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Estrogen Receptor β Isoform 5 Confers Sensitivity of Breast Cancer Cell Lines to Chemotherapeutic Agent–Induced Apoptosis through Interaction with Bcl2L12[1,2]

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Abstract

Alternative splicing of estrogen receptor β (ERβ) yields five isoforms, but their functions remain elusive. ERβ isoform 5 (ERβ5) has been positively correlated with better prognosis and longer survival of patients with breast cancer (BCa) in various clinical studies. In this study, we investigated the inhibitory role of ERβ5 in BCa cells. Although ERβ5 does not reduce proliferation of BCa cell lines MCF-7 and MDA-MB-231, its ectopic expression significantly decreases their survival by sensitizing them to doxorubicin- or cisplatin-induced apoptosis through the intrinsic apoptotic pathway. Moreover, we discovered Bcl2L12, which belongs to the Bcl-2 family regulating apoptosis, to be a specific interacting partner of ERβ5, but not ERβ1 or ERα, in an estradiol-independent manner. Knockdown of Bcl2L12 enhanced doxorubicin- or cisplatin-induced apoptosis, and this process was further promoted by ectopic expression of ERβ5. Whereas Bcl2L12 was previously shown to inhibit apoptosis through binding to caspase 7, such interaction is reduced in the presence of ERβ5, suggesting a mechanism by which ERβ5 sensitizes cells to apoptosis. In conclusion, ERβ5 interacts with Bcl2L12 and functions in a novel estrogen-independent molecular pathway that promotes chemotherapeutic agent–induced *in vitro* apoptosis of BCa cell lines.

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Introduction

Breast cancer (BCa) is the leading cause of cancer-related death in women worldwide. Estrogen receptors (ERs) are one of the most important biomarkers for the prediction of prognosis and response to therapy among patients with BCa [1]. Hormonal therapy through estrogen depletion or with selective ER modulators is widely used to block the action of estrogen on its receptors and to induce cell death. Nonetheless, this therapy can be applied only in patients with estrogen-sensitive BCa [2]. Even worse, some patients with advanced BCa eventually are unresponsive to selective ER modulators [3,4] and require chemotherapy as second-line treatment, with its severe adverse effects, especially at high dosage [5,6].

In contrast to ERα, which has a proliferative action in BCa, ERβ has been found during the last few years to be protective. Although ERα is generally known to promote BCa tumorigenesis [7,8], ERβ was found to antagonize ERα by negating ERα activity [9]. A decrease in ERβ expression during the progression of BCa suggests that ERβ is antiproliferative and suppresses carcinogenesis [10–12]. ERβ also can inhibit the survival of BCa cells by promoting apoptosis and enhancing the efficacy of apoptotic chemotherapeutic agents [13–16]. For example, ERβ expression triggers the activation of p53 through

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phosphorylation and enhances apoptosis [17,18]. A genome-wide study showed that ERβ downregulates antiapoptotic factors in either the absence or presence of estradiol (E2) [19]. Its expression also sensitizes BCa cells to doxorubicin and cisplatin [20,21], an effect independent of ligand. Moreover, various studies showed that ERβ agonists confer resistance of BCa cells to chemotherapeutic agents [22–24], suggesting that ERβ may enhance the chemosensitivity of cells in a ligand-independent manner.

Alternative splicing of *ESR2* gene produces ERβ1 (or wild-type ERβ) and its four isoforms, including ERβ isoform 2 (ERβ2) to ERβ5, which possess unique amino acid sequences at their carboxyl (C) terminus [9]. Although ~90% of their sequences are identical with that of ERβ1, their binding to estrogen is either weak (ERβ4 and ERβ5) or absent (ERβ2) [25]. Our previous study demonstrated that the activation function 2 (AF-2) domain at C termini is responsible for their estrogen independence [25]. Therefore, these isoforms are considered to be transcriptionally inactive but capable of modulating ERβ1- or ERα-mediated transcription when heterodimerized with them [26,27]. ERβ5 expression, similar to that of ERβ1, was shown to be protective in patients with BCa [28,29] and may inhibit tumor growth [30]. Other studies reported a positive association of ERβ5 expression with a longer relapse-free survival (RFS) [31] and a significant correlation of its nuclear expression with overall survival (OS) [29], suggesting that ERβ5 expression may be a powerful prognostic marker for BCa. Thus, we are interested in clarifying its functions in BCa.

Our current study revealed the role and molecular mechanism of ERβ5 in apoptosis of BCa cells. To investigate functions of ERβ5, we performed yeast two-hybrid screening and isolated *Bcl2L12*, which is a Bcl-2 family member, and found that Bcl2L12 interacts specifically with ERβ5 but not with other ER subtypes. Bcl2L12 has opposing roles in apoptosis in different cancers [32,33]. Therefore, we also determined the effect of Bcl2L12 expression on BCa cell lines treated with apoptosis-inducing drugs. Moreover, we investigated the relationship between ERβ5-mediated sensitization of cells to apoptosis and the interaction of ERβ5 with Bcl2L12 and found that ERβ5 confers sensitivity of BCa cells to apoptosis-inducing chemotherapeutic agents through direct interaction with Bcl2L12.

Materials and Methods

Cell Cultures and Reagents

MCF-7, MDA-MB-231, 293T, and 293FT cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained according to the manufacturer's protocols. Stably expressed cell lines were also supplemented with blasticidin (10 μg/ml; Life Technologies, Carlsbad, CA).

Plasmids were transfected into 293T or 293FT cells by Lipofectamine 2000 (Life Technologies). DharmaFECT 1 was used as the small interfering RNA (siRNA) transfection reagent (Thermo Scientific Dharmacon, Florence, KY) for MCF-7 and MDA-MB-231. Procedures of transfection were those recommended by the manufacturer. The chemotherapeutic agents doxorubicin hydrochloride and cisplatin [*cis*-diamminedichloroplatinum(II)] were purchased from Sigma-Aldrich (St Louis, MO).

Plasmids and siRNAs

Full-length *ERβ1*, *ERβ5*, *ERα*, and *Bcl2L12* were cloned into pcDNA-HisMax (Life Technologies). The siRNA oligonucleotides specific to *Bcl2L12*, siL12-1 (AAGCUGGUCCGCCUGUCCU), and siL12-2 (UGGUGGAGCUGUUCUGUAG) were used for the knockdown of *Bcl2L12* (Thermo Scientific Dharmacon). The sequences were based on the published data of Stegh et al. [34]. ON-TARGET*plus* nontargeting siRNA (siNT) was used as the negative control (Thermo Scientific Dharmacon).

Antibodies

Rabbit polyclonal anti-ERβ (H-150) and goat polyclonal anti–caspase 7 (N-17) were purchased from Santa Cruz Biotechnology (Dallas, TX). Mouse monoclonal anti-His (THE His) was purchased from GenScript (Piscataway, NJ). Mouse monoclonal anti-ERβ (14C8) was purchased from Abcam (Cambridge, MA). Rabbit anticleaved poly (ADP-ribose) polymerase (PARP), anticleaved caspase 3, anticleaved caspase 7, anticleaved caspase 8, and anti–caspase 9 were purchased from Cell Signaling Technology (Danvers, MA). EZview anti-HA affinity gel was purchased from Sigma-Aldrich. Two custom rabbit polyclonal anti-Bcl2L12 (anti–L12-1 and anti–L12-2) were kindly provided by Dr Alexander H. Stegh at Northwestern University (Evanston, IL). All control IgGs were purchased from Santa Cruz Biotechnology.

Construction of ERβ5 Stably Expressed Cell Lines

Procedures of constructing stably expressed cell lines have been described previously [35]. In brief, full-length *ERβ5* or *LacZ* (negative control), respectively, was subcloned into pLenti6 lentiviral vector by Multisite Gateway Cloning (Life Technologies) and then transfected into 293FT cells for production of lentivirus. The titer of each lentivirus was measured, and the multiplicity of the infection of MCF-7 and MDA-MB-231 cells was determined. Lentivirus-infected MCF-7 and MDA-MB-231 cells were selected with blasticidin (10 μg/ml) for 3 weeks. Stable expression of ERβ5 or LacZ was confirmed by quantitative reverse transcription–polymerase chain reaction (RT-PCR), Western blot analysis, and β-galactosidase assay.

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted with TRIzol reagent (Life Technologies), and cDNA was synthesized with SMART MMLV reverse transcriptase with poly d(T) primer (Promega, Fitchburg, WI). All the procedures followed the manufacturer's instructions. Quantitative RT-PCR reactions were performed with ABI7900 real-time PCR system (Life Technologies). Intron-spanning primers of *ERβ5* (forward—5′-CGGAAGCTGGCTCACTTGCT-3′ and reverse—5′-CTTCACCCTCCGTGGAGCAC-3′) and *Bcl2L12* (forward—5′-CCTGTTCCAACTCCACCTAGAA-3′ and reverse—5′-GACTCAGAGGGGGCTGCT-3′) were used. The primers of *Bcl2L12* were designed specifically for amplification of the sequence in the wild type of *Bcl2L12* but not in the truncated form, *Bcl2L12A*.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium Cell Proliferation Assay

MCF-7 and MDA-MB-231 were seeded in 96-well plates at 3×10^3. After 24 hours, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed to normalize the number of cells in each well. The CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega), following the manufacturer's protocol, was used for the experiment. The reading was taken at 24, 72, and 120 hours for cell proliferation experiments or after 24 and 48 hours of drug treatments for cell viability experiments.

Treatment with Apoptosis-Inducing Chemotherapeutic Agents

Cells were seeded on 6-well plates at 3.3×10^5 (MCF-7) or 1.8×10^5 (MDA-MB-231). They were stably transfected with expression plasmids and/or transiently transfected with siRNAs as described. Medium was added with or without doxorubicin or cisplatin at different concentrations after 24 hours. The adherent and nonadherent cells were harvested and lysed with M-PER lysis buffer (Thermo Scientific Pierce, Florence, KY) containing complete EDTA-free protease inhibitor cocktail (Calbiochem, Billerica, MA). Equal amounts of total protein lysates were used in Western blot analysis. Different antibodies to apoptotic markers (cleaved PARP, cleaved caspase 3, cleaved caspase 7, cleaved caspase 8, and cleaved caspase 9) and other relevant antibodies were used as stated. Target proteins were detected with IRDye secondary antibody, and signals were obtained with the Odyssey Infrared Imaging System (Li-Cor Biosciences, St Lincoln, NE). Relative band intensities were measured by ImageJ analysis (National Center for Biotechnology Information, Bethesda, MD).

Staining with Annexin V and 7-Aminoactinomycin D

MCF-7 and MDA-MB-231 cells were seeded on 12-well plates at 2×10^5 (MCF-7) or 1×10^5 (MDA-MB-231). The medium was added, with doxorubicin or cisplatin at different concentrations, for 18 hours. The adherent and nonadherent cells were collected. Cells were washed with phosphate-buffered saline, resuspended in the binding buffer, and then incubated with annexin V–Alexa Fluor 488 (Life Technologies) and 7-aminoactinomycin D (7-AAD; BD Biosciences, San Jose, CA) for 10 minutes in the dark. FACS analysis was performed with FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). Data analysis was done by CellQuest Pro version 5.2 (BD Biosciences).

Yeast Two-Hybrid Screening

Human prostate MATCHMAKER cDNA library (Clontech Laboratories, Mountain View, CA) was used as prey library for screening. C-terminal ERβ was cloned into the bait vector pGBKT7 (Clontech Laboratories). The screening procedures followed the manufacturer's protocol. In brief, the bait vector and prey library were transformed to yeast strain AH109 and Y187, respectively. Yeast mating was performed at 30°C at a low shaking speed. Clones were selected on quadruple nutrient dropout agar (QDO; Clontech, Mountain View, CA; SD/–Ade-His-Leu-Trp). Positive clones were isolated, and the presence of coding sequences was confirmed by PCR screening. The interaction was confirmed by cotransforming bait and prey plasmids into yeast strain Y187 using the polyethylene glycol–lithium acetate method of the Yeastmaker Yeast Transformation System (Clontech Laboratories). Full-length *ERβ1*, *ERβ5*, and *ERα* were subcloned into pGBKT7 vector as baits in the cotransformation assay. Transformed cells were grown on QDO agar with X-α-galactosidase for 4 days until blue yeast colonies appeared.

In Vitro *Coimmunoprecipitation*

The yeast plasmid containing the partial sequence of *Bcl2L12* was extracted. T7 promoter and hemagglutinin (HA) tag were added to the N terminus of the coding sequence by the PCR reaction. Plasmids of full-length *ERβ1*, *ERβ5*, and *ERα* and PCR products containing *Bcl2L12* were respectively translated *in vitro* by the TNT T7-reticulocyte system (Promega) labeled with EasyTag EXPRESS ^{35}S Protein Labeling Mix (PerkinElmer, Hebron, KY). *In vitro*–translated bait and prey proteins were incubated together in the absence or presence of E2 at 4°C for 1 hour. Lysates were then immunoprecipitated with 20 μl anti-HA affinity gel (Sigma-Aldrich) at 4°C overnight. The samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Dried gels were exposed to X-ray films for 72 hours with intensifying screen for signal enhancement (Kodak, Rochester, NY). The films were scanned with the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Mammalian Coimmunoprecipitation

The 293T cells were seeded on 60-mm plates at 3×10^6 in charcoal-stripped serum medium and transfected with different plasmids for 24 hours; the culture medium was added with DMSO (as vehicle control) or 10 nM E2 as indicated. MCF-7 cells grown in full-serum medium were used in the coimmunoprecipitation (co-IP) experiment. Cells were lysed with IP Lysis Buffer (Thermo Scientific Pierce) containing protease inhibitor. One milligram of lysate was immunoprecipitated with 2 μg of the appropriate antibodies at 4°C overnight and then with protein G Dynabeads (Life Technologies). Control IgG was used as the negative control. The samples were subjected to Western blot analysis. IRDye secondary antibodies were used to detect protein bands, and the Odyssey Infrared Imaging System (Li-Cor Biosciences) was used to obtain the signals.

Immunofluorescence Staining

The 293T cells were seeded on round coverslips and transfected with *ERβ5* and *Bcl2L12*; MCF-7–ERβ5 cells were also seeded on coverslips without any transfection. Cells were fixed in 10% formalin at room temperature for 20 minutes and permeabilized with 1% NP-40. Normal chicken serum (10%) was used for blocking. Mouse ERβ (14C8) and rabbit Bcl2L12 antibodies (anti–L12-2) were incubated with the cells at room temperature for 1 hour. Different fluorescent secondary antibodies were then incubated with the cells for 1 hour in the dark. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Prolong Gold antifade reagent (Life Technologies) was used for enhancing signals. The fluorescent images were obtained by an Axiovert 200M fluorescent microscope equipped with an AxioCam MRm camera and AxioVision 4.8 software (Carl Zeiss, Oberkochen, Germany).

Statistical Analysis

Student's *t* test was used for statistical analysis (GraphPad Software, La Jolla, CA). All *P* values were two-sided, and $P < .05$ was considered to be statistically significant.

Results

Ectopic Expression of ERβ5 in BCa Cell Lines Does Not Alter Cell Proliferation

To investigate the underlying mechanisms and functions of ERβ5 in BCa, we constructed BCa cell lines expressing either ERβ5 or LacZ. Two BCa cell lines, MCF-7 and MDA-MB-231, were chosen because of their different ERα and p53 status; MCF-7 is ERα and p53 positive, whereas MDA-MB-231 is ERα negative and possesses a mutant form of p53 [36,37]. The mRNA and protein expression were measured after the construction of ERβ5 stably expressed MCF-7 (Figure 1*A*) and MDA-MB-231 (Figure 1*B*). Unlike the antiproliferative role of ERβ1 in BCa [38], ectopic expression of ERβ5 did not alter cell proliferation of either cell line (Figure 1, *C* and *D*), suggesting that ERβ5 has no significant effect on cell proliferation in BCa cells.

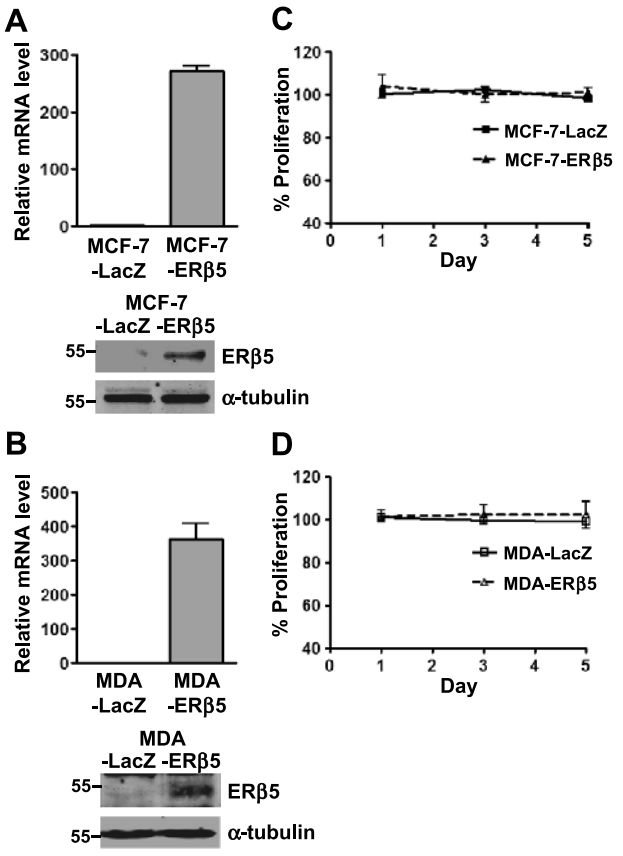

Figure 1. Ectopic expression of ERβ5 in BCa cell lines does not alter the rate of cell proliferation. (A and B) The mRNA and protein expression of ERβ5 were determined in ERβ5 or LacZ stably expressed MCF-7 (A) and MDA-MB-231 (B) cell lines by quantitative RT–PCR and Western blot analysis, respectively. *Glyceraldehyde 3-phosphate dehydrogenase* (*GADPH*) was used as the housekeeping gene in quantitative RT-PCR, whereas α-tubulin served as the loading control in Western blot analysis. The cell lines were stably transfected with either an *ERβ5* or *LacZ* expression plasmid. Results are the average of two independent experiments. Data are represented as means ± SD. (C and D) Ectopic expression of ERβ5 does not alter the proliferation rate of BCa cells. The proliferation rates of MCF-7 (C) and MDA-MB-231 (D) were measured by MTS assay. Equal numbers of cells (3×10^3) were seeded on 96-well plates. The data were recorded after days 1, 3, and 5 and represented as the percentage of cell proliferation relative to LacZ stably expressed cells on day 1. The results are the average of three independent experiments; each was performed in triplicate. Data are represented as means ± SD.

ERβ5 Functions in the Intrinsic Apoptotic Pathway and Sensitizes BCa Cell Lines to Doxorubicin-Induced Apoptosis

ERβ5 expression significantly correlates with better RFS and OS of patients with BCa [29,31]. Although ERβ5 expression does not alter the proliferation of BCa cells, it may determine cell survival through other molecular pathways, such as apoptosis. Doxorubicin induces apoptosis and is a standard chemotherapy for patients with BCa [39], whereas its high dosage increases the risk of cardiotoxicity and other adverse effects [40]. To determine whether the presence of ERβ5 can sensitize cells to doxorubicin treatment, we treated ERβ5 or LacZ stably expressed MCF-7 (MCF-7–ERβ5/–LacZ) with different concentrations of doxorubicin for 12 hours (data not shown) or 24 hours (Figure 2*A*). With doxorubicin treatment, MCF-7–ERβ5 showed an increase, compared with the LacZ control, in the expression of cleaved caspase 9 but not cleaved caspase 8, indicating activation of the intrinsic pathway. The expression of cleaved PARP and cleaved caspase 7, which are the late apoptotic markers, also increased (Figure 2*A*). To further determine ERβ5-mediated sensitization, we confirmed the apoptotic status of cells using annexin V/7-AAD FACS analysis. Again, we found an increase in apoptosis of MCF-7–ERβ5 cells with doxorubicin treatment compared with the LacZ control (Figure 2*B*). Moreover, after doxorubicin treatment, survival of MCF-7–ERβ5 cells was significantly lower than that of MCF-7–LacZ, as measured by the MTS assay (Figure 2*C*). Results were similar in ERβ5 or LacZ stably expressed MDA-MB-231 (MDA-ERβ5/–LacZ; Figure W1, *A–C*). Thus, we infer that ERβ5 sensitizes BCa cells to doxorubicin-induced apoptosis through an intrinsic apoptotic pathway.

ERβ5 Sensitizes BCa Cell Lines to Cisplatin-Induced Apoptosis

ERβ5 was shown to confer chemosensitivity of BCa cells to doxorubicin-induced apoptosis. Because cisplatin induces apoptosis through a mechanism distinct from that with doxorubicin [41,42] and is effective against triple-negative BCa [43–45], we next examined whether ERβ5 can improve the efficacy of cisplatin in BCa cells. Experiments were similar to those done for doxorubicin in BCa cell lines. MCF-7–ERβ5 showed greater sensitivity to cisplatin treatment than the LacZ control, as reflected by enhanced activation of PARP, caspase 9, and caspase 7 (Figure 3*A*) and a greater percentage of apoptotic cells in FACS analysis (Figure 3*B*). Cell survival was significantly decreased with cisplatin treatment in MCF-7–ERβ5 as measured by the MTS assay (Figure 3*C*). Results were similar in MDA-MB-231 ectopically expressed with ERβ5 (Figure W2, *A–C*). These findings, taken together, indicate that ERβ5 enhances the chemosensitivity of MCF-7 and MDA-MB-231 to cisplatin-induced apoptosis.

Bcl2L12 Specifically Interacts with ERβ5 in an E2-Independent Manner

To identify novel ERβ5-mediated molecular pathways, we employed yeast two-hybrid screening using ERβ as bait to isolate its interacting partners in a human total cDNA library. We isolated a clone that encodes amino acids 14 to 273 of human Bcl2L12. To eliminate a false-positive interaction and to determine whether Bcl2L12 interacts with other ERs, we performed yeast cotransformation of Bcl2L12 with full-length ERβ5, ERβ1, or ERα. Bcl2L12 showed a strong interaction with ERβ5 and a weak interaction with ERα but did not interact with ERβ1 (Figure W3*A*). The physical binding between the proteins was further verified by *in vitro* co-IP. ERβ5 interacted with Bcl2L12 (Figure W3*B*, *lane 2*), whereas ERα (Figure W3*B*, *lanes 6* and *7*) and ERβ1 (Figure W3*B*, *lanes 9* and *10*) could not be coimmunoprecipitated with Bcl2L12 in the absence or presence of E2. Next, we confirmed the interaction in mammalian cells. Full-length sequence of *Bcl2L12* was cloned from MCF-7. Consistent with the results in two-hybrid assays and *in vitro* co-IP, Bcl2L12 was coimmunoprecipitated with ERβ5 independent of E2 in 293T cells (Figure 4*A*, *lanes 3* and *5*) but not with ERβ1 or ERα (data not shown). The interaction was further confirmed in MCF-7–ERβ5 (Figure 4*B*). Moreover, immunofluorescence staining showed that ERβ5 and Bcl2L12 were localized in the same subcellular compartments. Colocalization was observed in both nucleus and cytoplasm of 293T (Figure W3*C*) and MCF-7–ERβ5 cells (Figure W3*D*). In conclusion, we determined that ERβ5, but not ERα or ERβ1, interacts with Bcl2L12 in an E2-independent manner.

Knockdown of Bcl2L12 Sensitizes BCa Cells to Doxorubicin and Cisplatin

Bcl2L12 acts as a proapoptotic or antiapoptotic factor in different cancers [33,46]. To explore the functions of Bcl2L12 in BCa, we used the gene knockdown approach, because Bcl2L12 is highly expressed in MCF-7 and MDA-MB-231 (Figure W4*A*). Moreover, ectopic expression of ERβ5 did not alter the expression of Bcl2L12 (Figure W4, *B* and *C*). Two *Bcl2L12* siRNAs (siL12-1 and siL12-2), validated by Stegh et al. [34], significantly decreased its mRNA and protein levels in MCF-7 (Figure W4, *D* and *E*) and MDA-MB-231 (Figure W4, *F* and *G*). PARP, caspase 9, caspase 7, and caspase 3 were moderately activated in the absence of apoptotic drugs after knockdown of *Bcl2L12* by either siL12-1 (Figure 5*B*, *lanes 4* and *10*) or siL12-2 (Figure W5*B*, *lanes 3* and *7*) in MDA-MB-231.

With doxorubicin treatment, depletion of Bcl2L12 by siL12-1 in MCF-7 increased the activation of PARP, caspase 9, and caspase 7 (Figure 5*A*, *lanes 5* and *11*). Although activation of caspase 7 and caspase 3 was enhanced with similar treatment in MDA-MB-231 (Figure 5*B*, *lanes 5* and *11*), cleavage of PARP and caspase 9 was similar with and without the knockdown by siL12-1 (Figure 5*B*, *lanes 2*, *5*, *8*, and *11*). MCF-7 and MDA-MB-231 with ectopic expression of ERβ5 and knockdown of *Bcl2L12* showed the highest expression of apoptotic markers (Figure 5, *A* and *B*, *lane 11*). The inhibitory effect of Bcl2L12 on doxorubicin-induced apoptosis was confirmed by its depletion with the use of its second siRNA, siL12-2 (Figure W5, *A* and *B*, *lanes 4* and *8*). The suppression of cisplatin-induced apoptosis by Bcl2L12 also was determined. Knockdown of *Bcl2L12* did not facilitate the activation of apoptotic markers in MCF-7–LacZ/–ERβ5 (Figure 5*A*, *lanes 6* and *12*). However, down-regulation of Bcl2L12 expression increased the level of cleaved caspase 9, cleaved caspase 7, and cleaved caspase 3 but not cleaved PARP in MDA-LacZ/–ERβ5 (Figure 5*B*, *lanes 6* and *12*). Next, FACS analysis was performed to confirm the inhibitory role of Bcl2L12 in drug-induced apoptosis. The percentage of apoptotic cells in both cell lines significantly increased on the knockdown of *Bcl2L12*, followed by treatment with doxorubicin or cisplatin (Figure 5, *C* and *D*). Consistent with the results of Western blot analysis, ectopic expression of ERβ5, together with Bcl2L12 depletion, resulted in the highest percentage of apoptotic cells (Figure 5, *C* and *D*, *lower right panel*). Our results not only reveal that Bcl2L12 confers chemoresistance to doxorubicin- or cisplatin-induced apoptosis in BCa cells but also imply that ERβ5 and Bcl2L12 play opposing roles in the apoptosis of BCa cells.

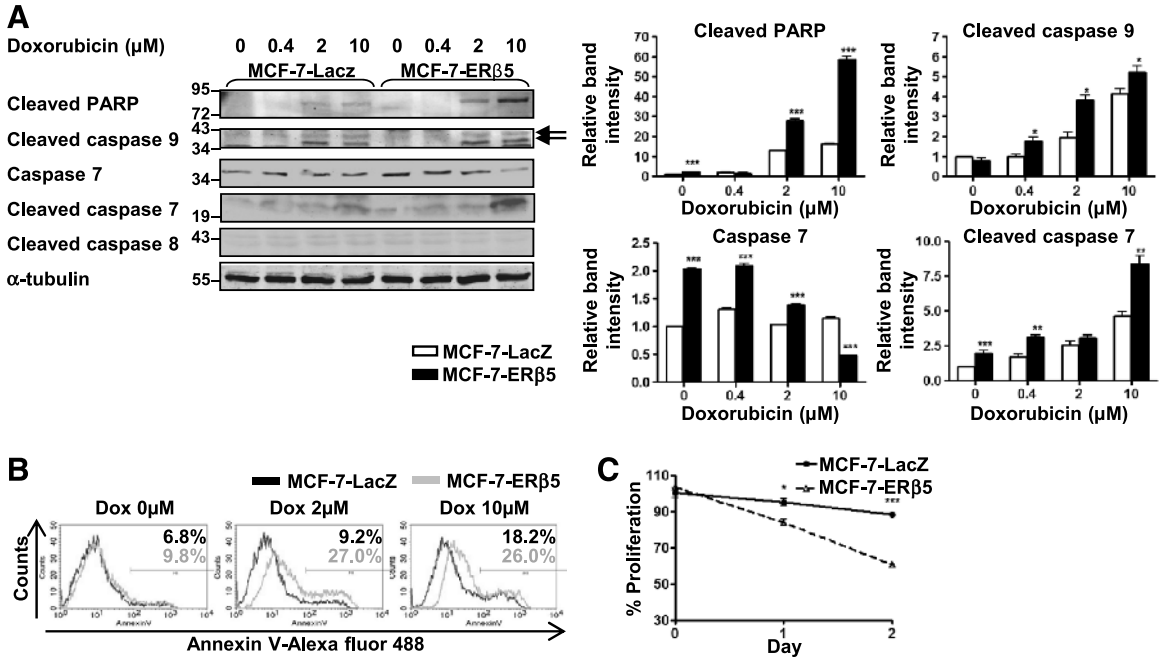

Figure 2. Ectopic ERβ5 sensitizes MCF-7 to doxorubicin-induced apoptosis. (A) ERβ5 and LacZ stably expressed MCF-7 (MCF-7–ERβ5 and MCF-7–LacZ) were treated with different concentrations of doxorubicin (0, 0.4, 2, and 10 μM) for 24 hours. Whole-cell lysates were extracted for Western blot analysis with antibodies as indicated. Band intensities were measured and normalized to the intensity of α-tubulin. The relative band intensities were compared with that of LacZ stably expressed cells without the treatment. Results of densitometric analysis are the average of three measurements in a representative experiment. Data are presented as means ± SD. The statistical significance of the difference in densitometric analysis between MCF-7–ERβ5 and MCF-7–LacZ at the same concentration of doxorubicin is shown as $*P < .05$, $**P < .01$, and $***P < .001$. (B) Annexin V/7-AAD staining assays were performed in ERβ5 (gray) and LacZ (black) stably expressed MCF-7 cells. Cells were incubated with different concentrations of doxorubicin (0, 2, and 10 μM) for 18 hours. The percentage of annexin V–positive (apoptotic) cells was determined by FACS. Three independent experiments were performed. The results shown are from a representative experiment. (C) Cell viability of MCF-7 stably expressed cell lines was measured by MTS assay. Equal numbers of cells (3×10^3) were seeded on 96-well plates. Cells were incubated with 2 μM doxorubicin after 24 hours. The data were recorded on the first and second days after drug treatment and represented as the percentage of cell viability relative to that of untreated cells. Results are the average of three independent experiments; each was performed in triplicate. Data are represented as means ± SD. The statistical significance of the difference in cell viability between ERβ5 and LacZ stably expressed cells is shown as $*P < .05$ and $***P < .001$.

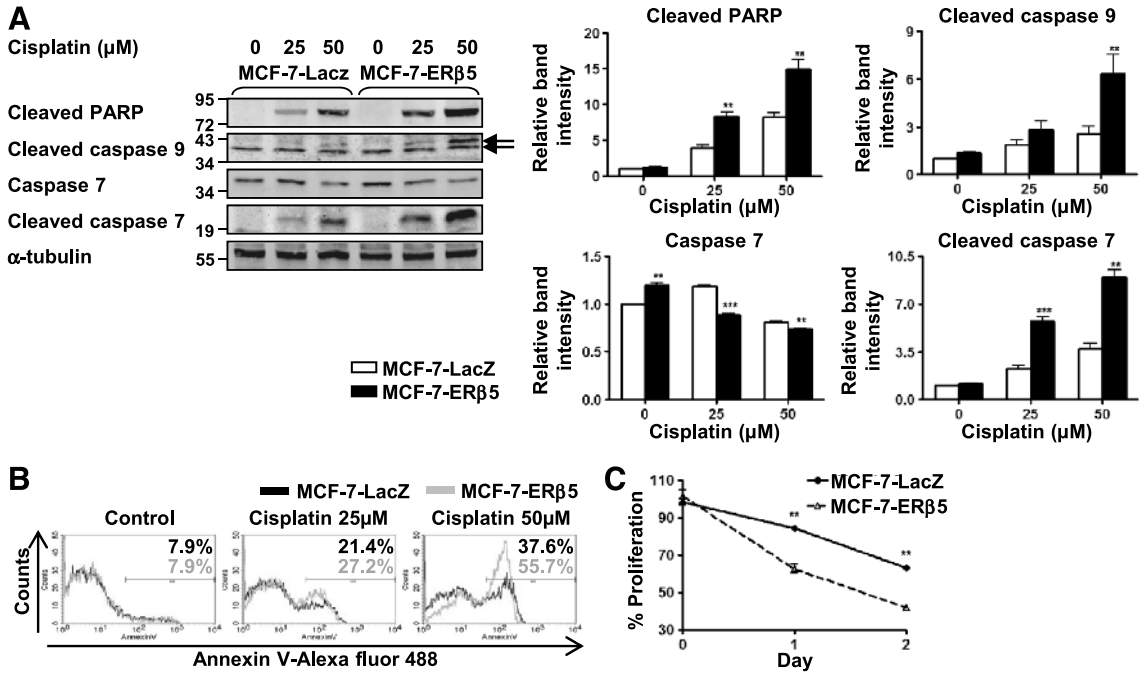

Figure 3. Ectopic ERβ5 sensitizes MCF-7 to cisplatin-induced apoptosis. (A) ERβ5 and LacZ stably expressed MCF-7 were treated with different concentrations of cisplatin (0, 25, and 50 μM) for 24 hours. Whole-cell lysates were extracted for Western blot analysis with antibodies as indicated. The measurement of relative band intensities and analysis of data were similar to those in Figure 2*A*. The statistical significance of the difference in densitometric analysis between MCF-7–ERβ5 and MCF-7–LacZ at the same concentration of cisplatin is shown as $*P < .05$, $**P < .01$, and $***P < .001$. (B) Annexin V/7-AAD staining assays were performed in ERβ5 (gray) and LacZ (black) stably expressed MCF-7 cells. Cells were incubated with different concentrations of cisplatin (0, 25, and 50 μM) for 18 hours. Other experimental details and analysis of data were similar to those in Figure 2*B*. (C) The cell viability of MCF-7 stably expressed cell lines was measured by MTS assay. Cells were incubated with 50 μM cisplatin. The procedures of experiment and analysis of data were similar to those described in Figure 2*C*. The statistical significance of the difference in cell viability between ERβ5 and LacZ stably expressed cells is shown as $**P < .01$.

ERβ5 Sequesters Bcl2L12 from Interacting with Caspase 7

In our current study, ERβ5 sensitized BCa cells to apoptosis induced by chemotherapeutic agents, whereas Bcl2L12 inhibited the response to treatment with different drugs. Because Bcl2L12 has been shown to repress apoptosis in glioblastoma by preventing the cleavage of caspase 7 through physical binding [34], we proposed that the interaction between Bcl2L12 and caspase 7 also occurs in BCa and that it may be inhibited by ERβ5. To understand how ERβ5-Bcl2L12 interaction regulates apoptosis in BCa cells, we performed co-IP of Bcl2L12 and caspase 7 in MCF-7. On immunoprecipitation with antibody to Bcl2L12, caspase 7 was coimmunoprecipitated in MCF-7–LacZ, whereas the binding of caspase 7 to Bcl2L12 was drastically reduced in MCF-7–ERβ5 (Figure 6). Because ERβ5 interacted with Bcl2L12 in MCF-7 (Figure 4*B*), our data suggest that ERβ5 inhibits the physical binding between Bcl2L12 and caspase 7.

Discussion

ERβ5 has been shown to be positively correlated with survival outcomes such as RFS and OS in patients with BCa [29,31], suggesting that ERβ5 is a good prognostic marker in BCa. In our study,

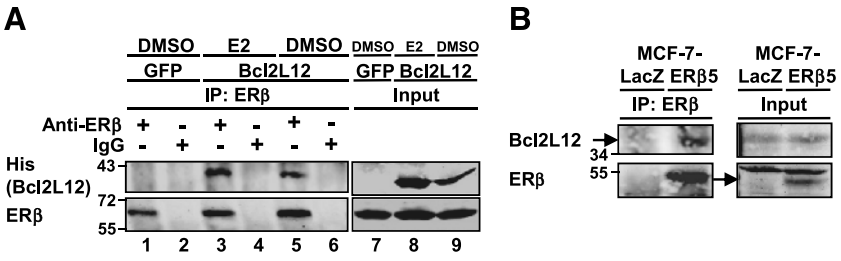

Figure 4. Bcl2L12 specifically interacts with ERβ5 in an E2-independent manner. (A) ERβ5 interacts with Bcl2L12 *in vivo* in an E2-independent manner. The 293T cells were grown in charcoal-stripped serum medium and transfected with ERβ5 and His-tagged Bcl2L12. After 24-hour transfection, DMSO or 10 nM E2 was added. Lysates were immunoprecipitated with ERβ antibody and then immunoblotted with His (Bcl2L12) or ERβ antibody. IgG was used as negative control. (B) ERβ5 interacts with Bcl2L12 in MCF-7. MCF-7–ERβ5 was grown in full-serum medium. Lysates were immunoprecipitated with ERβ antibody and then immunoblotted with Bcl2L12 (L12-1) or ERβ antibody.

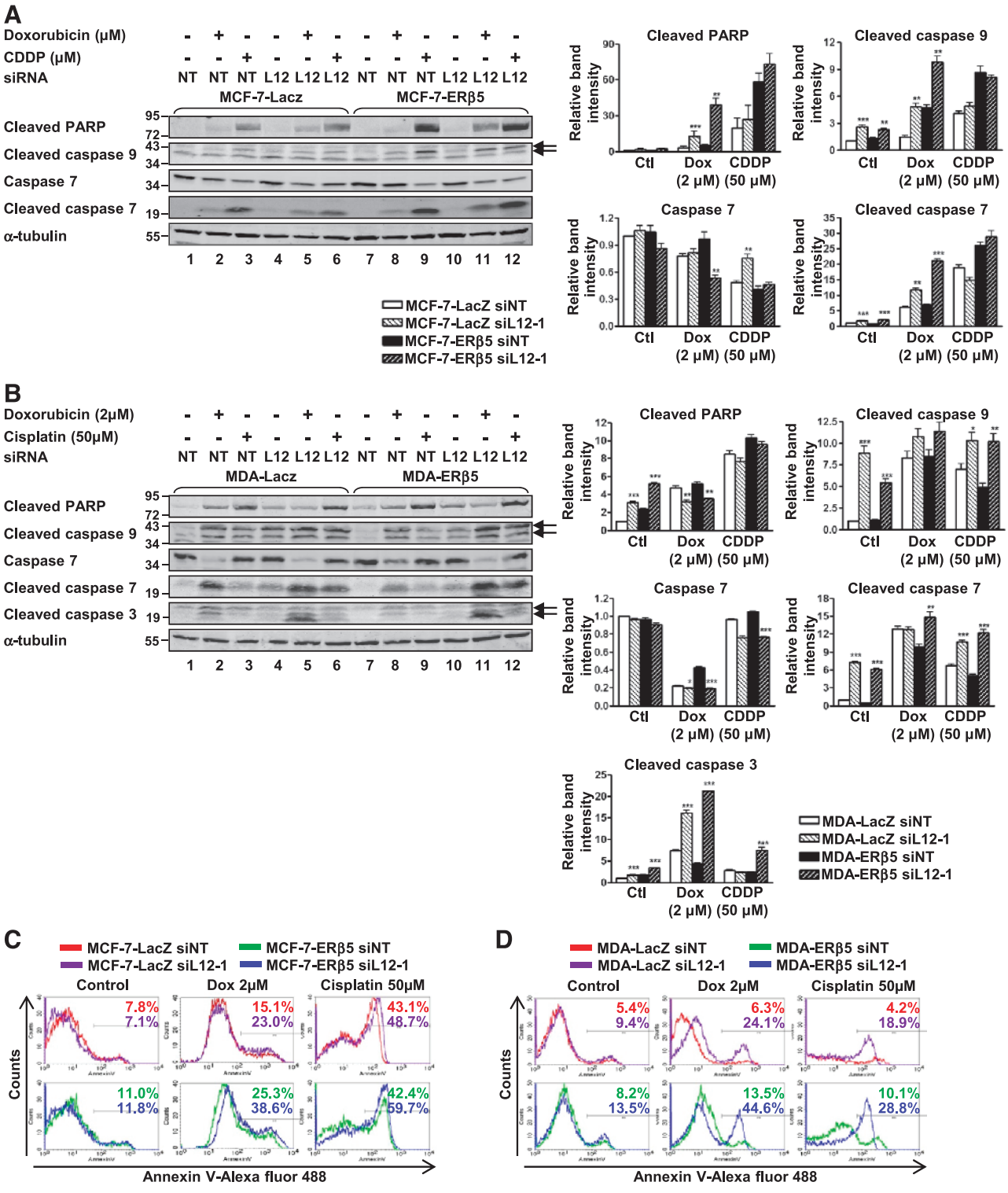

Figure 5. Knockdown of Bcl2L12 sensitizes MCF-7 and MDA-MB-231 to doxorubicin- and cisplatin-induced apoptosis. (A and B) ERβ5 and LacZ stably expressed MCF-7 (A) or MDA-MB-231 (B) were transfected with control siNT or *Bcl2l12* siRNA (siL12-1) twice. The cells were then treated with doxorubicin (2 μM) or cisplatin (50 μM) for 24 hours. Whole-cell lysates were extracted for Western blot analysis with antibodies as indicated. The measurement of relative band intensities and analysis of data were similar to those in Figure 2*A*. Cleavage of caspase 9 gives rise to a doublet band, which is indicated by arrows. The statistical significance of the difference in densitometric analysis between the cells treated with siNT and siL12-1 at the same concentration of drugs is shown as $*P < .05$, $**P < .01$, and $***P < .001$. (C and D) Annexin V/7-AAD staining assays were performed. MCF-7–LacZ (C) or MDA-LacZ (D) transfected with siNT (red) and siL12-1 (purple), as well as MCF-7–ERβ5 (C) or MDA-ERβ5 (D) transfected with siNT (green) or siL12-1 (blue) were used in the assays. Cells were incubated with doxorubicin (2 μM) and cisplatin (50 μM) for 18 hours. Other experimental details and analysis of data were similar to those in Figure 2*B*.

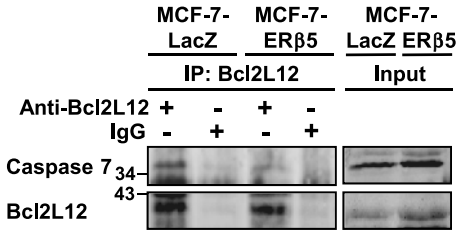

Figure 6. ERβ5 inhibits the interaction between Bcl2L12 and caspase 7. ERβ5 and LacZ stably expressed MCF-7 cells were grown in full-serum medium. Lysates were immunoprecipitated with Bcl2L12 (L12-2) antibody and then immunoblotted with caspase 7 or Bcl2L12 (L12-1) antibody. IgG was used as negative control.

expression of ERβ5, unlike that of ERβ1, had no effect on cell proliferation. Nonetheless, ERβ5 decreased the cell survival by sensitizing the BCa cell lines MCF-7 and MDA-MB-231 to doxorubicin- or cisplatin-induced apoptosis. In contrast, Bcl2L12, which was isolated as an ERβ5-specific interacting partner, conferred chemoresistance of BCa cells to drug-induced apoptosis. We also showed that ERβ5 prevented Bcl2L12 from interacting with caspase 7. Thus, ERβ5 is able to sensitize BCa cells to chemotherapeutic agents through Bcl2L12.

Ectopic expression of ERβ5 sensitized the two BCa cell lines to doxorubicin-induced apoptosis, as shown in Western blot analysis, FACS analysis, and cell viability assays. In addition, on treatment with cisplatin, ERβ5 enhanced apoptosis of MDA-MB-231, as well as MCF-7, which is resistant to the drug [47–49]. Because MCF-7 and MDA-MB-231 possess wild-type and mutant p53, respectively, our data indicate that the ERβ5-mediated apoptotic pathway is independent of p53. Moreover, ERα status did not affect the sensitivity of these cell lines to doxorubicin and cisplatin. Therefore, we suggest that ERβ5-mediated sensitization for chemotherapeutic agent–induced apoptosis is independent of its inhibition of ERα genomic signaling but probably acts through a novel pathway involving the interaction with an apoptotic protein, Bcl2L12.

We performed yeast two-hybrid screening to identify novel binding partners of ERβ5. One of the prey candidates, Bcl2L12, interacted specifically with ERβ5 but not with ERβ1 or ERα in either the absence or presence of E2. ERβ5 has the same N terminus sequence as ERβ1 but a different C-terminal region [25]. The observation is not uncommon for ERs and their interacting proteins. Physical binding between ERs and p160 coactivators requires synergy between N-terminal AF-1 and C-terminal AF-2 domains [50–55]. Moreover, our three-dimensional molecular models showed that the absence of helix 12 and the incomplete helix 11 of ERβ5 constitute a conformation of the C terminus different from that of ERβ1 [25]. Thus, the unique protein conformation of ERβ5 may be responsible for its interaction with Bcl2L12. Collectively; the data reveal that Bcl2L12 interacts specifically with ERβ5, but not ERα or ERβ1, in an E2-independent manner. In addition, this is the first discovery of an interaction between an ER and a Bcl-2 family member.

To determine the significance of the ERβ5-Bcl2L12 interaction for apoptosis, we first clarified the function of Bcl2L12 in BCa cells. Using two validated siRNAs [34], we showed that Bcl2L12 confers chemoresistance of BCa cells to doxorubicin or cisplatin. Moreover, ectopic expression of ERβ5 with Bcl2L12 depletion resulted in the highest degree of apoptosis in the two cell lines, as reflected in Western blot and FACS analyses. Although Bcl2L12 depletion followed by cisplatin treatment of MCF-7 dramatically increased apoptosis in FACS analysis, expression of apoptotic markers did not show a change in Western blot analysis. The discrepancy between the results of the two experiments may be due to differences in the apoptotic markers used in FACS (cell-surfaced phosphatidylserine) and Western blot (cleaved form of different caspases and PARP) analyses. Bcl2L12 plays distinct roles in the apoptosis of different cancers. It is antiapoptotic in glioblastoma [33,56,57] but promoted cisplatin-induced apoptosis in MDA-MB-231 in one study [58]. In our current study, similar to the studies of glioblastoma, we showed that Bcl2L12 inhibits apoptosis. The difference between the findings of our current study and that of a previous study in BCa [58] could be explained in several ways. In our study, the function of Bcl2L12 on doxorubicin- and cisplatin-triggered apoptosis was determined in MCF-7 and MDA-MB-231. Moreover, we do not believe that off-target effects are present because both Stegh et al. [34] and our group (Figure W4, *D*–*G*) validated two separate siRNAs. In addition, different passage numbers of cells and the use of different culture conditions may lead to differential cellular responses. Here, we conclude that Bcl2L12 confers chemoresistance of BCa cells to doxorubicin or cisplatin, whereas ectopic expression of ERβ5 with Bcl2L12 depletion further enhances the apoptosis.

In the presence of chemotherapeutic agents, ERβ5 promoted apoptosis, which was augmented when expression of Bcl2L12 was knocked down, implying that Bcl2L12 inhibits the ERβ5-mediated apoptotic pathway. Suppression of apoptotic signaling by Bcl2L12 has been studied extensively in glioblastoma [34,57,59,60] and found to inhibit the activity of caspase 7 through physical binding [34]. Our study showed that the interaction between Bcl2L12 and caspase 7 is reduced in the presence of ERβ5. This suggests that Bcl2L12 can no longer repress the cleavage of caspase 7 when ERβ5 is expressed, resulting in a mechanism by which ERβ5 sensitizes cells to apoptosis. However, we also observed an increase in the activation of caspase 9 in ERβ5-expressing BCa cells compared with the controls treated with same concentration of drugs (Figures 2*A* and 3*A*; Figures W1*A* and W2*A*). Because caspase 9 functions at an early stage of the intrinsic apoptotic pathway [61], ERβ5 may have additional mechanism(s) of enhancing apoptosis.

In conclusion, our study revealed a novel estrogen-independent molecular pathway of ERβ5 in BCa cells. To our knowledge, we are the first to illustrate that ERβ5 confers sensitivity of BCa cells to chemotherapeutic agent–induced apoptosis through the intrinsic pathway. Moreover, we discovered the estrogen-independent ERβ5-*Bcl2l12* interaction and uncovered the ERβ5-mediated sensitization stem from its inhibition of Bcl2L12–caspase 7 interaction. Our further research will focus on the functions of ERβ5 and Bcl2L12 *in vivo* and their prognostic values in BCa. Because the ERβ5-mediated sensitization occurred in BCa cell lines with different statuses of ERα and p53, ERβ5 may be a new therapeutic target for various types of BCa. Moreover, our study yields valuable information concerning the development of small molecules or peptide mimics targeting the ERβ5-Bcl2L12 interaction to enhance the efficacy of chemotherapeutic agents for patients with advanced BCa.

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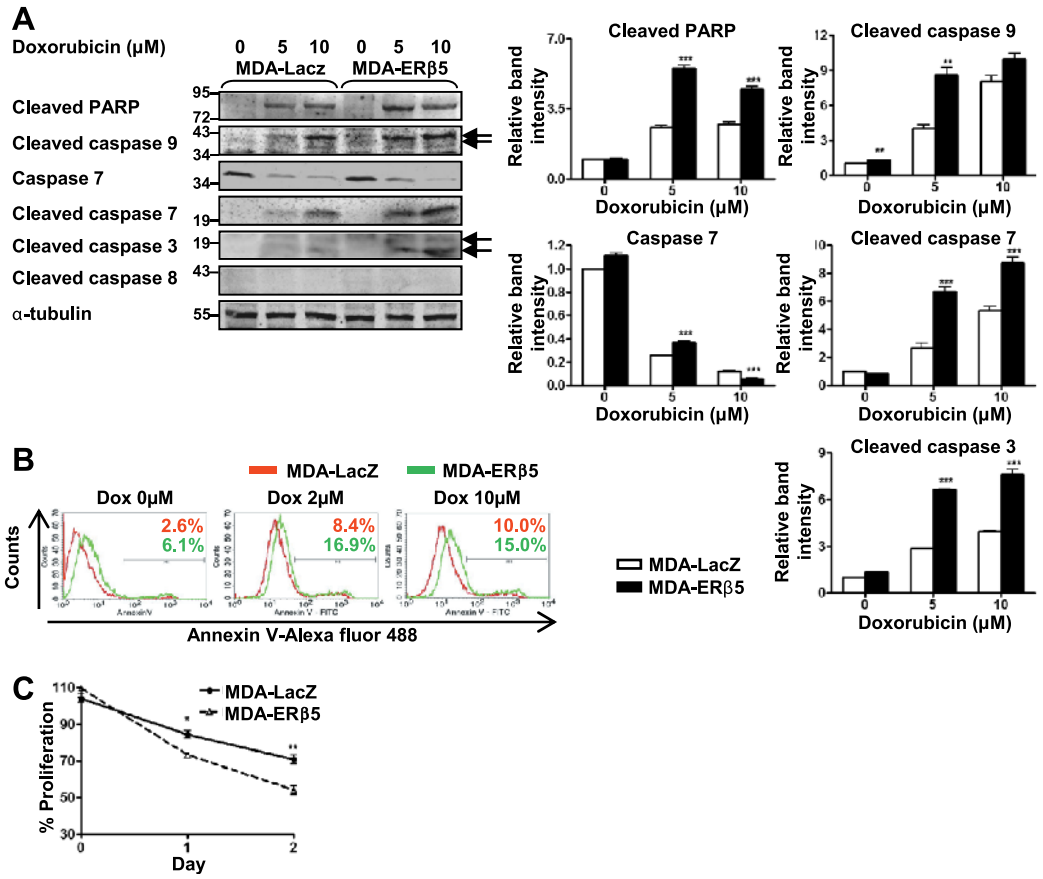

Figure W1. Ectopic ERβ5 sensitizes MDA-MB-231 to doxorubicin-induced apoptosis. (A) ERβ5 and LacZ stably expressed MDA-MB-231 (MDA-ERβ5 and MDA-LacZ) were treated with different concentrations of doxorubicin (0, 5, and 10 μM) for 24 hours. Whole-cell lysates were extracted for Western blot analysis with antibodies as indicated. Band intensities were measured and normalized to the intensity of α-tubulin. The relative band intensities were compared with that of untreated LacZ stably expressed cells. Results are the average of three measurements in a representative experiment. Data are presented as means ± SD. Similar results were obtained in cells incubated with doxorubicin for 12 hours. The statistical significance of the difference in densitometric analysis between MDA-ERβ5 and MDA-LacZ at the same concentration of doxorubicin is shown as $*P < .05$, $**P < .01$, and $***P < .001$. (B) Annexin V/7-AAD staining assays were performed in ERβ5 (green) and LacZ (red) stably expressed MDA-MB-231 cells. Cells were incubated with different concentrations of doxorubicin (0, 5, and 10 μM) for 18 hours. The percentage of annexin V–positive (apoptotic) cells was determined by FACS. Three independent experiments were performed. The results shown are from a representative experiment. (C) The cell viability of MDA-MB-231 stably expressed cell lines was measured by MTS assay. Equal numbers of cells (3×10^3) were seeded on 96-well plates. Cells were incubated with 5 μM doxorubicin after 24 hours. The data were recorded on the first and second days after drug treatment and represented as the percentage of cell viability relative to that of untreated cells. The results are the average of three independent experiments; each was performed in triplicate. Data are represented as means ± SD. The statistical significance of the difference in cell viability between ERβ5 and LacZ stably expressed cells is shown as $*P < .05$.

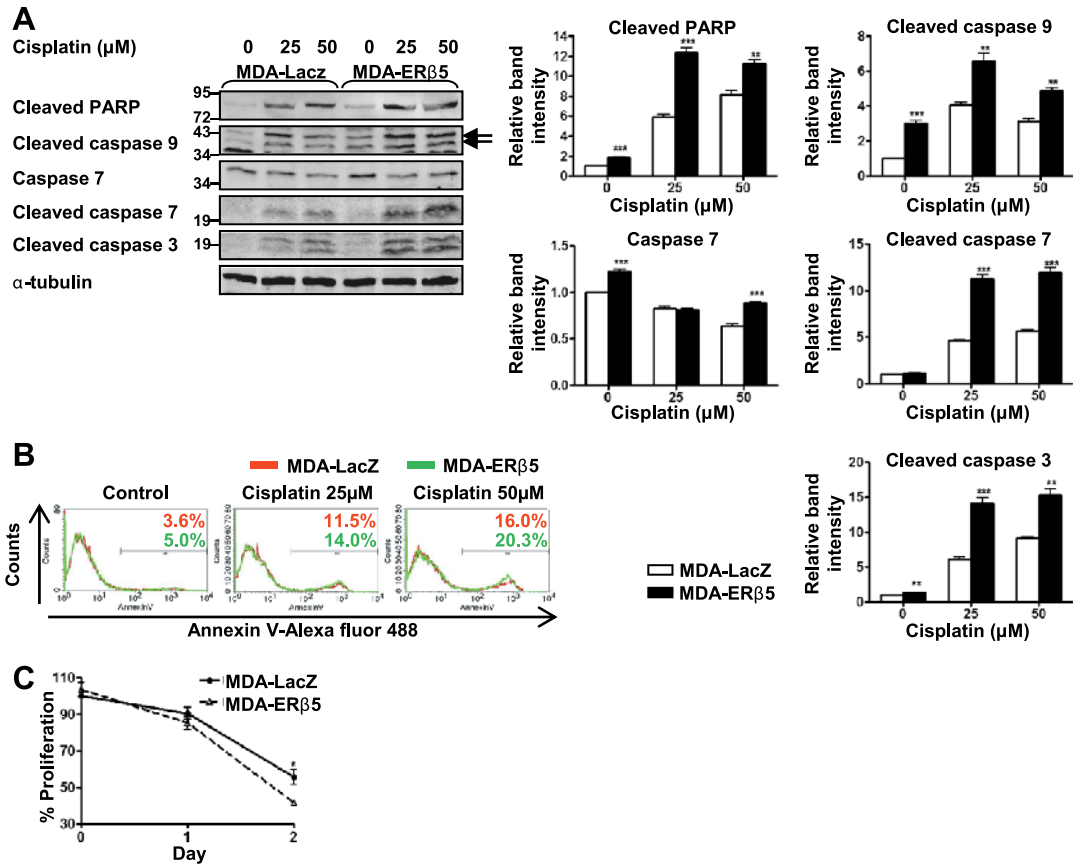

Figure W2. Ectopic ERβ5 sensitizes MDA-MB-231 to cisplatin-induced apoptosis. (A) ERβ5 and LacZ stably expressed MDA-MB-231 were treated with different concentrations of cisplatin (0, 25, and 50 μM) for 24 hours. Whole-cell lysates were extracted for Western blot analysis with antibodies as indicated. The measurement of relative band intensities and analysis of data were similar to those in Figure W1*A*. The statistical significance of the difference in densitometric analysis between MDA-ERβ5 and MDA-LacZ at the same concentration of cisplatin is shown as $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$. (B) Annexin V/7-AAD staining assays were performed in ERβ5 (green) and LacZ (red) stably expressed MDA-MB-231 cells. Cells were incubated with different concentrations of cisplatin (0, 25, and 50 μM) for 18 hours. Other experimental details and analysis of data were similar to those in Figure W1*B*. (C) The cell viability of MDA-MB-231 stably expressed cell lines was measured by MTS assay. Cells were incubated with 50 μM cisplatin. The procedures of experiment and analysis of data were similar to those described in Figure W1*C*. The statistical significance of the difference in cell viability between ERβ5 and LacZ stably expressed cells is shown as $^{**}P < .01$.

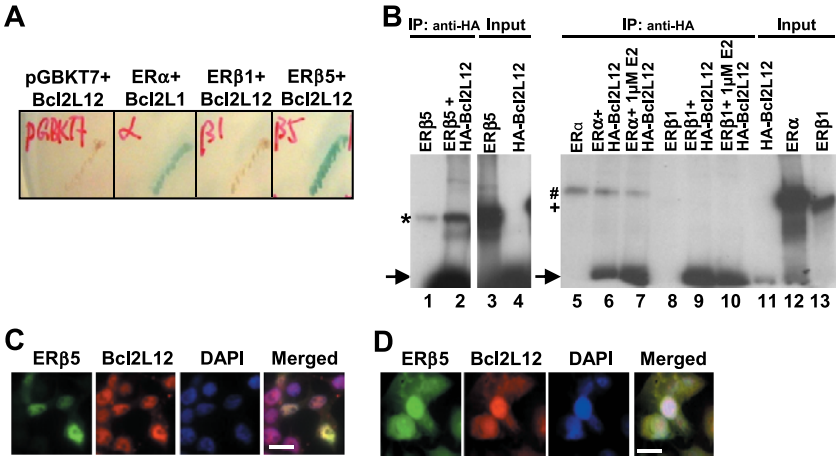

Figure W3. Bcl2L12 specifically interacts with ERβ5 but not ERβ1 or ERα. (A) Full-length ERβ5 and ERα, but not ERβ1, interact with Bcl2L12 in yeast. ERβ1, ERβ5, ERα, or empty vector (pGBKT7) was transformed with Bcl2L12 into yeast cells. Transformed cells were seeded on QDO containing X-α-galactosidase until growth of blue colonies. (B) Bcl2L12 interacts with ERβ5 but not ERβ1 or ERα *in vitro*. ERβ5, ERβ1, ERα, and HA-tagged Bcl2L12, respectively, were translated *in vitro* and labeled with [^{35}S] methionine. Lysates were mixed and incubated in the absence or presence of E2 as indicated, followed by immunoprecipitation with anti-HA affinity gel. The immunoprecipitates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by X-ray autoradiography. The asterisk (*), number sign (#), and plus sign (+) respectively indicate the positions of ERβ5, ERα, and ERβ1. (C and D) ERβ5 colocalized with Bcl2L12 in 293T and MCF-7–ERβ5 cells. The two cell lines were grown in full-serum medium. The 293T cells were transfected with ERβ5 and Bcl2L12. Antibodies to ERβ and Bcl2L12 were used for immunostaining. DAPI was used as a nuclear marker. Images in C and D were captured by fluorescence microscopy. Bar = 20 μm.

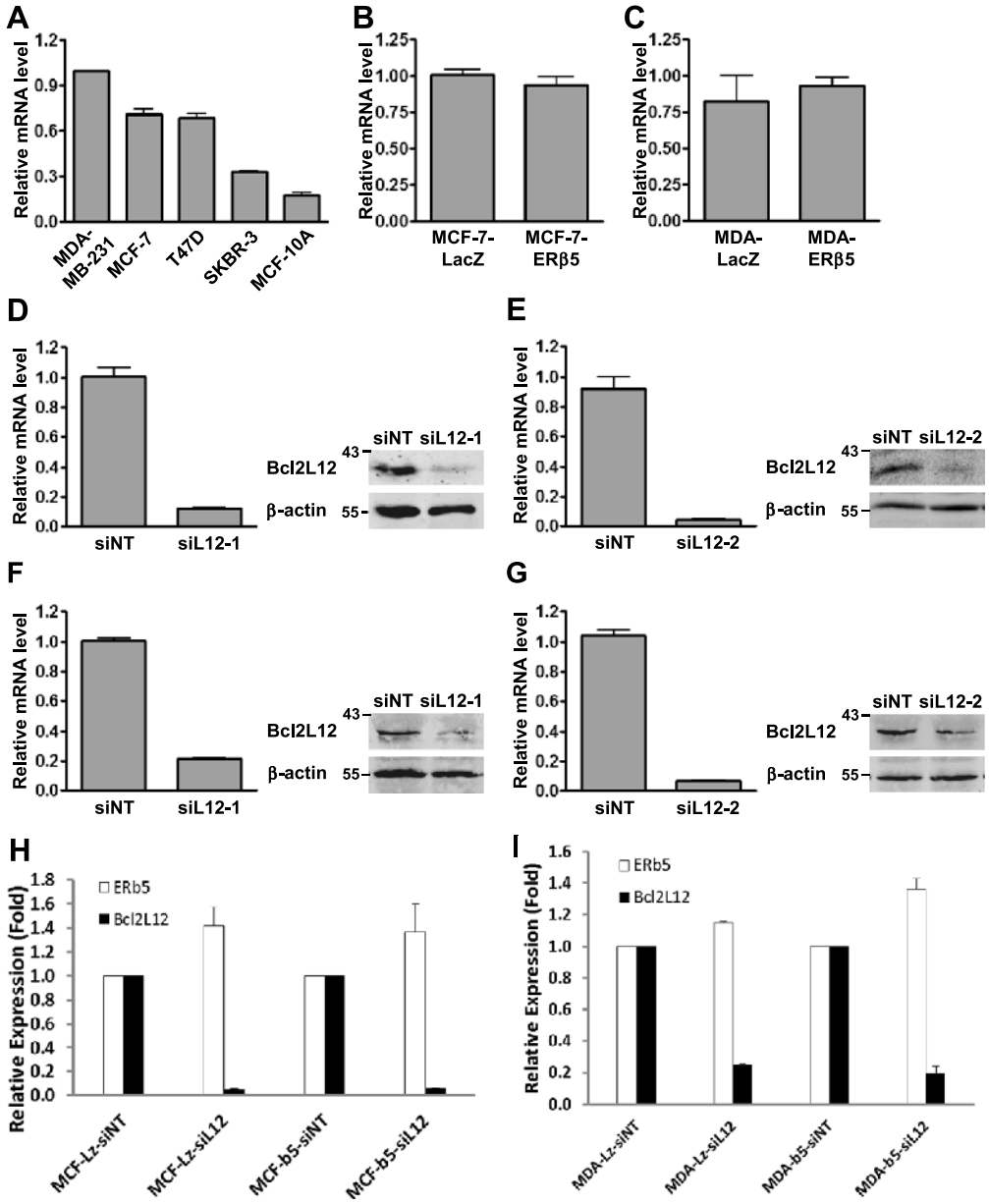

Figure W4. Expression of Bcl2L12 was significantly reduced by gene-specific siRNAs in MCF-7 and MDA-MB-231. (A) Expression level of Bcl2L12 in different mammary cell lines was determined. The cDNAs of BCa cell lines (MDA-MB-231, MCF-7, T47D, and SKBR-3) and mammary epithelial cells (MCF-10A) were used in quantitative RT-PCR. The results are the average of two independent experiments. (B and C) Expression of Bcl2L12 is not altered by the ectopic expression of ERβ5 in BCa cells. The cDNAs of MCF-7–ERβ5/−LacZ (B) and MDA-ERβ5/−LacZ (C) were used in quantitative RT-PCR. The results are the average of two independent experiments. (D–G) Bcl2L12 siRNAs efficiently reduced its expression in MCF-7 and MDA-MB-231. Two Bcl2L12-specific siRNAs (siL12-1 and siL12-2) were transfected into MCF-7 (D and E) and MDA-MB-231 (F and G). After 24-hour transfection, cells were transfected for the second time. siNT was used as negative control. The relative expression of Bcl2L12 was measured by quantitative RT-PCR and Western blot analysis. β-Actin was used as loading control in Western blot analysis. The results of quantitative RT-PCR are the average of three independent experiments. All data are represented as means ± SD. (H and I) ERβ5 and Bcl2L12 expression in MCF-7–ERβ5/−LacZ and MDA-ERβ5/−LacZ cells with or without Bcl2L12 siRNA knockdown.

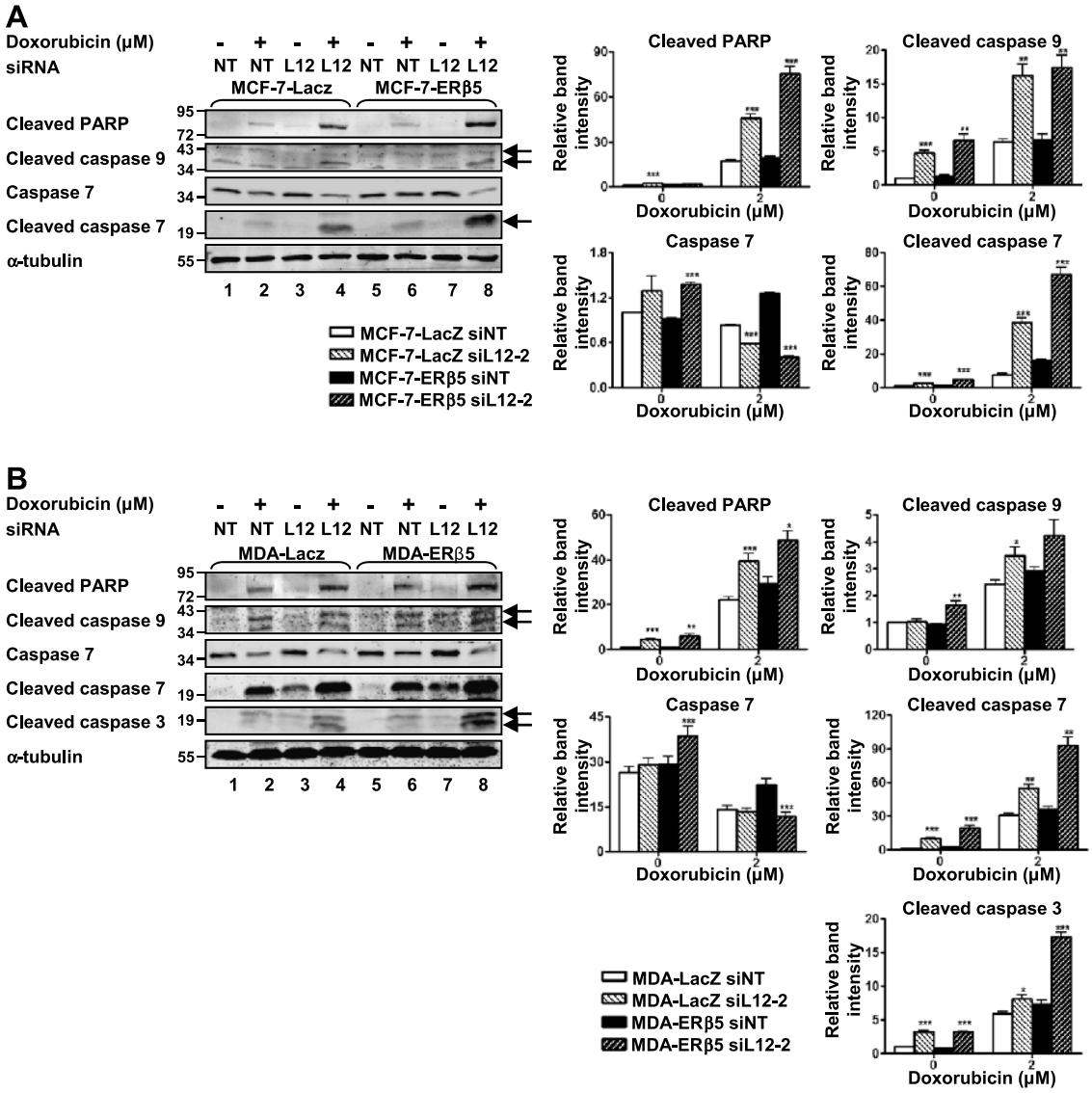

Figure W5. Knockdown of Bcl2L12 by siL12-2 sensitizes doxorubicin-induced apoptosis in MCF-7 and MDA-MB-231. (A and B) siNT or siL12-2 was transfected twice into MCF-7–ERβ5/−LacZ (A) and MDA-ERβ5/−LacZ (B). MCF-7 cells were incubated with 2 μM doxorubicin, and MDA-MB-231 cells were incubated with 5 μM doxorubicin. Whole-cell lysates were extracted for Western blot analysis with antibodies as indicated. The measurement of relative band intensities and analysis of data were similar to those in Figure W1*A*. The statistical significance of the difference in densitometric analysis between the cells treated with siNT and siL12-1 at the same concentration of doxorubicin is shown as $*P < .05$, $**P < .01$, and $***P < .001$.