RNA. The result revealed that FOXO1 knockdown led to a significantly decreased LC3II and a significant increased P62 protein expression level in PTX treated MDA-MB-231 cells, suggesting a reduced autophagy level (Fig. 6A-C). Many autophagy-related genes, such as *ATG5*, *VPS34 (PI3KC3)*, *ATG4B*, *BECN1* and *MAP1LC3B*, are transcriptionally regulated by FOXO1 [19]. In this study, we also found that these genes except ATG4B were suppressed after FOXO1 knockdown in the PTX-treated MDA-MB-231 cells (Fig. 6D). Importantly, knockdown of FOXO1 enabled to enhance PTX-induced apoptotic cell death (Fig. 6E). These results illustrated that FOXO1 played a critical role for PTX-induced autophagy in MDA-MB-231 cells and targeting FOXO1 might improve the efficacy of PTX in TNBC therapy.

Discussion

Although PTX has been widely used in the treatment of various solid tumors including ovarian, lung and breast cancer [20], of which chemotherapy efficacy is varied from different types of cancers even develop resistance to it. Recently, it has also been found that the TNBC frequently acquires resistance to this drug and is involving in a number of regulating pathways [21, 22]. For example, PTX triggered BRCA1-IRIS expression, a product of the oncogene BRCA1, which enhanced AKT-related signaling thus developed the PTX resistance phenotype in TNBCs [23]. Blanchard et al. demonstrated that aurora kinase A also

promoted PTX resistance in TNBCs by stabilizing FOXM1 [3]. Targeting these pathways or inhibiting tumor-associated factors might be hopeful for improving PTX efficacy in TNBC [24–28]. Furthermore, a recent study indicated that inhibition of autophagy can enhance paclitaxel-induced cell death in the TNBC MDA-MB-231 cells with PTX resistance [29]. Here, we showed that PTX enabled to induce both autophagy and apoptosis, consistently, inhibition of autophagy contributed to promote PTX-induced apoptosis in MDA-MB-231 cells. And elevated autophagy flux was implicated in the significantly increasing nuclear FOXO1. Furthermore, knockdown of FOXO1 enhanced PTX-induced cell apoptosis. These findings might provide a potential anti-cancer target and be important to improve PTX efficacy in TNBC treatment.

Autophagy and apoptosis are two antagonistic and interconnected molecular mechanisms to various cellular stresses. Autophagy, as a pro-survival regulating process, often attenuates apoptotic cell death [30, 31]. PTX as a broad-spectrum anti-cancer agent exerts its activity by often inducing cytotoxic apoptosis or inhibiting autophagy [32, 33]. However, a previous study reported that PTX also induced autophagy of tumor cells [34], which might have an adverse effect on PTX efficacy. A further study indicated that PTX induced both autophagy and apoptosis in several cancer cells. And the up-regulated autophagy was related to autophagosome-regulatory genes ATG5 and Beclin1. After 3-MA treatment or knocking down Beclin1, the tumor cell response occurred switch from autophagy to apoptosis [10]. Similarly, our study also demonstrated that autophagy was induced in MDA-MB-231 cells accompanied with PTX-induced apoptosis, and autophagic inhibition with 3-MA enhanced the apoptotic cell death.

FOXO1 is a representative member of the forkhead transcription factors (FOX) family, which plays a crucial role in tumor proliferation inhibition and energy metabolism regulation and the induction of cellular response [35]. In this study, we found that FOXO1 plays an important role in PTX-induced autophagy in MDA-MB-231 cells. And the increase of FOXO1 was accompanied with the attenuating phosphorylated AKT1, which suggested that PTX induces the elevated FOXO1 expression by inhibiting the AKT1-related signaling pathway. A previous study also determined that phosphorylated AKT1 catalyzed the phosphorylation of FOXO1, resulting in the loss of transcription activity and translocating from the nucleus to the cytoplasm [36]. However, we showed that FOXO1 was accumulated in the nucleus rather than translocated to the cytoplasm. Therefore, we detected the expression of core autophagy-related genes that are transcriptionally regulated by FOXO1. The results showed that *ATG5*, *BECN1* and *MAP1LC3B* were significantly up-regulated when treatment with PTX, which was consistent with previous study where nuclear FOXO1 transcriptionally activated *ATG5* and *BECN1* to promote autophagy [37]. After inhibition of FOXO1 combined with PTX treatment, *ATG5*, *BECN1*, *VPS34* and *MAP1LC3B* were markedly down-regulated. This suggests that the FOXO1-mediated autophagy in the PTX-treated MDA-MB-231 cell is to regulate its downstream target genes by transactivation, which is different from the cytosolic FOXO1 inducing autophagy by binding to ATG7 gene [38]. In addition, FOXO1 can transcriptionally inhibit mTOR activity by increasing *SENSE3* expression to promote autophagy [39]. Importantly, autophagy was inhibited after knockdown of FOXO1, at the same time, the apoptotic cell death induced by PTX was increased in MDA-MB-231 cells. This finding is consistent with the concept that regulation of apoptosis by autophagy to enhance cancer therapy [7] and provides a potential therapeutic target for TNBC.

In summary, PTX induced both autophagy and apoptosis, and inhibition of autophagy promoted apoptosis in MDA-MB-231 cells. Furthermore, the elevated FOXO1 was correlated with the increasing autophagy flux. And knockdown of FOXO1 enhanced PTX-induced cell death, which might be important to improve PTX efficacy in TNBC (Fig. 7).

Declarations

Authors' contributions

H-YZ and H-JW conceived and designed the experiments. AY, KX, WZ, ZX, DZ, DJ, HZ, MAJ and YQ performed the experiments. H-YZ, KX, and AY analyzed the data. KX, WZ, and H-YZ wrote the paper. All authors reviewed the manuscript and approved the submission.

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Conflict of interest

The authors do not have any conflicts of interest.

Ethical approval

Not applicable.

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Figures

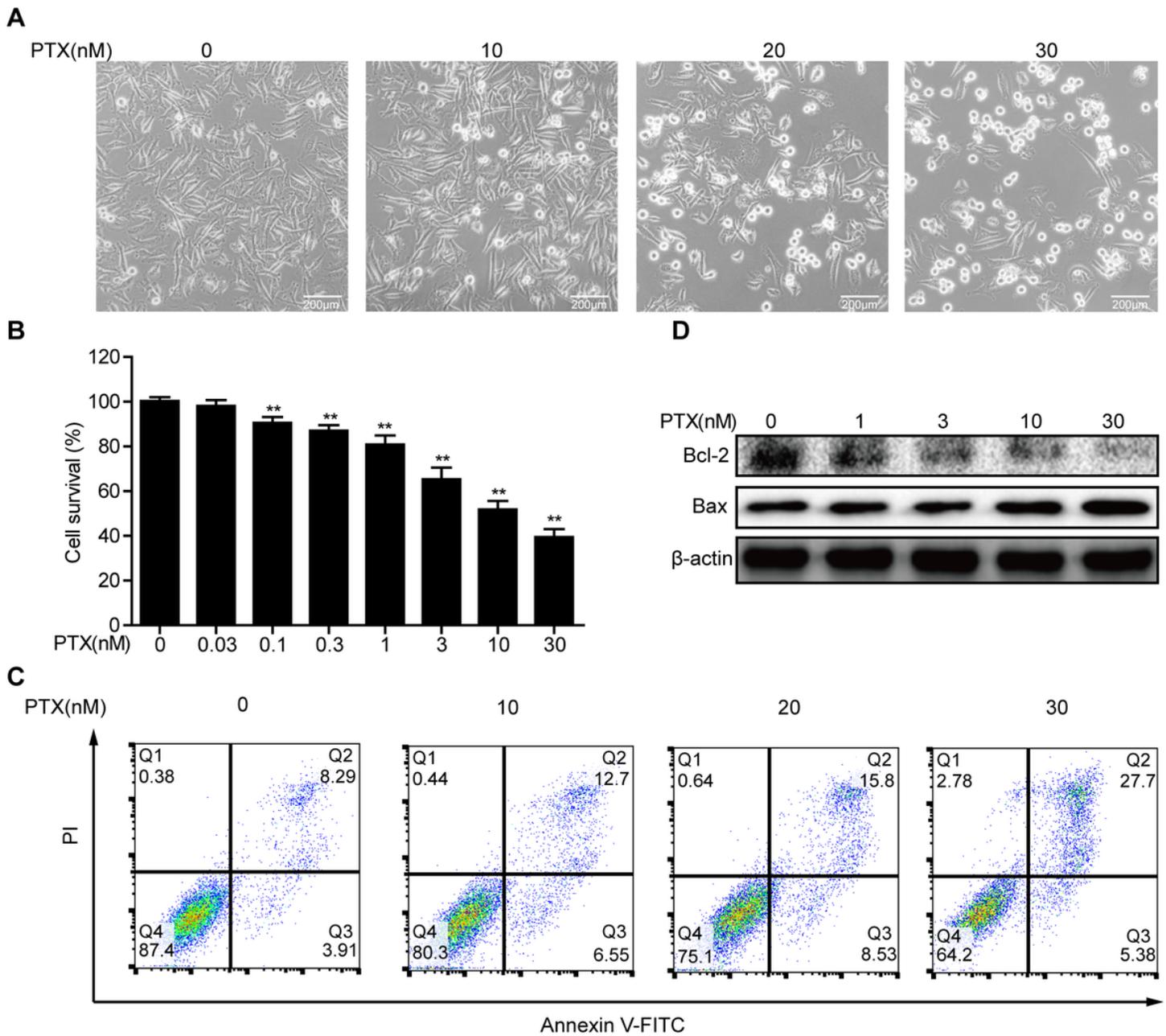


Figure 1

PTX exerts cytotoxicity and induces apoptosis in MDA-MB-231 cells. (A) Photomicrographs of MDA-MB-231 cells exposed to 10, 20 and 30 nM PTX for 24 hours (scale bar = 200 μ m). (B) Quantification of cell viability in different dose of PTX. (Error bars represent SD of three independent experiments, * $p < 0.05$ and ** $p < 0.01$ compared with control). (C) Effect of different-dose PTX on the expression levels of apoptosis proteins Bcl-2 and Bax, β -actin as a loading control. (D) Effect of different-dose PTX on the apoptosis of MDA-MB-231 cells.

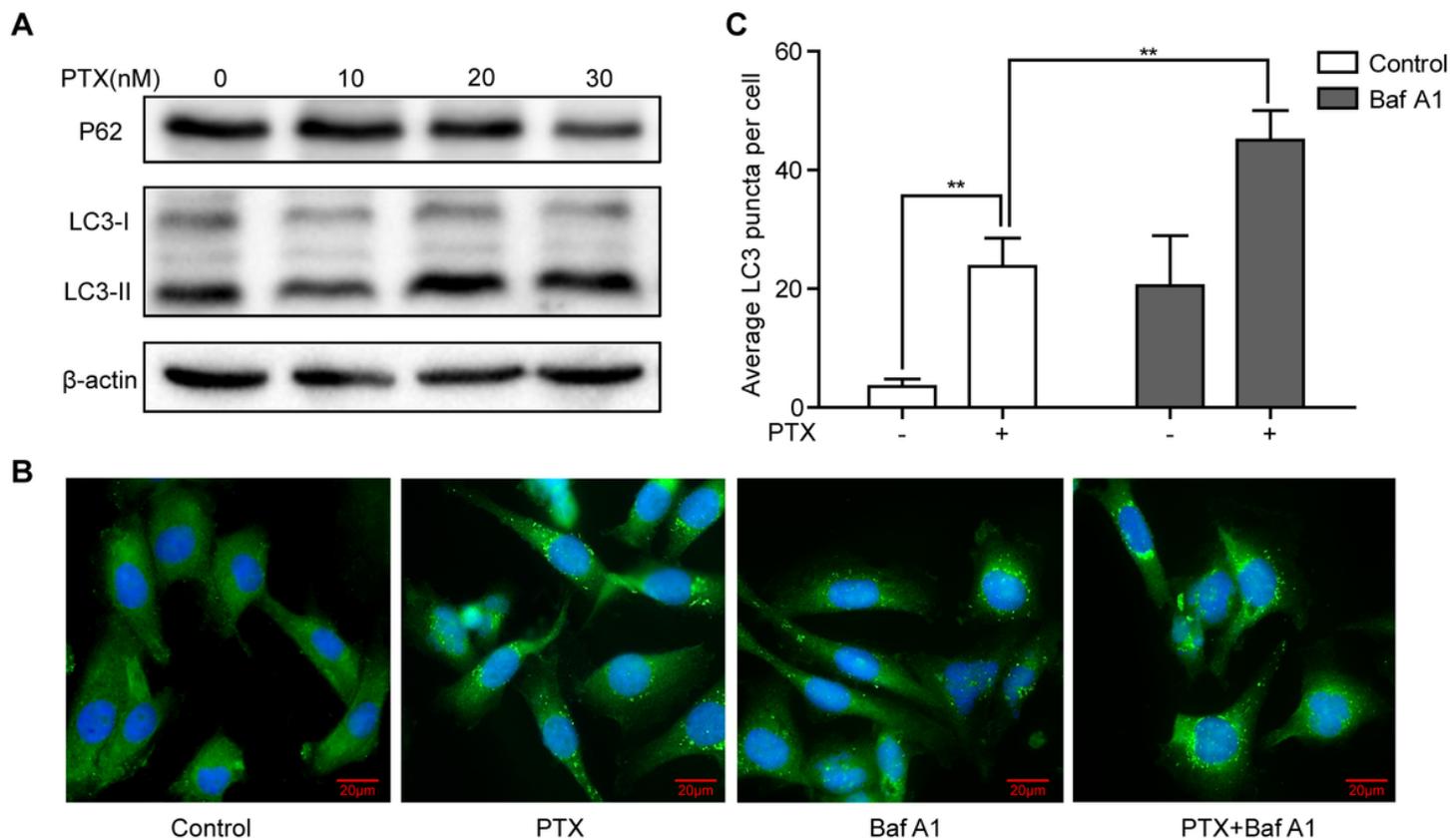


Figure 2

PTX induces autophagy in MDA-MB-231 cells. (A) The protein expression levels of autophagy markers p62, LC3-I and LC3-II at the different-dose PTX, β -actin as a loading control. (B) Representative images of LC3 punctate. Cells were stained with antibodies against LC3 (green), and nuclei were stained with DAPI (blue). Scale bars =20 μ m. (C) Quantification of LC3 punctate, error bars represent SD of three independent experiments (** $p < 0.01$).

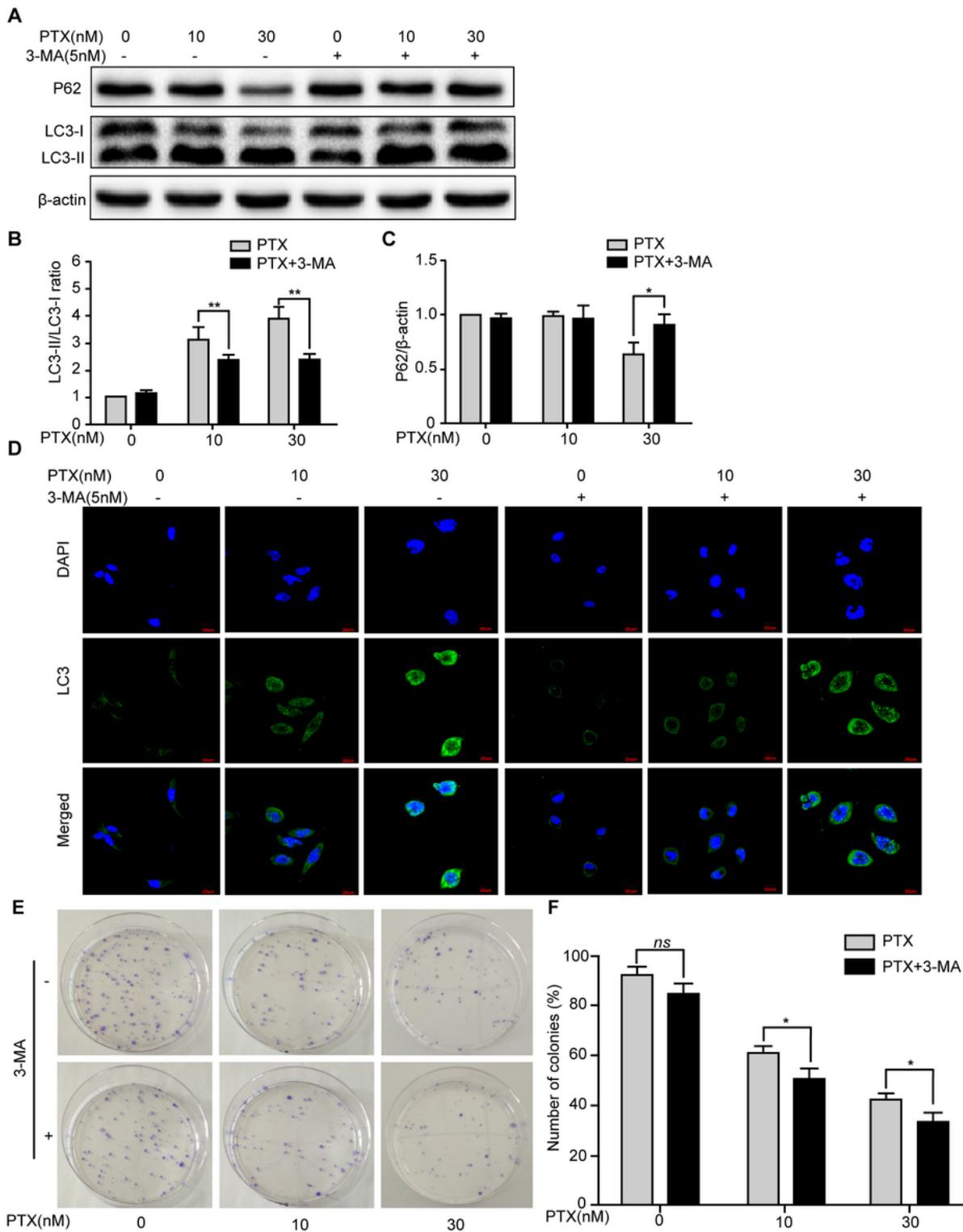


Figure 3

Inhibition of autophagy with 3-MA promotes PTX-induced apoptotic cell death. (A) Effect of 3-MA on the protein expression levels of autophagy markers p62, LC3-I and LC3-II, β -actin as a loading control. (B, C) Quantification of LC3-II/LC3-I ratio (B) and p62 protein (C) in A. (D) Representative images of LC3 punctate after treatment with or without 3-MA and/or PTX. (E) Detection of cell viability by colony

formation assay. (F) Quantification of cellular colony formation rate in G. Data was presented at means and SD of three independent experiments, * $p < 0.05$ and ** $p < 0.01$.

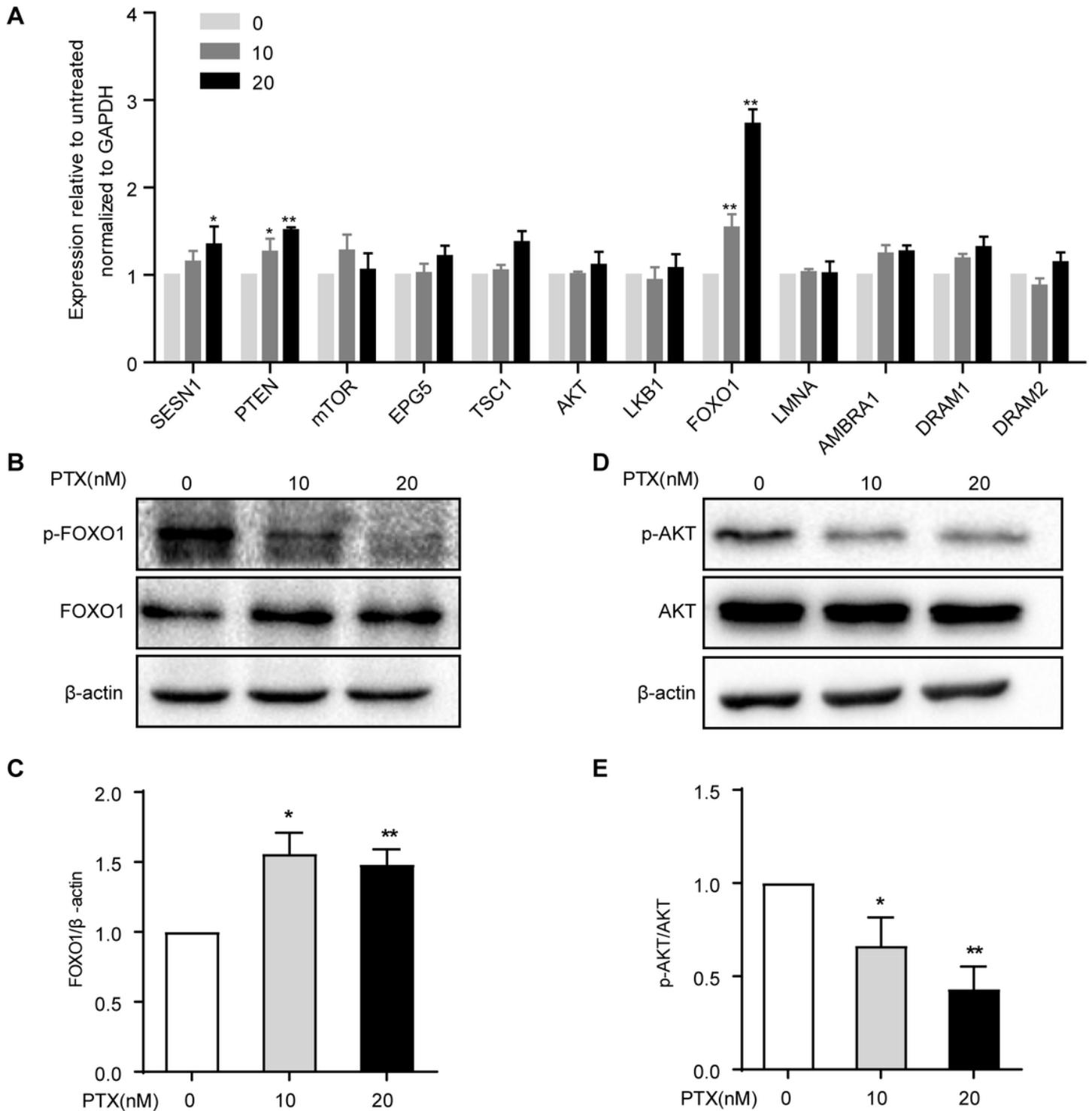


Figure 4

Autophagy regulating pathway induced by PTX in MDA-MB-231 cells. (A) The mRNA expression levels of autophagy signaling pathway-related genes after treatment with 10 and 20 nM PTX, GAPDH as a loading control. (B, D) The protein expression of FOXO1, phosphorylated FOXO1 (p-FOXO1) (B), AKT and

phosphorylated AKT (p-AKT) (D), β -actin as a loading control. (C, E) Quantification of FOXO1 protein in B and p-AKT/AKT ratio in D. Data was represented at means and SD of three independent experiments, * $p < 0.05$ and ** $p < 0.01$.

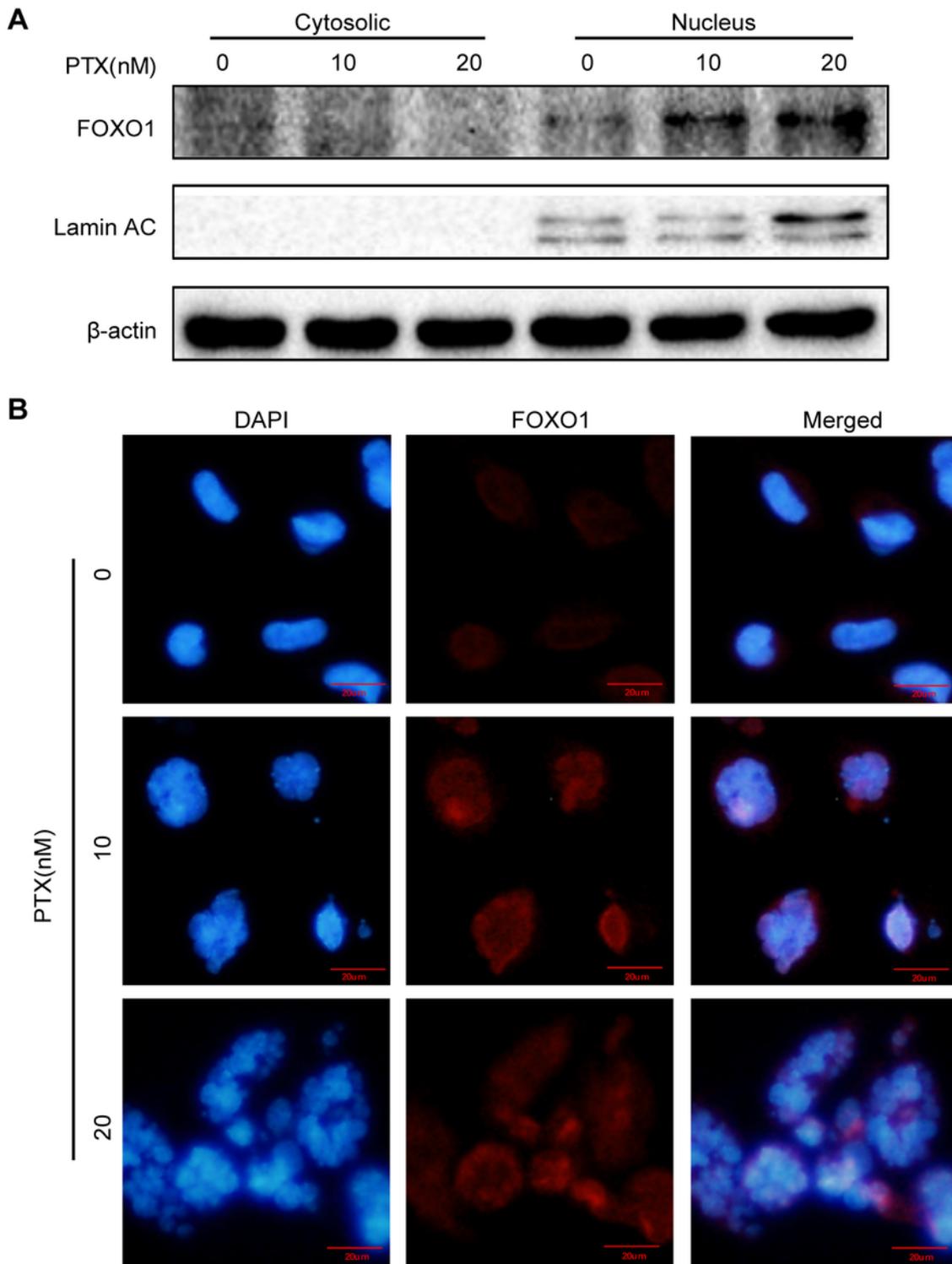


Figure 5

PTX induces the increase of nuclear located FOXO1 in MDA-MB-231 cells. (A) The localization of FOXO1 by western blotting, Lamin AC, a nucleus-specific marker, β -actin as a loading control. (B) The location of

FOXO1 was detected by immunofluorescence, scale bars = 20 μ m.

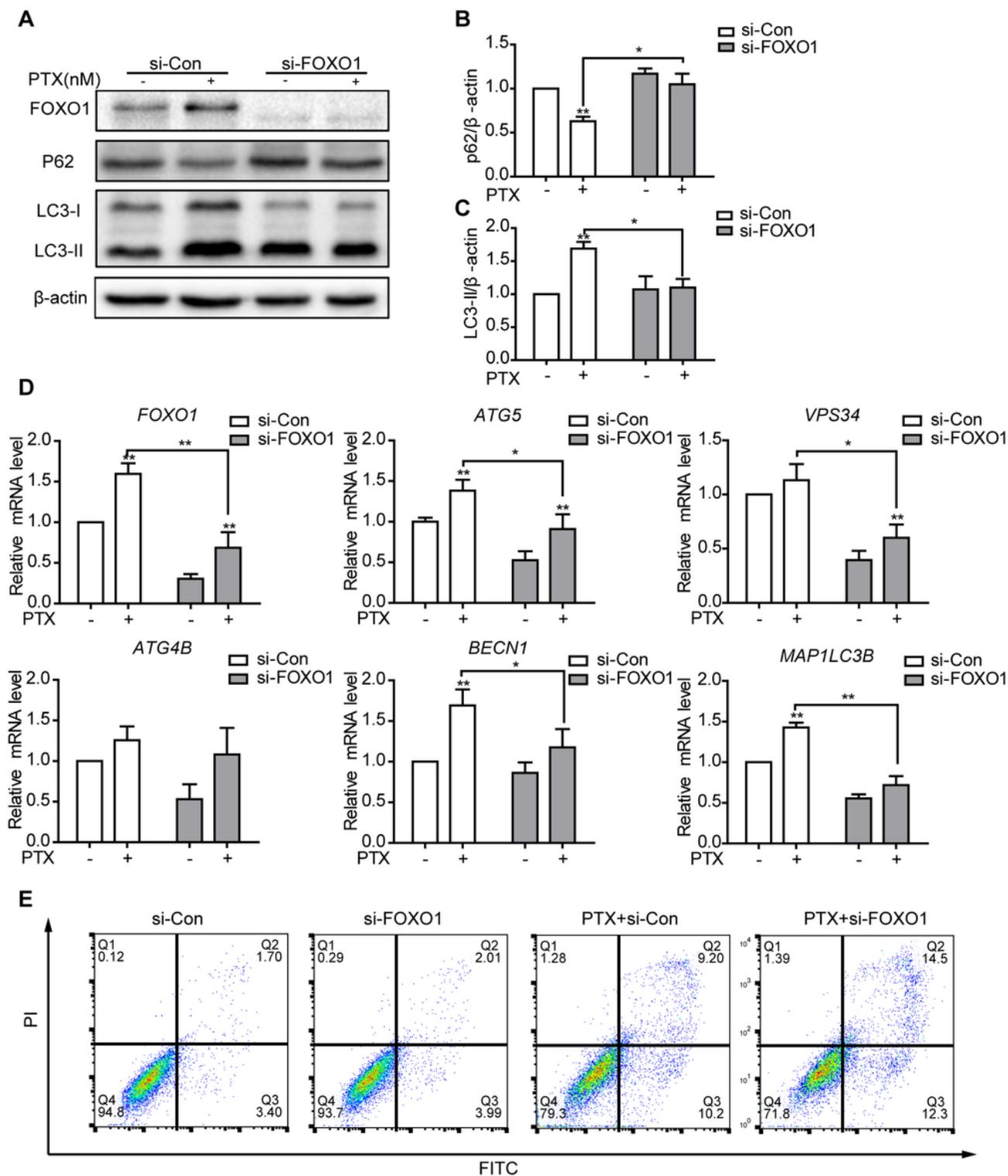


Figure 6

Knockdown of FOXO1 attenuates PTX-induced autophagy and promotes apoptotic cell death in MDA-MB-231 cells. (A) The protein expression of autophagy markers p62, LC3-I and LC3-II after treatment with small interference RNA targeting FOXO1 (si-FOXO1), β -actin as a loading control. (B, C) Quantification of p62 and LC3-II proteins in A. (D) The mRNA expression levels of FOXO1 and its downstream target genes

after treatment with siRNA and/or PTX. (E) Effect of FOXO1 knockdown on PTX-induced cell apoptosis was analyzed by flow cytometry. PTX = 20 nM. Data was represented at means and SD of three independent experiments, * $p < 0.05$ and ** $p < 0.01$.

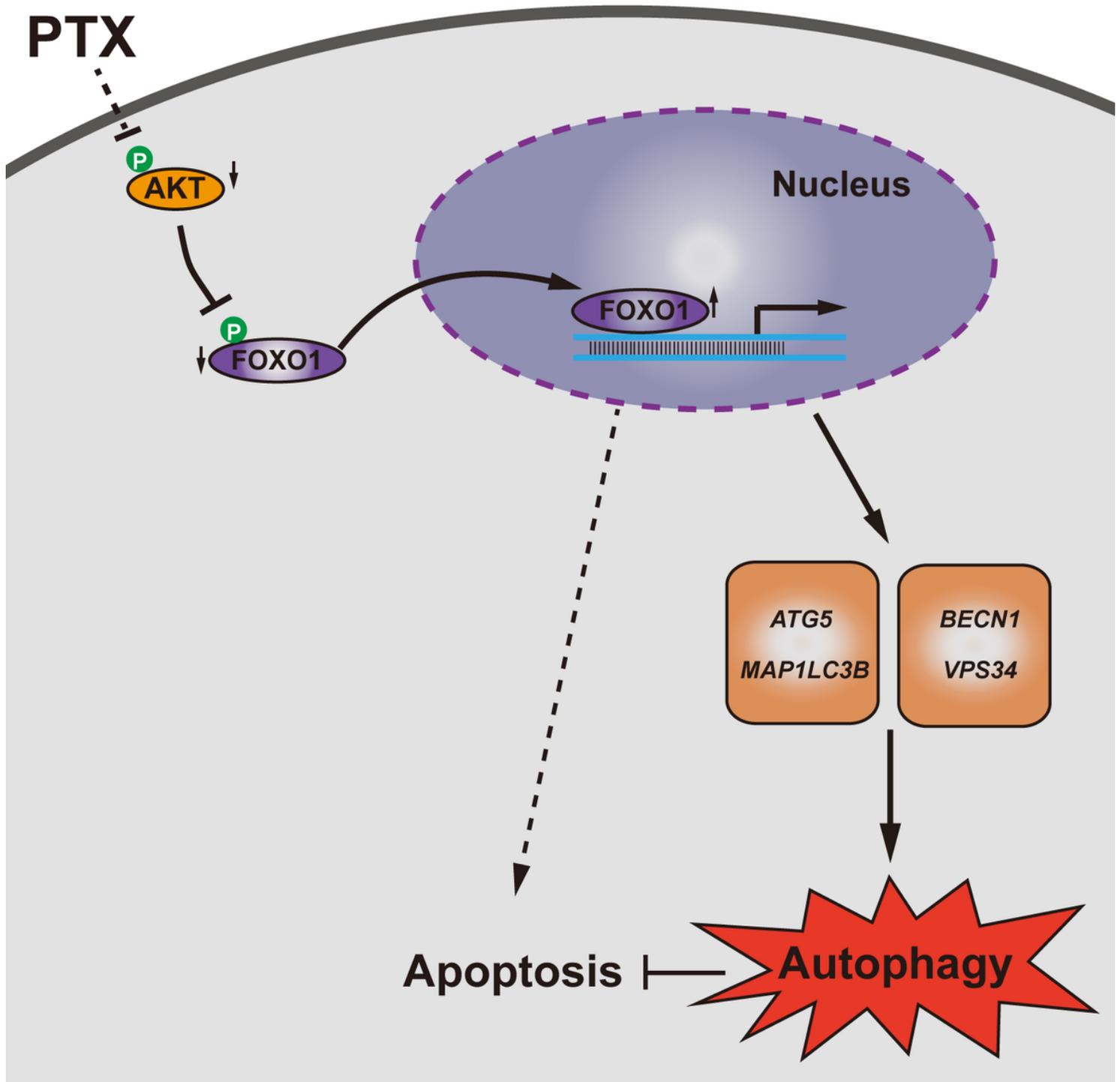


Figure 7

A schematic model of FOXO1-mediated autophagy and apoptosis induced by PTX in MDA-MB-231.

Supplementary Files

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- [Suppltable1.docx](#)