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Ambra1 inhibits paclitaxel-induced apoptosis in breast cancer cells by modulating the Bim/mitochondrial pathway

W.-L. SUN*, L. WANG, J. LUO, H.-W. ZHU, Z.-W. CAI*

Department of Medical Oncology, The Second Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi, China

**Correspondence: swl20022001@hotmail.com, czw001967@sohu.com*

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Cancer cells often evade apoptosis induced by anti-cancer drugs, thereby reducing their efficacy. Autophagy/Beclin 1 regulator 1 (Ambra1) is a crucial pro-autophagic protein. It also plays an important role in the execution of apoptosis. However, the mechanism by which Ambra1 regulates apoptosis has not been fully clarified. Moreover, whether Ambra1 participates in the regulation of paclitaxel-induced apoptosis in breast cancer cells is not clear. Herein, we show that Ambra1 inhibits paclitaxel-induced apoptosis in breast cancer cells. Moreover, Bim and mitochondria are key effectors of Ambra1 in this process. Thus, Ambra1 is a protein that makes breast cancer cells resistant to apoptosis by modulating the Bim/mitochondrial pathway and may therefore be a potential target for the breast cancer treatment.

Key words: Ambra1, Bim, apoptosis, paclitaxel, breast cancer

Breast cancer is the most common malignancy in women worldwide and is one of the leading causes of cancer-related deaths [1]. As an effective drug against breast cancer, paclitaxel (PTX) kills cancer cells mainly by inducing apoptosis [2, 3]. However, escape from apoptosis is a hallmark of cancer cells, which reduces the effectiveness of the drug [4, 5]. Autophagy/Beclin 1 regulator 1 (Ambra1) is an essential protein upstream of the autophagic pathway; it is also an important factor in the execution of apoptosis [6–12]. *In vivo*, functional deficiency of Ambra1 causes excessive apoptotic cell death in mouse embryos [6]. *In vitro*, the down-regulation of Ambra1 enhances the sensitivity of cells to apoptotic stimuli in several cell lines, and its overexpression inhibits apoptosis [11, 12]. Therefore, Ambra1 may protect cells from apoptosis. The relationship between Ambra1 and autophagy and apoptosis has been comprehensively reviewed [13–16]. In fact, the dynamic interaction with anti-apoptotic protein Bcl2 is an important mechanism through which Ambra1 is thought to regulate autophagy and apoptosis [10]. In addition, in the process of apoptosis, Ambra1 is degraded by apoptotic proteins, which destroys its autophagic function and promotes apoptosis [11]. However, the C-terminal fragment of Ambra1, which is formed by cleavage by caspases, plays a pro-apoptotic role by inhibiting Bcl2 [17]. Therefore, the mechanism through which Ambra1 regulates apoptosis is complex and has not yet been fully elucidated. Moreover, whether Ambra1 is involved in PTX-induced apoptosis of breast cancer cells is also not clear.

In this work, we explored the role of Ambra1 in PTX-induced apoptosis in MDA-MB-231, SK-Br-3 and MCF-7 breast cancer cells, as well as the relationship between Ambra1 and Bim, a pro-apoptotic protein belonging to the BH3-only group of Bcl-2 family members [18–21], in regulating apoptosis. We found that down-regulation of Ambra1 activates the mitochondrial apoptosis pathway by increasing the expression of Bim, thereby enhancing PTX-induced apoptosis in breast cancer cells. To summarize, we propose a model in which Ambra1 modulates PTX-induced apoptosis of breast cancer cells by regulating the Bim/mitochondrial pathway. In addition, our findings suggest that Ambra1 may be a potential target for treatment of breast cancer.

Materials and methods

Cell lines. MCF-7, MDA-MB-231 and SK-Br-3 cells were obtained from the cell bank of Chinese Academy of Sciences (Shanghai, China). MCF-7 and SK-Br-3 cells were cultured in MEM media (Thermo Fisher, Waltham, MA, USA) and MDA-MB-231 cells were cultured in L15 media (Thermo Fisher, Waltham, MA, USA), supplemented with 10% fetal calf serum (Thermo Fisher, Waltham, MA, USA),

100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified incubator with 5% CO_2. Before the study, the cells were passaged for 6 generations. The identity of the cell lines was determined by short tandem repeats (STR) profiling.

Assay kits and antibodies. PTX was from Hospira Australia Pty Ltd. (Victoria, Australia). The annexin V-FITC/PI apoptosis assay kit was from Nanjing KeyGen Biotech. Co., Ltd. (Nanjing, China). The caspase-9 activity assay kit and cell counting kit-8 (CCK-8) were from the Beyotime Institute of Biotechnology (Shanghai, China). Anti-Ambra1 antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Anti-GAPDH antibody was from MultiSciences (Lianke) Biotech Co., Ltd. (Shanghai, China). Anti-Bim and anti-cytochrome c (cyt c) antibodies were from CST (Danvers, USA).

Cell viability assay. For the cell viability assay, cells were seeded at 8×10^3 cells per well in 96-well flat-bottomed plates and were allowed to attach overnight at 37 °C. Afterwards, medium containing the assay agents was added to each well and cells were further cultured at 37 °C for the indicated times. The number of viable cells was estimated by CCK-8 assay. The absorbance was measured at 450 nm with a microplate reader.

Apoptosis assay. For the apoptosis assay, cells were seeded at 5×10^5 cells in 12.5 cm^2 tissue culture flasks and were treated as described for the CCK-8 assay for the indicated times. Afterward, cells were trypsinized at the indicated time and dyed with annexin V-FITC and PI according to the manufacturer's instructions. Then, apoptotic cells were detected with a flow cytometer.

Caspase-9 activity assay. Cells were collected after treatment with the assay agents for the indicated time, and 30 μl of lysis buffer was added to the collected cells. The cells were resuspended in the lysis buffer and incubated on ice with light agitation for 30 min. Lysates were centrifuged at 13 000 rpm for 5 min; 10 μl of supernatant was used to assay the protein concentration with Bradford reagent, and another 10 μl was used to assay caspase-9 activity. The activity of caspase-9 was assayed with Ac-LEHD-pNA as a substrate; the samples were incubated at 37 °C for 2 h, and OD values were detected at 405 nm with a microplate reader.

Western blotting. For western blotting analyses, cells were seeded in 25-cm^2 tissue culture flasks and were allowed to reach approximately 80% confluency in fresh medium before treatment with the agents. After treatment, detached and attached cells were collected by centrifugation, and whole-cell lysates were obtained using a lysis buffer (1×PBS pH 7.6, 1% NP-40, 0.1% sodium dodecyl sulfate and 0.5% sodium deoxycholate supplemented with inhibitor cocktails). Approximately 30–50 μg of total protein from each group was electrophoretically separated on 12 or 15% SDS-PAGE gels and electrotransferred to polyvinylidene fluoride membranes (PVDF membranes, Pierce). The PVDF membranes were blocked with 5% nonfat dry milk in Tris-buffered saline-Tween 20 (TBST, pH 7.6) for 1 h at room temperature, incubated with the primary antibodies diluted in 5% nonfat dry milk in TBST with light agitation overnight at 4 °C, washed with TBST three times, and incubated with the secondary antibodies diluted in 5% nonfat dry milk in TBST with light agitation for 1 h at room temperature; the proteins were then detected with electrochemiluminescence (Bio-Rad, California, USA).

Lentiviral vector and siRNA construction and transfection. A lentiviral vector-*AMBRA1* transfected with full-length human *AMBRA1* cDNA (abbreviated as OE) and an empty vector (Empty, as a control) were constructed by Genechem (Shanghai, China).Two target-specific *AMBRA1* siRNAs (si #1 and si #2), a target-specific *BIM* siRNA and control scrambled siRNA were synthesized by GenePharma (Shanghai, China). The sequence of the si #1 was GCT GGA ATC TTC CCT CAT TTC, the sequence of si #2 was GGA GAC ATG TCA GTA TCA ACT, and the sequence of si-*BIM* was GAC CGA GAA GGU AGA CAA UUG (21). The siRNAs were transfected into cells by Lipofectamine™ 2000 (Invitrogen, California, USA); the transfection was performed according to the instructions of the manufacturer.

Colony-formation assay. Cells were seeded at 8×10^2 cells per well in a 6-well plate and cultured for 16 days. Cells were washed twice with PBS and fixed in 4% paraformaldehyde for 30 min. Then, the cells were washed once with PBS. Colonies were stained with GIMSA for 20 min, washed twice with ddH2O, allowed to dry, and the number of colonies was counted.

Real-time Quantitative PCR (qRT-PCR). RNA was extracted by using Trizol reagent (Generay Biotech (Shanghai) Co., Ltd., Shanghai, China) as instructed by the supplier. cDNA synthesis was generated using a reverse transcription kit (Vazyme Biotech Co., Ltd., Shanghai, China) according to the manufacturer's recommendations. cDNA from cell samples was amplified by qRT-PCR with specific primers for *AMBRA1* (upper: TGGGGAGGTTAGGATTTGGGA, lower: GAGCCGTAGGGTGGAAAGC), *BIM* (upper: CAGACAGGAGCCCAGCACC, lower: TCCAATACGCCGCAACTCTT) and *GAPDH* (upper: TGACTTCAACAGCGACACCCA, lower: CACCCTGTTGCTGTAGCCAAA) with the ChamQ SYBR Color qPCR Master Mix (Vazyme Biotech Co., Ltd., Shanghai, China). The primers were synthesized by Shanghai Sunny Biotechnology (Shanghai, China) Data were normalized to *GAPDH* expression.

Statistical analysis. Statistical comparisons of the mean values were performed by the Student 2-tailed t test. Data are shown as the means ± SD of three independent experiments. Values of $p<0.05$ were considered statistically significant.

Results

Down-regulation of Ambra1 enhances PTX-induced apoptosis in breast cancer cells. To investigate the role of Ambra1 in PTX-induced apoptosis in MDA-MB-231,

SK-Br-3, and MCF-7 breast cancer cells, we examined the effect of PTX on Ambra1 expression. The cells were treated with 10.0 μM PTX for 24 h. Then, the mRNA and protein of Ambra1 were detected by qRT-PCR and western blotting, respectively. It was revealed that Ambra1 mRNA and protein increased after treatment with PTX (Figure 1A, *p<0.05). Next, we knocked down Ambra1 using two target-specific *AMBRA1* siRNAs (si #1 and si #2). An irrelevant siRNA (scramble) was used as a control. Si #1, si #2, and scrambled siRNA were transfected into the cells for 72 h. Si #1 and si #2 significantly reduced the expression of Ambra1 in both protein and mRNA (Figure 1B, *p<0.05). Upon Ambra1 being knocked down, the cells were treated with 10.0 μM PTX for an additional 24 h. Then, cell viability, caspase-9 activity

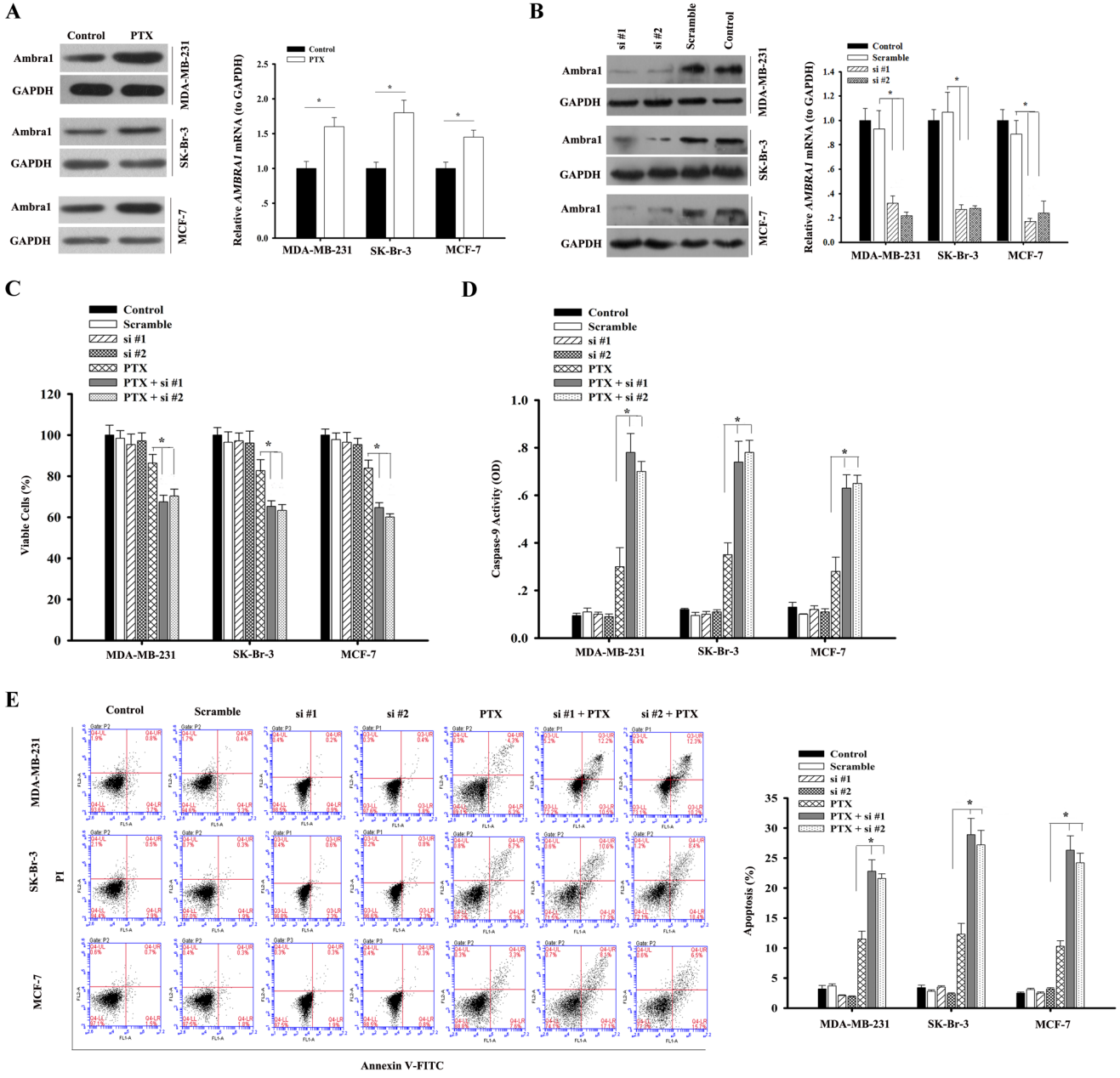

Figure 1. Knockdown of Ambra1 increases PTX-induced apoptosis in breast cancer cells. A) MCF-7, MDA-MB-231 and SK-Br-3 cells were treated by 10.0 μM PTX for 24 h; then Ambra1 protein and mRNA were tested by western blotting and qRT-PCR, respectively. B) The cells were treated by target-specific AMBRA1 siRNAs (si #1 or si #2) and scrambled siRNA for 72 h, and then Ambra1 was detected by western blotting. At the same time, AMBRA1 mRNA was examined by qRT-PCR. C) Cells were incubated with scrambled siRNA or si #1 or si #2 for 72 h, followed by treatment with 10.0 μM PTX for another 24 h; then cell viability was analyzed by CCK-8. D) Caspase-9 activity was assayed. E) Apoptosis was determined using annexin V-FITC/PI and flow cytometry. All the results (means ± SD) come from three independent experiments (*p<0.05).

and apoptosis were analyzed. The treatment of si #1 or si #2 plus PTX resulted in a significant decrease in cell viability, whereas caspase-9 activity and apoptosis increased significantly (Figures 1C–E, *p<0.05). Cell viability was further confirmed by colony-formation assay (Figure 2, *p<0.05). Therefore, Ambra1 reduced PTX-induced apoptosis in breast cancer cells, and its down-regulation enhanced intrinsic apoptosis by activating caspase-9.

Ambra1 negatively regulates Bim expression. To explore the potential mechanism by which Ambra1 regulates PTX-induced apoptosis, we analyzed the effect of Ambra1 on Bim expression. Bim (also known as BCL2L11) belongs to the BH3-only Bcl2 family and is an essential protein for mitochondrial apoptosis [18–20]. First, MDA-MB-231, SK-Br-3, and MCF-7 cells were treated with si #1, si #2, or scrambled siRNA for 72 h. Next, the mRNA of *BIM* was tested by qRT-PCR. As expected, knockdown of *AMBRA1* resulted in a significant increase in *BIM* mRNA levels (Figure 3A, *p<0.05). Correspondingly, Bim protein was also elevated, as determined by western blotting (Figure 3B). Thus, knockdown of Ambra1 promoted the expression of Bim in breast cancer cells. To further probe the effect of Ambra1 on Bim expression, a lentiviral vector-*AMBRA1* transfected with full-length human *AMBRA1* cDNA (abbreviated as OE) and an empty vector (Empty, as a control) were constructed to overexpress Ambra1 in MDA-MB-231 cells. After transfection with LV-*AMBRA1* for 72 h, the expression of Ambra1 was significantly increased in both mRNA and protein (Supplementary Figure 1A, *p<0.05). At the same time, the overexpression of Ambra1 led to a significant decrease in the expression of Bim (Supplementary Figure 1B, *p<0.05). Therefore, Ambra1 negatively regulates the expression of Bim in breast cancer cells.

To determine whether Bim had a regulatory effect on Ambra1 expression, we knocked down Bim by si-*BIM*. *BIM* mRNA and protein were significantly decreased after

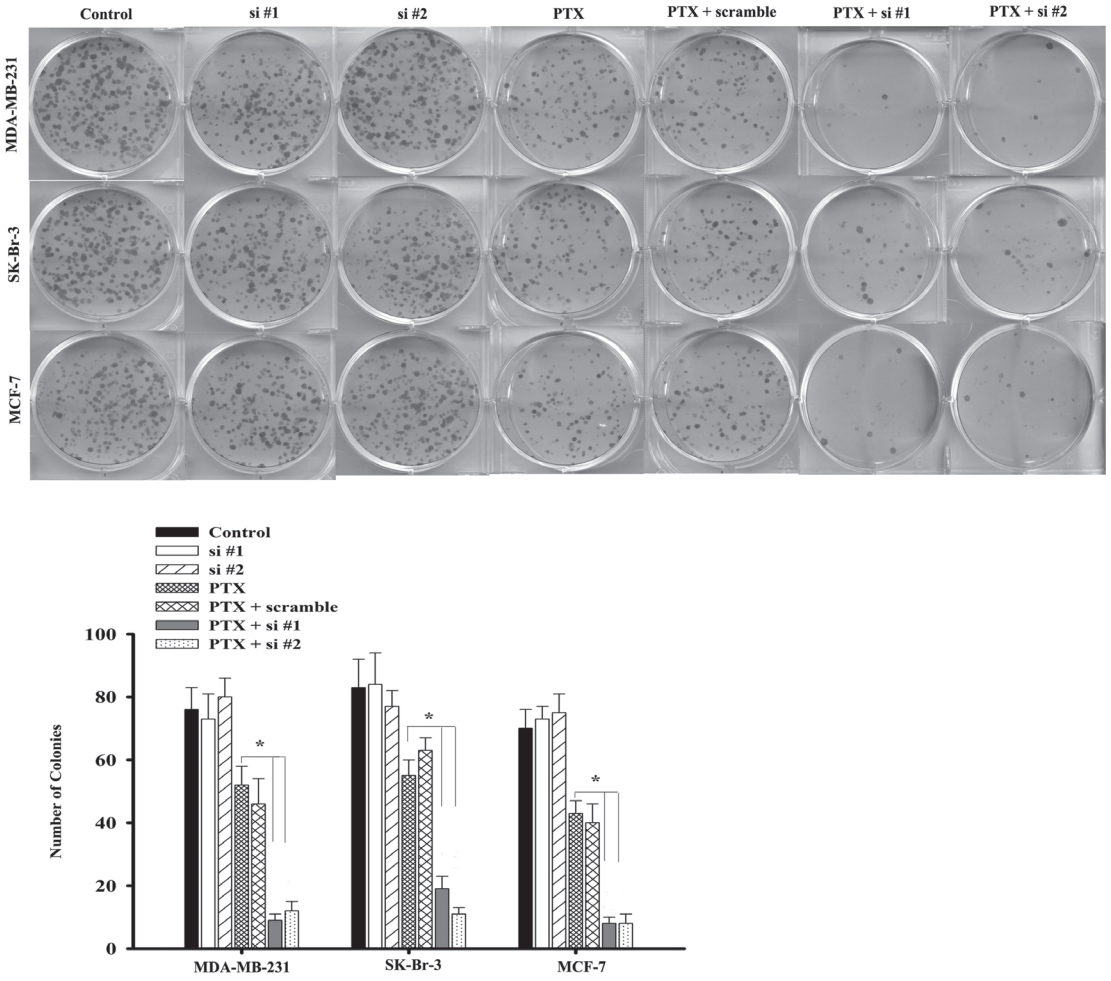

Figure 2. Knockdown of Ambra1 inhibits colony formation in breast cancer cells. MCF-7, MDA-MB-231 and SK-Br-3 cells were treated by target-specific *AMBRA1* siRNAs (si #1 or si #2) and scrambled siRNA for 72 h, followed by treatment with 10.0 μM PTX for another 24 h. Then, cells were seeded in 6-well plate and cultured for 16 days, and the number of colonies was counted. All the data (means ± SD) are from three independent experiments (*p<0.05).

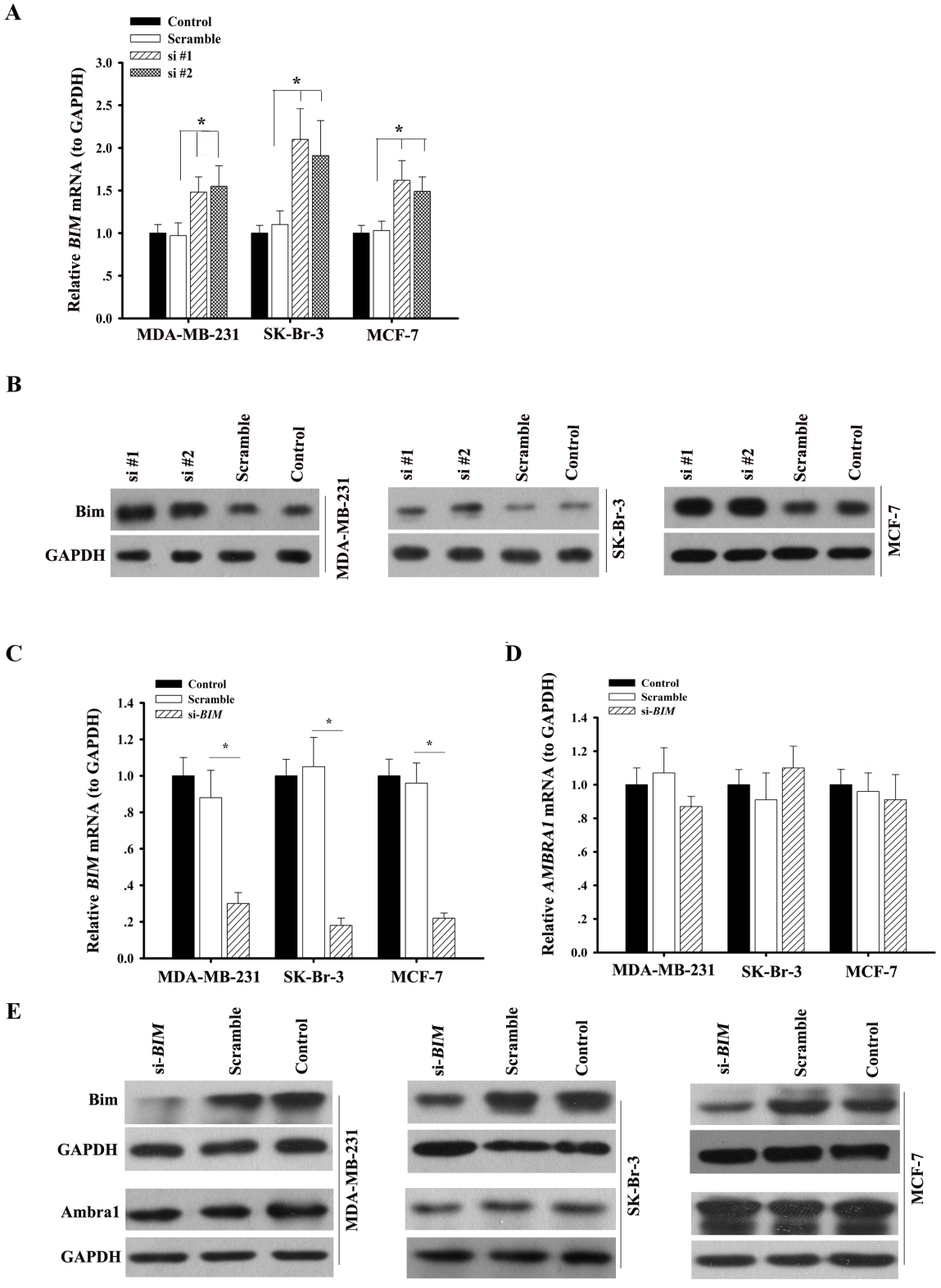

Figure 3. Ambra1 negatively regulates Bim expression. Cells were transfected with scrambled siRNA or si #1 or si #2 for 72 h, the mRNA (A) and protein (B) of Bim were tested by qRT-PCR and western blotting respectively. C) Cells were incubated with target-specific *BIM* si-*BIM* and scrambled siRNA for 72 h, and then the mRNA of *BIM* was examined by qRT-PCR; *AMBRA1* mRNA was detected concurrently (D). Then, the proteins of Bim and Ambra1 were analyzed by western blotting (E). All the data (means ± SD) come from three independent experiments (*p<0.05).

transfection with si-*BIM* for 72 h (Figure 3C and upper panel of 3E, *p<0.05). However, there was no significant change in the mRNA and protein of Ambra1 (Figure 3D and lower panel of 3E, **p>0.05). Therefore, there was no mutual regulation between Ambra1 and Bim.

PTX induces intrinsic apoptosis through the Bim/mitochondrial pathway. To confirm the role of Bim in PTX-induced apoptosis of MDA-MB-231, SK-Br-3 and MCF-7 breast cancer cells, we knocked down Bim by si-*BIM*. Then, the cells were treated with 10.0 μM PTX for another 24 h. Subsequently, cytochrome c (cyt c) release was detected by western blotting. The release of cyt c is an indicator of mitochondrial membrane potential (MMP) damage and is required for caspase-9 activation and intrinsic apoptosis [22, 23]. The results showed that the knockdown of *BIM* inhibited cyt c release induced by PTX treatment (Figure 4A). At the same time, the caspase-9 activity and apoptosis caused by PTX treatment also decreased significantly (Figures 4B and 4C, *p<0.05). Thus, PTX induces apoptosis through the Bim/mitochondrial pathway in breast cancer cells.

Bim is required for Ambra1 to regulate PTX-induced apoptosis. To confirm whether Bim was involved in the regulation of apoptosis by Ambra1, we knocked down Bim and Ambra1 concurrently. MDA-MB-231 cells were incubated with scrambled siRNA, si #1, si-*BIM* or si #1 combined with si-*BIM* for 72 h. The combination of si#1 and si-*BIM* reduced the mRNA and protein levels of both Ambra1 and Bim simultaneously (Figures 5A and 5B, *p<0.05). Next, the cells that were transfected with scrambled siRNA, si #1, si-*BIM*, or si #1 combined with si-*BIM* were treated with PTX for additional 24 h, and cyt c release was tested by western blotting. The increase of cyt c release caused by PTX or PTX plus si #1 was inhibited by si-*BIM* with or without si #1 (Figure 5C). In addition, si-*BIM* alone or in combination with si #1 had a similar effect on cyt c release (Figure 5C). In fact, the similar effect also occurred in the regulation of caspase-9 activity and apoptosis by si-*BIM* alone or in combination with si #1 (Figure 5D, *p<0.05). Therefore, Bim is required for Ambra1 to regulate PTX-induced apoptosis in breast cancer cells.

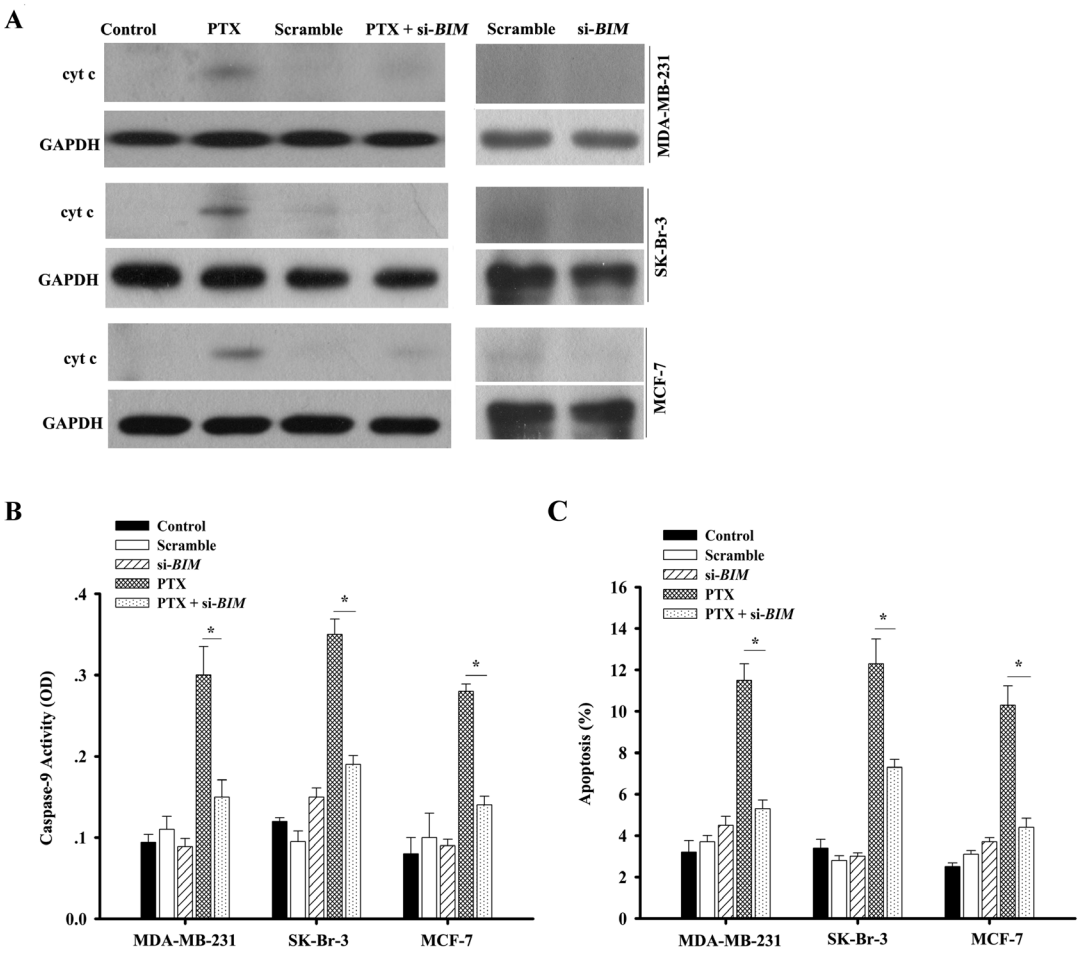

Figure 4. PTX induces intrinsic apoptosis via the Bim/mitochondrial pathway. A) Cells were incubated with scrambled siRNA or si-*BIM* for 72 h, followed by treatment with 10.0 μM PTX for another 24 h; then cyt c was detected by western blotting. B) Caspase-9 activity and C) apoptosis were assayed respectively. All the data (means ± SD) are from three independent experiments (*p<0.05).

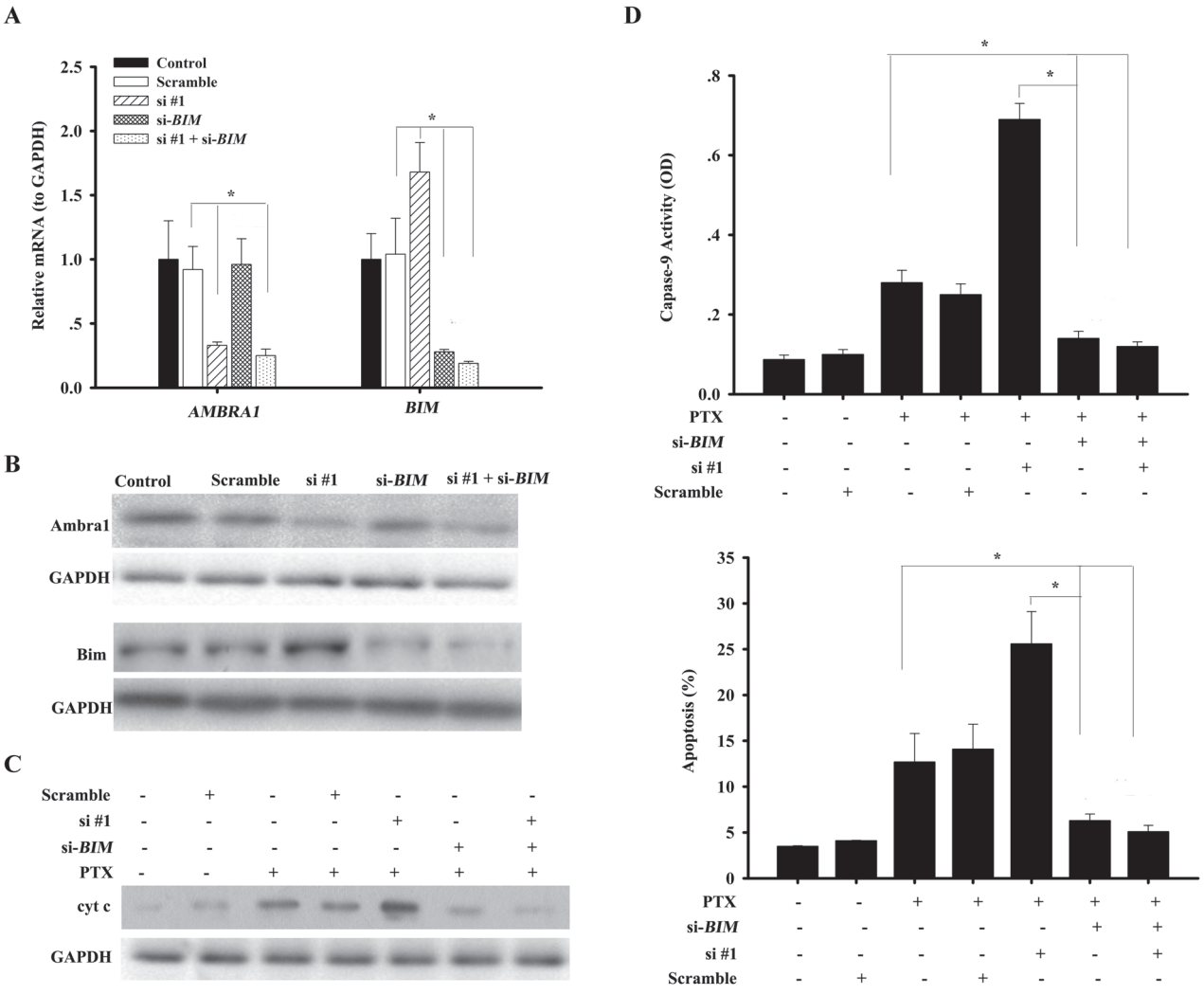

Figure 5. Bim is required for Ambra1 to regulate PTX-induced apoptosis. A) MDA-MB-231 cells were transfected with scrambled siRNA, si #1, si-*BIM* or si #1 combined with si-*BIM* for 72 h; the mRNA of *BIM* and *AMBRA1* were tested by qRT-PCR, and B) the proteins of Bim and Ambra1 were detected by western blotting. C) Cells were incubated with scrambled siRNA, si #1, si-*BIM*, or si #1 combined with si-*BIM* for 72 h, followed by treatment with 10.0 μM PTX for another 24 h. Then cyt c was detected by western blotting, and D) caspase-9 activity and apoptosis were assayed respectively. All the data (means ± SD) are from three independent experiments (*p<0.05).

Discussion

Our results indicate that Ambra1 inhibits PTX-induced apoptosis in breast cancer cells and that Bim is a crucial protein in this process. Based on our results, we propose a model in which Ambra1 modifies PTX-induced apoptosis of breast cancer cells by modulating the Bim/mitochondrial pathway.

As mentioned above, Ambra1, a key factor in the autophagic pathway, is also closely related to apoptosis. Indeed, there is a complex crosstalk between autophagy and apoptosis [24]. Usually, numerous autophagic proteins are involved in apoptosis regulation and vice versa. Ambra1 is one of the key proteins in this crosstalk [15]. It regulates autophagy and apoptosis mainly through dynamic binding to Bcl2 [10]. Normally, Ambra1 preferentially binds to the mitochondria-resident Bcl2 to block autophagy. The mitochondria-resident Bcl2 exerts an anti-apoptotic function [25]. Upon the autophagy initiation, Ambra1 is released from Bcl-2 and combines with Beclin1 to promote autophagy [6]. Interestingly, the combination of Ambra1 and mitochondria-resident Bcl2 is also disturbed by apoptosis [10]. Thus, Ambra1 has a dual function both in autophagy and cell death control. Pagliarini et al. has demonstrated that Ambra1 is rapidly degraded by caspases and calpains during apoptosis, which destroys its pro-autophagic function and promotes apoptosis [11]. Therefore, Ambra1 is a protective protein. In line with the above results, we found that down-regulation of Ambra1

enhanced PTX-induced intrinsic apoptosis by activating caspase-9 in breast cancer cells. Thus, Ambra1 makes the cells resistant to PTX-induced apoptosis. However, Strappazzon et al. found that the C-terminal part of Ambra1, generated by caspases-mediated cleavage, acts as a positive mediator for mitochondrial apoptosis by inactivating the anti-apoptotic factor Bcl2 [17]. This suggests that the effect of Ambra1 on apoptosis depends on whether the full-length or cleaved form of the protein is present.

Up to now, the mechanism by which Ambra1 regulates apoptosis has not been fully clarified. Bim belongs to the pro-apoptotic group of the Bcl2 family and is a key protein in mitochondrial apoptosis [18–20]. Thus, we decided to explore the relationship between Ambra1 and Bim in PTX-induced apoptosis. Our results show that down-regulation of Ambra1 brought about an increase in Bim expression, which was parallel to the increase of PTX-induced apoptosis; whereas up-regulation of Ambra1 expression had opposite effect. In turn, Bim did not significantly modify Ambra1 expression. Therefore, Ambra1 negatively regulates Bim expression in breast cancer cells. In fact, Bim was required for PTX to induce mitochondrial apoptosis in breast cancer cells, because its down-regulation inhibited release of the cyt c, caspase-9 activity and apoptosis caused by the drug. Furthermore, the down-regulation of Bim also inhibited increased PTX-induced apoptosis caused by Ambra1 knock-down. A similar effect was achieved when Ambra1 and Bim were knocked-down simultaneously. Therefore, Bim is required for Ambra1 to regulate PTX-induced apoptosis in breast cancer cells.

In summary, Ambra1 is involved in the regulation of PTX-induced apoptosis in breast cancer cells. In this process, Bim and the mitochondrial apoptotic pathway are crucial effectors of Ambra1. Therefore, we propose a novel model of Ambra1 in the regulation of PTX-induced apoptosis in breast cancer cells. In addition, our results also indicate that Ambra1 may be a potential target for the treatment of breast cancer.

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

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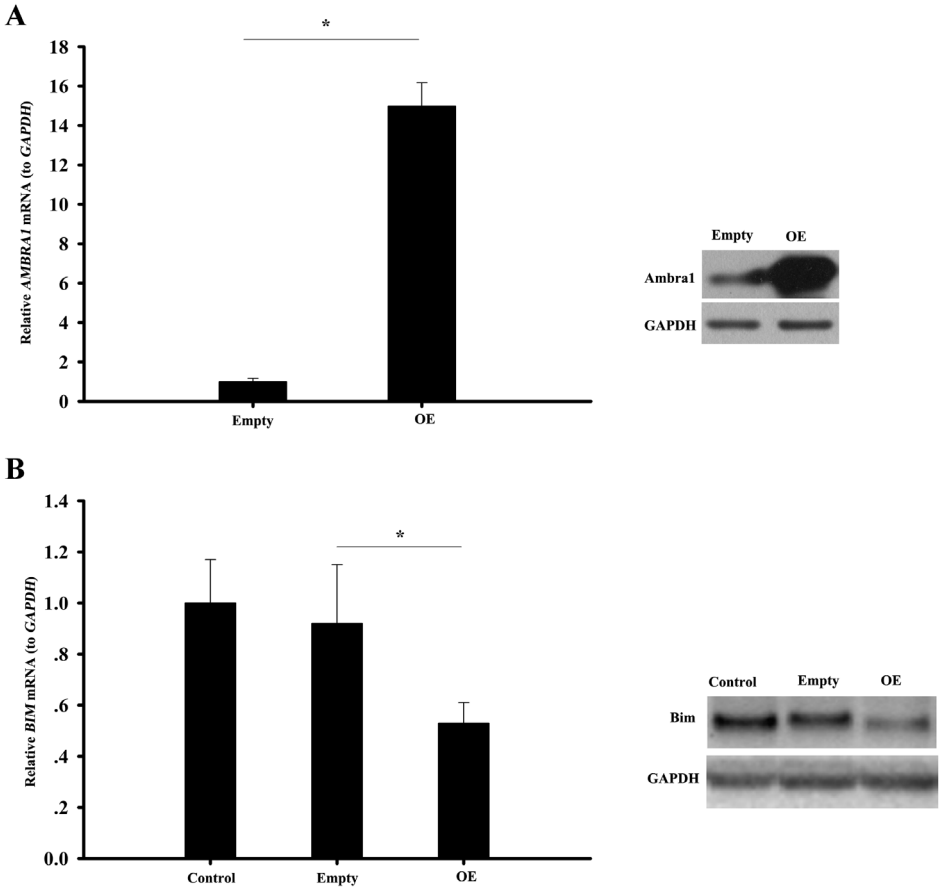
Supplemental material



Supplementary Figure 1. Overexpression of Ambra1 inhibited Bim expression in MDA-MB-231 cells. A) MDA-MB-231 cells were treated by the lentiviral vector-*AMBRA1* transfected with full-length human *AMBRA1* cDNA (abbreviated as OE) and an empty vector (Empty, as a control) for 72 h; and then the mRNA of *AMBRA1* was tested by qRT-PCR, and the protein of Ambra1 was detected by western blotting. B) Cells were incubated with OE or Empty for 72 h; the mRNA and protein of Bim were tested by qRT-PCR and western blotting respectively. All the data (means ± SD) are from three independent experiments (*p<0.05).