

Suppression of the death gene BIK is a critical factor for resistance to tamoxifen in MCF-7 breast cancer cells

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Abstract. Apoptosis is controlled by the BCL-2 family of proteins, which can be divided into three different subclasses based on the conservation of BCL-2 homology domains. BIK is a founding member of the BH3-only pro-apoptotic protein family. BIK is predominantly localized in the endoplasmic reticulum (ER) and induces apoptosis through the mitochondrial pathway by mobilizing calcium from the ER to the mitochondria. In this study, we determined that suppression of the death gene *Bik* promotes resistance to tamoxifen (TAM) in MCF-7 breast cancer cells. We utilized small interfering (siRNA) to specifically knockdown BIK in MCF-7 cells and studied their response to tamoxifen. The levels of cell apoptosis, the potential mitochondrial membrane ($\Delta\Psi_m$), and the activation of total caspases were analyzed. Western blot analysis was used to determine the expression of some BCL-2 family proteins. Flow cytometry studies revealed an increase in apoptosis level in MCF-7 cells and a 2-fold increase in relative BIK messenger RNA (mRNA) expression at a concentration of 6.0 μ M of TAM. BIK silencing, with a specific RNAi, blocked TAM-induced apoptosis in 45 \pm 6.78% of cells. Moreover, it decreased mitochondrial membrane potential (Ψ_m) and total caspase activity, and exhibited low expression of pro-apoptotic proteins BAX, BAK, PUMA and a high expression of BCL-2 and MCL-1. The above suggests resistance to TAM, regulating the intrinsic pathway and indicate that BIK comprises an important factor in the process of apoptosis, which may exert

an influence the ER pathway, which regulates mitochondrial integrity. Collectively, our results show that BIK is a central component of the programmed cell death of TAM-induced MCF-7 breast cancer cells. The silencing of *BIK* gene will be useful for future studies to establish the mechanisms of regulation of resistance to TAM.

Introduction

Apoptosis, a genetically programmed cell death, is conserved among eukaryotes. It is important during embryonic development to ensure organogenesis and adulthood for maintenance of cellular homeostasis (1,2). Alterations in this process can lead to pathological conditions such as cancer and degenerative diseases. Apoptosis is controlled by the BCL-2 family of proteins, this family can be divided into three different subclasses based on conservation of the BCL-2 homology (BH1-4) domains: multidomain anti-apoptotic proteins (BCL-2, BCL-XL, MCL-1, BCL-W, and Bfl-1/A1); multidomain pro-apoptotic proteins (BAX and BAK), and BH3-only pro-apoptotic proteins (BID, BAD, BIM, PUMA, NOXA and BIK) (3).

BH3-only proteins may function as death sensors that mediate the activation of the mitochondrial apoptosis pathway in response to oncogenic stress signals or DNA damage (4). Notably, BH3-only proteins are not able to kill cells that lack BAX and BAK, indicating that BH3-only proteins function upstream of these proteins (5). BIK induces apoptosis in a variety of eukaryotic cells and is non-essential for animal development (6,7). BIK is a pro-apoptotic tumor suppressor in several human tissues and its expression in cancers is prevented by chromosomal deletions of the BIK locus or by epigenetic silencing (3,8,9).

Several anticancer drugs transcriptionally activate the *BIK* gene through transcriptional pathways dependent on factors such as E2F and p53 (8,10-14). *Bik* has also been used as a therapeutic molecule in gene therapy-based approaches to treat difficult cancers. However, the relation between BIK and the resistance to TAM is poorly understood. TAM is widely

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employed in chemotherapy for breast cancer. In MCF-7 breast cancer cells, TAM inhibits cell proliferation and induces oxidative stress (OS) and apoptosis via mitochondria-dependent mechanisms by estrogen receptor-dependent modulation of gene expression (14,15). In the present study, we investigated the relationship between BIK and treatment with TAM in MCF-7 human breast cancer cells.

Materials and methods

Cell cultures. MCF-7 human breast cancer cells (American Type Culture Collection, ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium F:12 (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) containing penicillin (100 U/ml) and streptomycin (100 µg/ml) (Life Technologies Inc. BLR, Grand Island, NY, USA).

The cells were grown in 75-cm² tissue culture flasks in (5% CO₂) at 37°C and routinely passaged when confluent. Before each experiment, cells were seeded in 3.5-cm diameter tissue culture plates (5% CO₂).

Half maximal effective concentration (EC₅₀) of TAM. TAM (TAM citrate; Sigma Chemical Co., St. Louis, MO, USA) stock solution was prepared in 2% ethanol. MCF-7 cells were exposed to different concentrations of TAM (1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 µM) at 37°C for 24 h. Apoptosis of the cells was measured by flow cytometry by Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining (BioLegend, San Diego, CA, USA).

Suppression of Bik expression with small interfering RNA interference (siRNAi). Cells were plated in antibiotic-free DMEM-F12 at a density of 2.5x10⁵ cells and when 50% confluence was reached, the cells were transfected with oligofectamine reagent (Invitrogen) and 100 nmol/l BikRNAi (oligoduplex 5'-AAG ACCCCUCUCCAGAGACAU-3', N5' or AAAUGUCUCUGG AGAGGGGUC-3') (Labs and Integrated DNA Technologies, IDT) or control sequence-scrambled (Silencer Negative Control #3 siRNA, Ambion) composed of a 19 bp scrambled sequence without significant homology to any known gene sequences from mouse, rat or human. Briefly, 10 µl oligofectamine was diluted 7.5-fold in Opti-MEM and incubated at room temperature for 10 min. In parallel, a separate tube, 5 µl of 50 µmol/l siRNA, was diluted in 425 µl of Opti-MEM. Diluted oligofectamine (75 µl) was added to the diluted siRNA and the complex was incubated for 20 min at room temperature. Cells were washed with 2 ml of Opti-MEM. The siRNA + oligofectamine complex (500 µl) was added gently to the dish. The final concentration of siRNA was 100 nmol/l. After 6 h, 1.25 ml of 3X serum medium was added to the dish without removing the transfection mix. The medium was exchanged for serum-containing medium after 6 h and the cells were further cultured for 48 h. The experiments were repeated two to three times.

Western blot analysis. MCF-7 cell groups (non-treated, scrambled, siRNA BIK and siRNA BIK with TAM) were cultured overnight at room temperature in 3.5-cm diameter tissue culture plates at a density of 1x10⁵ cells/plate. Cells

were transferred into 100 µl of lysis buffer (RIPA-Tris buffer:EGTA 2 mM; NaCl 316 mM; Na₂MoO₄ 20 mM; NaF 50 mM; Tris-HCl 20 mM; Na₃VO₄ 100 mM, PMSF 100 mM, and EDTA 100 mM; 0.1% of leupeptin and aprotinin, 0.2% SDS and 2% Triton X-100) and maintained under constant shaking for 2 h at 4°C. Subsequently, the sample was centrifuged for 5 min at 20,800 rpm and the supernatant (30 µg of protein) was denatured in Laemmli sample buffer, resolved through 12% SDS polyacrylamide gels, and electroblotted onto polyvinylidene difluoride (PVDF) membranes. Blots were stained with Ponceau S to confirm that protein loading was identical in all lanes. Membranes were soaked in PBS to remove the Ponceau S and incubated for 90 min in Tris-buffered saline (TBS) containing 5% dried skimmed milk and 0.1% Tween-20 to block the non-specific protein binding sites. Subsequently, the membranes were incubated for 14 h at 4°C with the primary antibody 1:1,000: BCL-2; MCl-1; BAX; BAK; PUMA, and cytochrome *c* (Cyt C), from Santa Cruz Biotechnology (Santa Cruz, CA, USA). BIK 1:100 from Abcam was diluted in 0.1% TBS-Tween-20 including 5% dried skimmed milk, then washed and incubated with peroxidase-conjugated secondary antibodies 1:10,000. Protein was detected using an ECL Western blot detection kit (Millipore). The blots were subjected to densitometry analysis and data were analyzed using GraphPad Prism5 software (GraphPad Software, San Diego, CA, USA). Western blot analyses were repeated three times.

Real-time quantitative RT-PCR. RNA from all samples was amplified by RT-PCR assay in a rotor gene Real-Time apparatus (Cobbett Research 2004) utilizing the Superscript III Platinum One-step qRT-PCR kit (Invitrogen). The 25 µl reaction buffer contained 100 ng of total RNA, 1X Superscript III Platinum One-step qRT-PCR reaction mix, and 0.4 µM of each of the primers. The primers were employed for *HPRT* gene amplification (13) and *BIK* gene (forward 5' GAG ACA TCT TGA TGG AGA CC3', reverse 5' TCT AAG AAC ATC CCT GAT GT3'). The following thermal profile was used: a single cycle of reverse transcription (RT) for 15 min at 50°C; another cycle of 2 min at 95°C followed by 45 amplification cycles of 20 sec at 95°C, and 1 min at 57°C. Threshold cycle (TC) value of BIK was normalized to *HPRT* (16).

Flow cytometry. Annexin V-FITC/PI double staining was used to detect the apoptosis index. Briefly, the MCF-7 human breast cancer cells (1x10⁶ cells/ml) were harvested by trypsinization and washed twice with cold PBS (0.15 mol/l, pH 7.2). The cells were centrifuged at 2,500 rpm for 5 min; then, the supernatant was discarded and the pellet was resuspended in 1X binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl₂), at a density of 1.0x10⁶ cells/ml, 100 µl of the sample was transferred into a 5 ml culture tube and incubated in the dark with 5 µl of FITC-conjugated Annexin V staining solutions (Biolegend, San Diego, CA, USA) and 5 µl of PI staining solutions; for 15 min at room temperature. Later, 400 µl of 1X binding buffer was added to each sample tube and the samples were analyzed by FACSCalibur flow cytometry (Becton-Dickinson) using Cell Quest Research software (Becton-Dickinson).

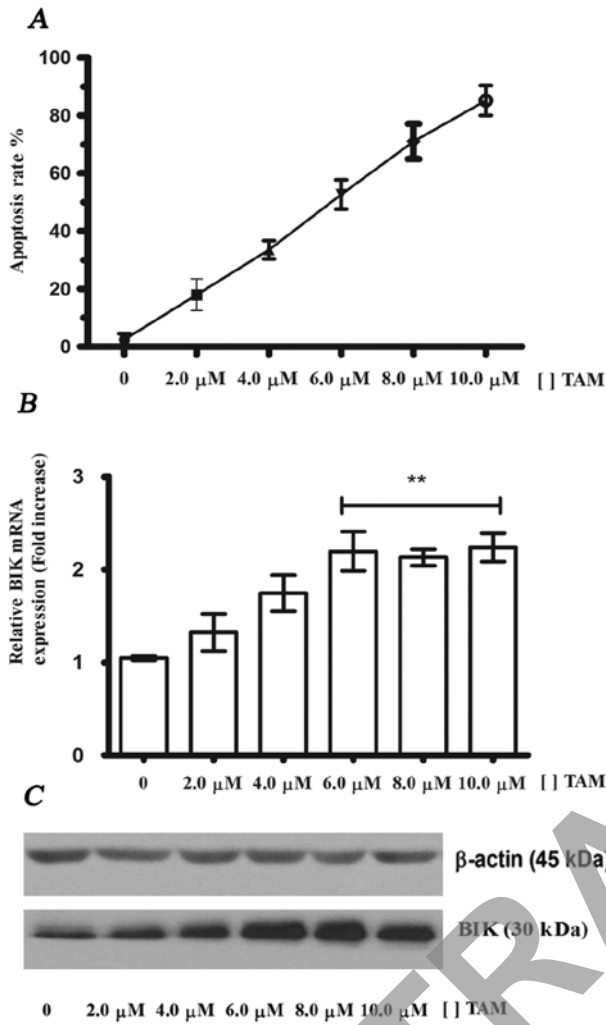


Figure 1. The messenger RNA (mRNA) and protein levels of Bik were increased in MCF-7 cells treated with tamoxifen (TAM). (A) MCF-7 cells were treated with TAM by determining EC_{50} . (B) MCF-7 cells were treated with TAM for 24 h and subjected to RT-PCR analyses. (C) Cell lysates were prepared and subjected to western blot analyses for Bik and β -actin as a control; one representative of at least three independent experiments with similar results is shown. Each value represents the mean \pm standard error of the mean (SE) (bars) of three independent experiments. $p < 0.05$, according to the Student's t-test.

Mitochondrial membrane potential of MCF-7 cells. The mitochondrial membrane potential ($\Delta\Psi_m$) was measured with JC-1 (5, 5,6, 6-tetrachloro-1, 1, and 3, 3-tetraethyl-benzimidazolyl-carbo cyanine iodide) to signal the loss of ($\Delta\Psi_m$). MCF-7 cells (non-treated, scrambled, siRNA BIK, and siRNA BIK with TAM) were harvested; equal numbers of cells (1×10^6) were incubated with JC-1 at 2.5 g/ml in 1 ml of PBS for 30 min at 37°C with moderate shaking. Cells were then centrifuged 1,600 rpm at 4°C for 5 min, washed twice with ice-cold PBS, resuspended in 200 μ l of PBS, and analyzed on a flow cytometer (Becton-Dickinson). We detected green fluorescence at excitation/emission wavelengths of 485/530 nm and red fluorescence at excitation/emission wavelengths of 550/595 nm utilizing CellQuest Research software.

Caspase assay. Caspases were detected by means of the Vybrant FAM Poly Caspases assay kit, which detects active

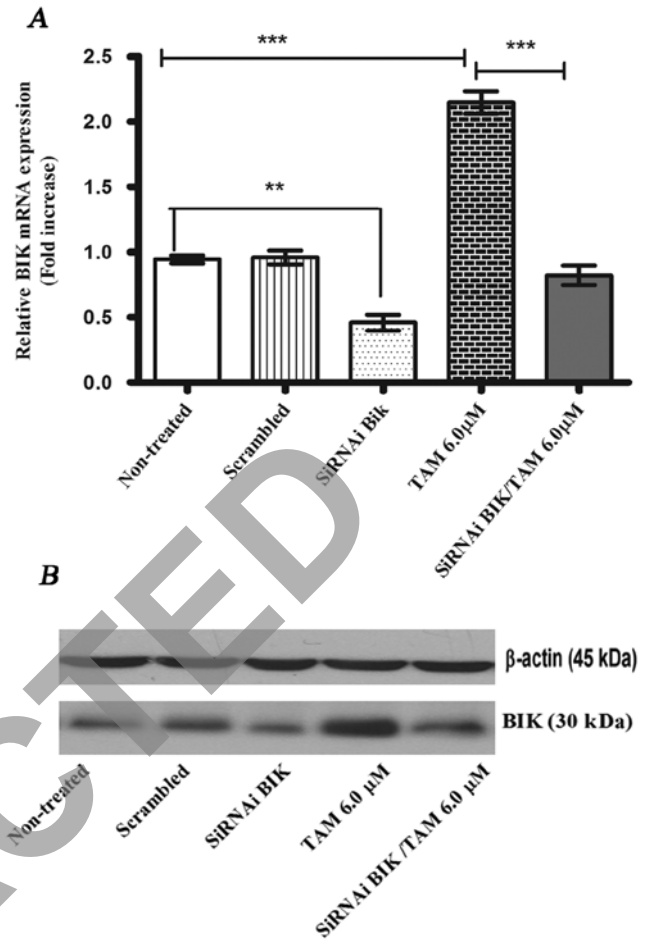


Figure 2. Transfection and silencing of Bik in the human breast cancer line MCF-7. (A and B) Cells were transiently transfected with Bik-specific (BIK) or sequence-scrambled control and then cultured in the presence or absence of 6.0 μ M of tamoxifen (TAM) for 24 h, followed by evaluation of expression of Bik and β -actin (as control) by real-time RT-PCR and western blot analysis. The groups were analyzed by one-way ANOVA with the Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. non-treated. Representative figures of three independent experiments carried out in triplicate are shown.

caspases by employing the FAM-VAD-FMK reagent, a fluorescently labeled inhibitor of such enzymes (FLICA). Briefly, 10 μ l aliquots of 30X FLICA working solution was added to MCF-7 cells in suspension and these were incubated in the dark for 1 h at 37°C and 5% CO_2 . After two washes with Wash Buffer 1X (supplied by the manufacturer), the samples were fixed by adding 40 μ l of 10% formaldehyde solution (supplied by the manufacturer) for 10 min at room temperature. Then, MCF-7 cells were again washed and resuspended in 400 μ l of Wash Buffer 1X containing 8 μ l of PI for FACS analysis.

Statistical analysis. Results were expressed as the means \pm standard error of the mean (SEM). All data were statistically analyzed using one- or two-way analysis of variance (ANOVA) for repeated measurements, followed by the post hoc Tukey's test. Fisher's post hoc analysis was also utilized to analyze differences between groups. Analysis was performed employing GraphPad Prism5 statistical software (GraphPad Software). Differences of $p < 0.05$ were considered statistically significant.

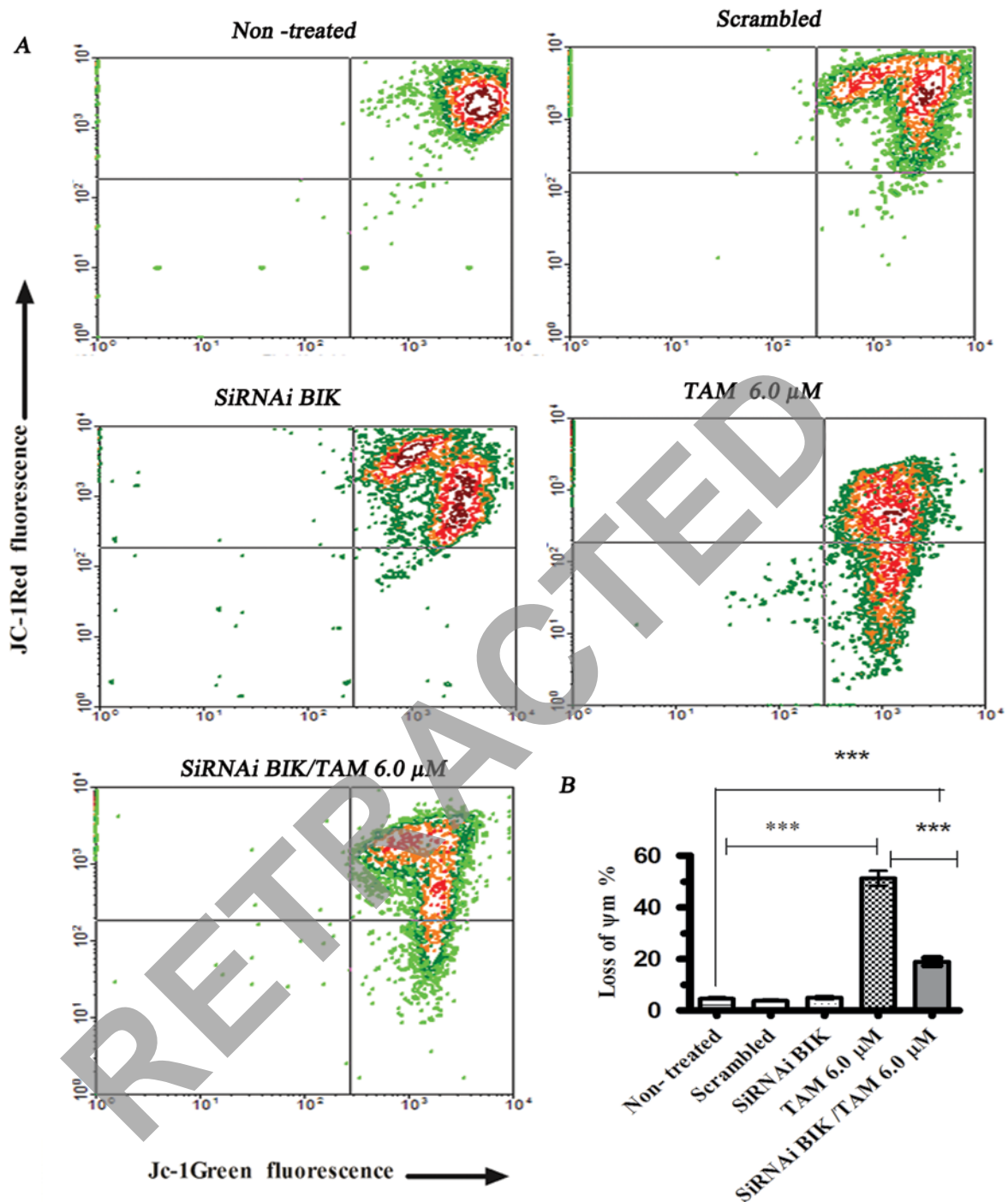


Figure 3. Suppression of Bik expression does not alter the mitochondrial membrane potential. (A) MCF-7 cells [non-treated, scrambled, siRNAiBIK, siRNAiBIK/tamoxifen (TAM), and TAM] were stained with JC-1 for loss of $\Delta\Psi_m$ using flow cytometry. JC-1 fluorescence is seen in both the FL-2 (red) and FL-1 channels (green). In FL-1 channels are cells with low levels of $\Delta\Psi_m$, while FL-2 channel indicates cells with stable levels of $\Delta\Psi_m$. (B) Representative graphic of fluorescence intensities. One representative of at least three independent experiments with similar results is shown. The groups were analyzed by one-way analysis of variance (ANOVA) with the Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. non-treated. Representative figures of three independent experiments carried out in triplicate are shown.

Results

TAM induces apoptosis and increases the levels of BIK mRNA and its protein in MCF-7 cells. In breast cancer and breast cell lines, TAM-induced apoptosis is mediated by the estrogen receptor. To identify whether the expression of the *BIK* gene

and *BIK* protein in MCF-7 cells are enhanced during TAM induced apoptosis, we incubated these cells for 24 h at different concentrations of TAM (range, 1-10 μ M). Flow cytometry data indicated that TAM increased the levels of apoptosis (Fig. 1A) and an EC_{50} of 6.0 μ M was obtained. RT-PCR and western blot analysis revealed that the expression of *BIK* mRNA and

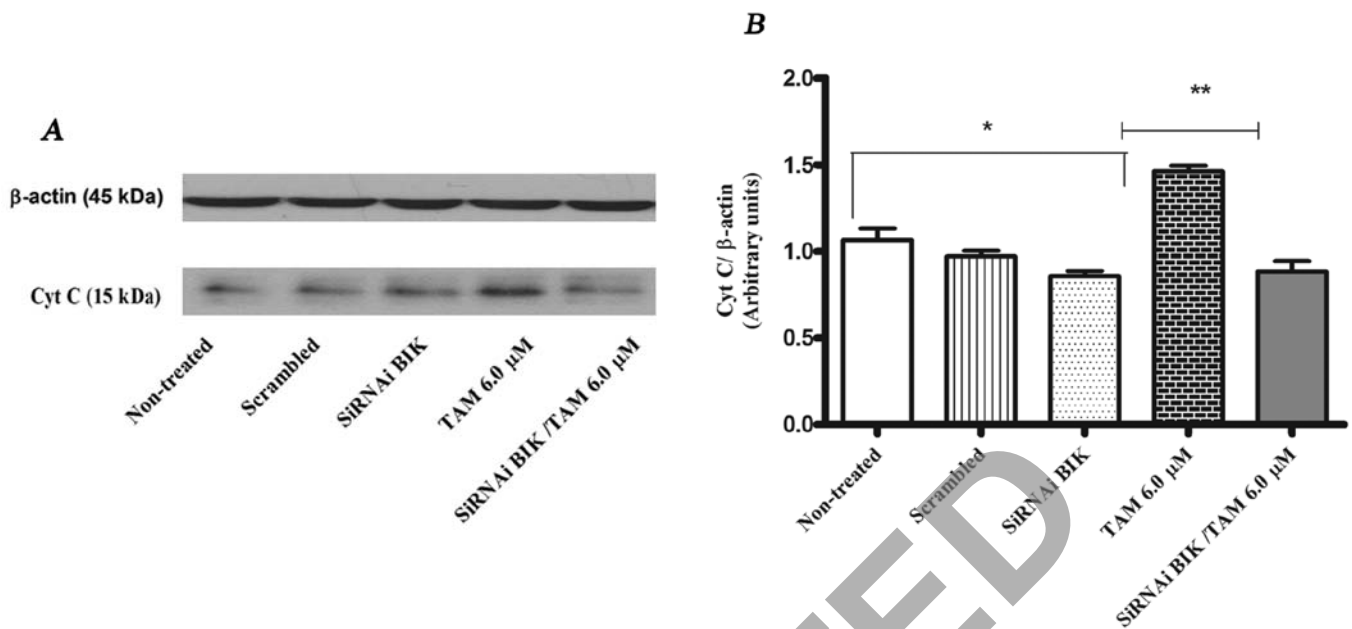


Figure 4. Silencing of Bik does not promote the release of cytochrome c in response to tamoxifen. (A) Cell lysates were prepared and subjected to western blot analyses for cytochrome *c* (Cyt C) and β -actin as control. (B) Densitometry analysis of the expression levels of Cyt C. One representative of at least three independent experiments with similar results is shown. The groups were analyzed by one-way analysis of variance (ANOVA) with the Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. non-treated. Representative figures of three independent experiments carried out in triplicate are shown.

its protein increases significantly at 6-10 μ M (Fig. 1B and C). These data indicate that BIK expression is also induced by TAM and suggest the participation of BIK in TAM-induced apoptosis in MCF-7 cells.

BIK interference protects against apoptosis. In order to evaluate if BIK expression was blocked using siRNA, we compared the levels of BIK mRNA and BIK protein among the following MCF-7 cell groups: non-treated; scrambled; siRNA BIK, and siRNA BIK with TAM (Fig. 2). The transient transfection of BIK siRNA reduced the expression levels of BIK mRNA by about $55 \pm 0.106\%$ in MCF-7 cells compared with the controls (non-treated and scrambled). In the TAM group, mRNA levels increased 2-fold with respect to control groups. We confirmed these results with the western blot analysis (Fig. 2).

We conducted the $\Delta\Psi_m$ assay in different MCF-7 cell groups for comparison. The groups comprised cells exposed or not to TAM, after previous transfection or non-transfected. In cells exposed to TAM 6 μ M but not transfected, the $\Delta\Psi_m$ decreased to $52.3 \pm 4.56\%$ with respect to cells not exposed to TAM. We did not find significant differences between the non-treated cells and cells transfected with scrambled RNA or with BIK siRNA; however, in cells exposed to TAM and transfected with BIK siRNA, the $\Delta\Psi_m$ decreased $20.2 \pm 3.59\%$ (Fig. 3). These results indicate that BIK could participate in the loss of $\Delta\Psi_m$ modulating anti-apoptotic and pro-apoptotic proteins that regulated mitochondrial pore formation.

The relation between $\Delta\Psi_m$ and apoptotic initiation is uncertain; however, a change in $\Delta\Psi_m$ might be associated with the release of cytochrome *c* (Cyt C), and probably with apoptotic initiation (17). The level of Cyt C protein was significantly higher in TAM-exposed cells; however, in TAM-exposed but

BIK siRNA-transfected cells, the level was similar to that of the controls, inhibiting the apoptosis process (Fig. 4). To corroborate these data, we measured total caspase activation.

In TAM-treated MCF-7 cells, total caspase activity was $50.81 \pm 9.17\%$, while in TAM-infected BIK-exposed MCF-1 cells, total caspase activity was $20.2 \pm 3.59\%$ and in control group showed no change (Fig. 5). With the aim of determining the percentage of apoptosis the cells were transfected with BIK and treated with TAM. We utilized flow cytometry staining non-viable cells with PI and Annexin V. TAM-induced apoptosis was $50.1 \pm 6.78\%$ at 24 h, and the percentage of apoptosis in siRNAi BIK TAM cells was $14.53 \pm 3.22\%$. These data suggest resistance to TAM-induced apoptosis in BIK siRNA-transfected cells (Fig. 6).

Low expression of BIK generates resistance to TAM in MCF-7 regulating pro-apoptotic and anti-apoptotic family members.

To investigate the molecular mechanisms of the Bik protein in the process of tamoxifen (TAM)-induced apoptosis, we determined the protein expression of anti-apoptotic and pro-apoptotic proteins by western blot analysis. The expression levels of BCL-2 and MCL-1 in Bik-transfected MCF-7 cells in response to TAM were higher in comparison with those of TAM only-treated MCF-7 cells (Fig. 7). Whereas the levels of pro-apoptotic proteins BAX, BAK and PUMA increased their expression in TAM treated non-transfected cells in comparison with TAM-exposed BIK-transfected MCF-7 cells (Fig. 8).

These experiments suggest that low expression of Bik may generate a process of resistance to apoptosis due to the low expression of molecules that promote mitochondrial pore formation, such as BAX and BAK, and induction of the expression of anti-apoptotic proteins like BCL2 and MCL-1.

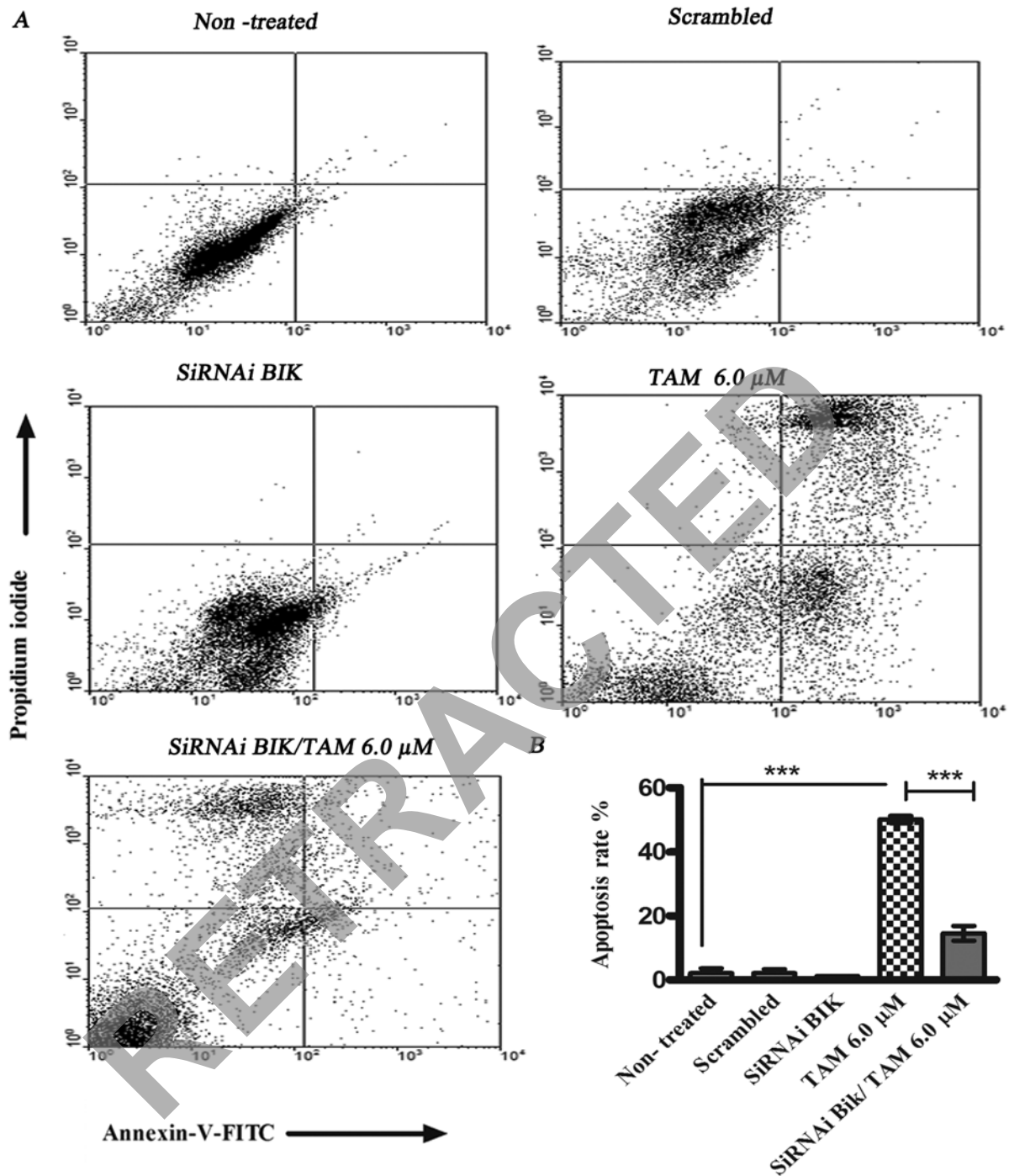


Figure 5. Suppression of the Bik gene promotes inhibition of caspase activity. (A) MCF-7 [non-treated, scrambled, siRNAiBIK, siRNAiBIK/tamoxifen (TAM), and TAM] were then labeled with the FAM-VAD-FMK probe and PI for determination of caspase activation. The samples were analyzed on a flow cytometer with 488 nm excitation using 530 nm band pass and 670 nm long pass emission filters. (B) Representative graphic of fluorescence intensities. Results are representative of three independent experiments. The groups were analyzed by one-way ANOVA with the Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. non-treated. Representative figures of three independent experiments carried out in triplicate are shown.

Discussion

The *Bik* gene has been associated with tumor reversion in different cell lines and was proposed as therapeutic for inducing apoptosis in cancer, including breast tumors (6,9,18); however, our group and others have obtained high BIK levels in breast cancer, non-small cell lung cancer (NSCLC), and

lymphoblastoid cell lines derived from patients with Fanconi anemia (19,20).

The BH3-only BIK protein, which is inducible by estrogen starvation and fulvestran treatment, has been suggested to play a critical role in anti-estrogen-induced apoptosis in breast cancer cells (18,21). The anti-estrogen TAM is the most commonly used treatment for estrogen receptor-positive

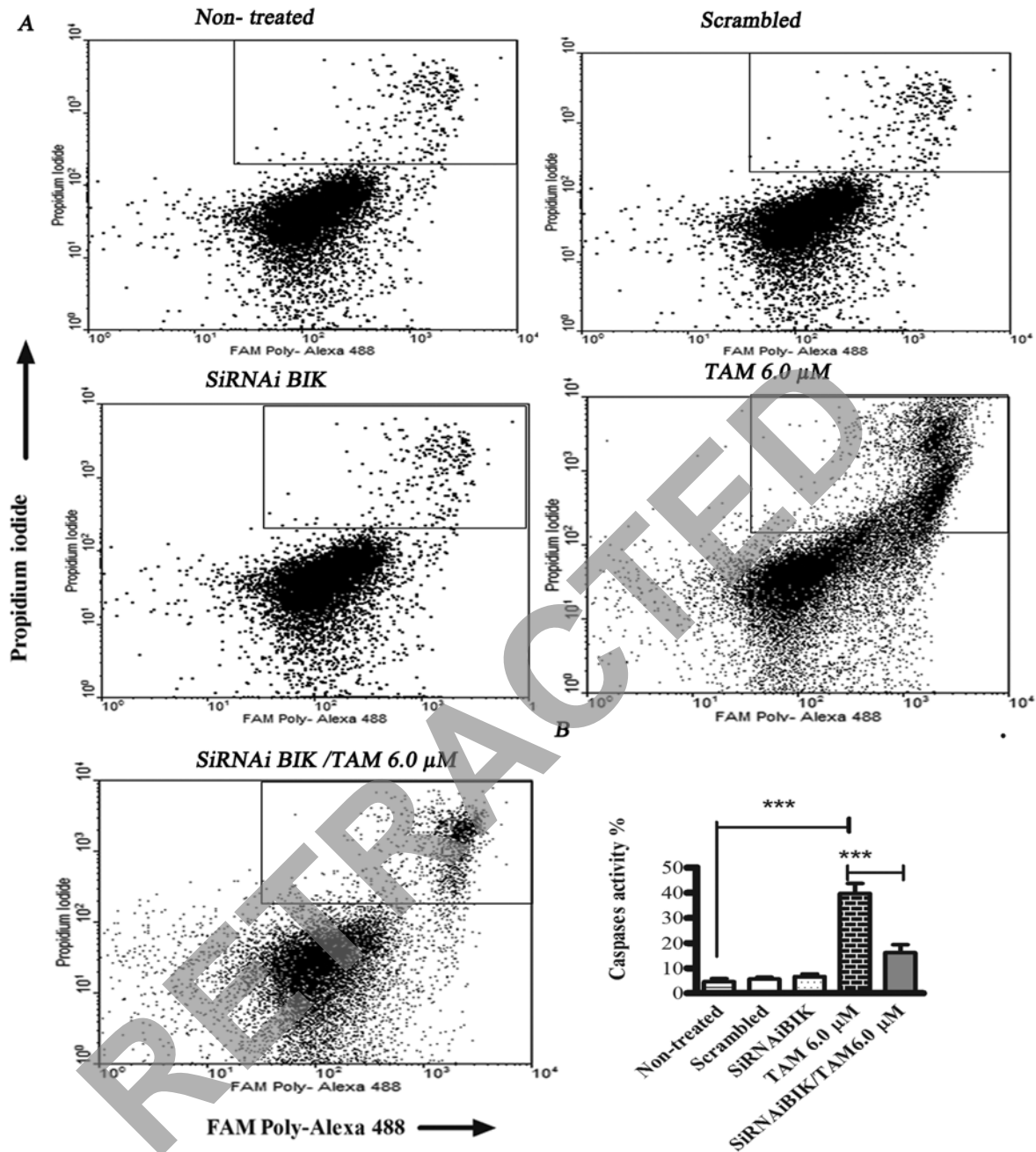


Figure 6. Suppression of the Bik gene promotes resistance in the process of apoptosis. (A) For corroborate apoptosis MCF-7 cells were stained with Annexin V-FITC and PI and analyzed by flow cytometry. (B) Representative graphic of fluorescence intensities for apoptosis. Results are representative of three independent experiments. The groups were analyzed by one-way ANOVA with the Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. non-treated. Representative figures of three independent experiments carried out in triplicate are shown.

patients with breast cancer. Although the efficacy of TAM has been attributed to the induction of tumor cell growth arrest and apoptosis by inhibition of estrogen receptor signaling (22-25), the molecular mechanism is not well understood to date. In the present study, we show that suppression of the BIK gene promotes resistance to TAM in breast cancer MCF-7 cells.

First, we showed that exposure to different concentrations of TAM led to the increase of *Bik* gene expression possibly by transcriptional pathways. Previous studies have shown

that certain drugs, cytokines and virus infection affected transcription factors, such as E2F and P53, or removal of epigenetic marks on the chromatin, which promotes the transcriptional activation of the *BIK* gene (11,13). It is noteworthy that Mathai *et al* found that BIK expression in KB human oral epithelial cells depends on P53, but the authors did not identify functional p53-interacting elements in the BIK promoter (11). In TAM-treated MCF-7 cells, we studied the effect of BIK interference. We found that siRNAi BIK-transfected cells were resistant to apoptosis, using the

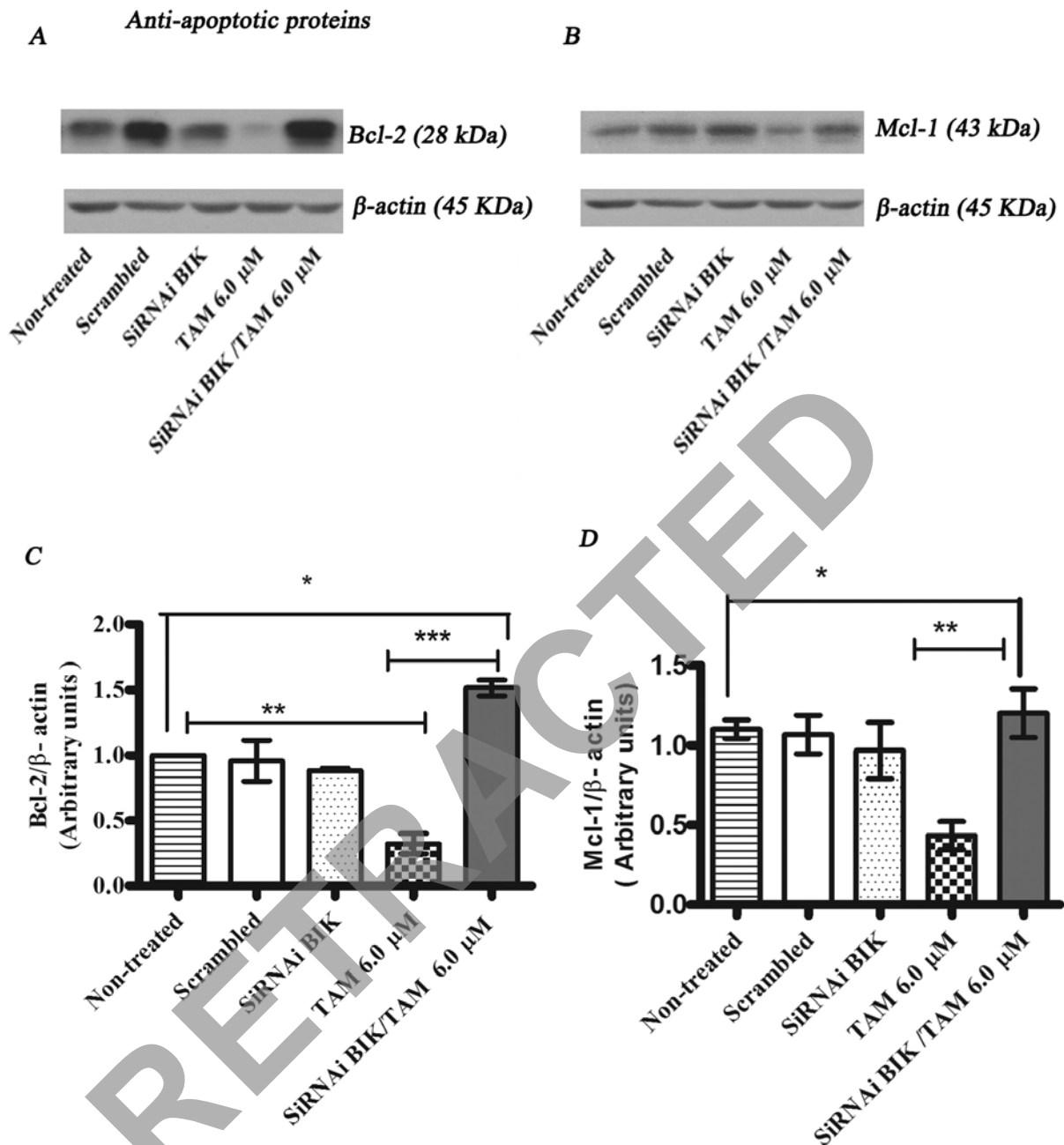


Figure 7. Anti-apoptotic proteins are highly expressed in MCF cells transfected with BIK and treated with TAM. (A and B) MCF-7 cells were incubated for 48 h, cell lysates were prepared from the cells, and subjected to western blot analyses for Bcl-2, and Mcl-1. One representative of at least three independent experiments with similar results is shown. (C and D) Band intensity in Bcl-2, and Mcl-1 was quantified by densitometric analysis. The groups were analyzed by one-way analysis of variance (ANOVA) with the Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. non-treated. Representative figures of three independent experiments carried out in triplicate are shown.

Annexin V and PI test, $\Delta\Psi_m$ and caspase activation. Because the relation between $\Delta\Psi_m$ and apoptosis is uncertain, we measured the Cyt C, levels of protein expression were found to be similar in transfected and control cells and that TAM treatment increases Cyt C in non-transfected cells, but not in BIK-siRNA transfected cells. With the aim of determining the molecular mechanisms of resistance to TAM mediated by BIK, we evaluated some BCL-2 family proteins. We found low expression of BAX, BAK and PUMA pro-apoptotic proteins and high expression of some anti-apoptotic proteins, such as BCL-2 and MCL-1 in BIK siRNA-transfected cells

after treatment with TAM, the latter two proteins have been shown to be involved in the prevention of Cyt C release (26,27). Our present data demonstrated that Bik is an important factor in the apoptosis process induced by TAM, which may regulate mitochondrial integrity by modulation of pro- and anti-apoptotic proteins; however, it is necessary to conduct more studies in order to understand BIK-mediated resistance to TAM-induced apoptosis.

Our results showed that suppression of the *BIK* gene exhibited anti-apoptotic effects in TAM-treated MCF-7 cells. Our data would be useful for future studies to establish

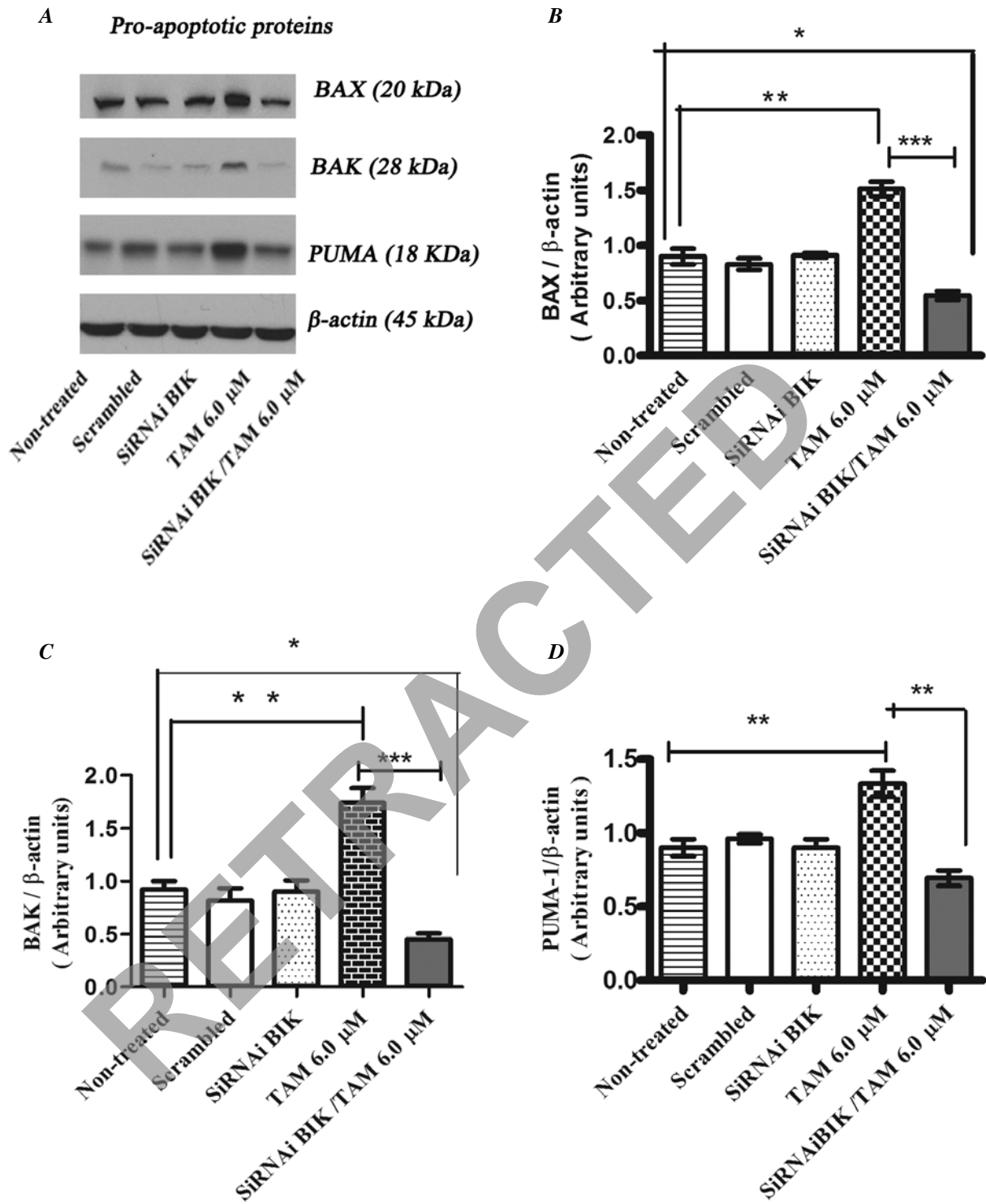


Figure 8. Suppression of BIK regulated negatively pro-apoptotic proteins in MCF-7 cells treated with TAM. (A) MCF-7 cells were incubated for 48 h, cell lysates were prepared from the cells, and subjected to western blot analyses for BAX, BAK and PUMA. One representative of at least three independent experiments with similar results is shown. (B, C and D) Band intensity in BAX, BAK and PUMA was quantified by densitometric analysis. The groups were analyzed by one-way analysis of variance (ANOVA) with the Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. non-treated; n.s., not significant. Representative figures of three independent experiments carried out in triplicate are shown.

the mechanisms of regulation of TAM resistance in breast cancer. In women with this neoplasm and with positive estrogen receptor, it would be important to determine BIK protein levels to define whether or not TAM would be the appropriate treatment.

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