Paclitaxel resistance is mediated by NF-κB on mesenchymal primary breast cancer cells

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Abstract. Paclitaxel has been used widely to treat breast cancer and other types of cancer. However, resistance is a major cause of failure for treatment and results in cancer progression. The present study investigated the association between paclitaxel resistance and the mesenchymal phenotype, using a model of primary breast cancer cells and employing four different cultures, two with an epithelial phenotype (MBCDF and MBCD17) and two with a mesenchymal phenotype (MBCDF-D5 and MBCD3). Epithelial-mesenchymal markers were evaluated by western blotting; MBCDF and MBCD17 cells expressed E-cadherin, SNAIL, Slug, and Twist, low levels of N-cadherin, but not vimentin. MBCDF-D5 and MBCD3 cells expressed N-cadherin, vimentin, and higher levels of SNAIL, and low levels of E-cadherin, Slug, and Twist. Cell viability was evaluated using a crystal violet assay after paclitaxel treatment; primary breast cancer cells with mesenchymal phenotype were resistant to paclitaxel compared with the epithelial primary breast cancer cells. Furthermore, using western blotting, it was revealed that mesenchymal cells had elevated levels of nuclear factor-κB (NF-κB) p65 and IκB kinase (IKK). Additionally, it was demonstrated that paclitaxel-induced degradation of the inhibitor of NF-κB, activation of NF-κB in a dose-dependent manner, and Bcl-2 and Bcl-xL upregulation. Finally, employing western blotting and crystal violet assays, the effects of the proteasome inhibitor ALLN were assessed. ALLN inhibited paclitaxel-induced NF-κB activation and restored the sensitivity to paclitaxel. Together, these data suggest that targeting the NF-κB/IKK axis might be a promising strategy to overcome paclitaxel resistance.

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Abbreviations: NF-κB, nuclear factor-κB; IKK, IκB kinase; IκB, inhibitor of NF-κB; EMT, epithelial-mesenchymal transition

Key words: breast cancer, paclitaxel, NF-κB, mesenchymal phenotype

Introduction

In 2020, breast cancer was both the most frequently diagnosed malignancy worldwide, with 11.7% of all new cancer diagnoses, and the most common cause of cancer death among women, with 15.5% of all fatalities; therefore, it remains a public health problem (1). The identification of breast cancer subtypes (Luminal A, Luminal B, HER2-enriched, basal and normal-like) is crucial for the selection of the most effective therapy (2-4). Depending on the breast cancer subtype, a specific treatment can be selected from among antimetabolites, and endocrine, immunological, alkylating or antimitotic drugs (5,6). Anthracyclines (doxorubicin) and taxanes (paclitaxel) are widely used for breast cancer treatment.

Paclitaxel is a taxane that binds to microtubules, promoting tubulin polymerization and inhibiting its depolymerization (7,8). The interference in the normal microtubule dynamics caused by paclitaxel activates the spindle assembly checkpoint (SAC), therefore inducing mitotic arrest and apoptosis (8-11). Paclitaxel has been widely used to treat several types of solid tumors, such as ovarian and breast cancer (12-15). Resistance to this drug is a major cause of treatment failure, and in consequence, tumor progression. Several mechanisms have been associated with paclitaxel resistance; one mechanism is the overexpression of the efflux protein P-glycoprotein, which increases the outflow of paclitaxel from the cells (16). Furthermore, the overexpression of Aurora A kinase, a regulator of SAC, has been implicated in paclitaxel resistance in triple-negative breast cancer cells (17). Another mechanism that contributes to this resistance is the overexpression of IκB kinase β (IKKβ), an upstream regulator of nuclear factor-κB (NF-κB) signaling (18). Although several mechanisms of paclitaxel chemoresistance have been described, they are not completely understood.

The transcription factor NF-κB family contains five members: NF-κB1 (p50), NF-κB2 (p52), Rel A (p65), c-Rel

and RelB. These transcription factors form homo- and hetero-dimers that under normal conditions reside in the cytoplasm bound to inhibitors, known as inhibitors of NF-κB (IκBs). The activation of NF-κB requires the IKK-dependent phosphorylation of IκBs (α, β and ε), which leads to polyubiquitination and subsequent degradation by the proteasome. Next, NF-κB is translocated to the nucleus where it regulates the expression of its target genes (19). NF-κB is activated by a wide variety of stimuli that include growth factors, cytokines, infectious agents and chemotherapy drugs, among others. Once activated, NF-κB is involved in the regulation of biological processes, such as proliferation, differentiation and apoptosis, and some pathologies, such as inflammation, cancer and chemotherapy resistance (20). The epithelial-mesenchymal transition (EMT) is a biological process in which a polarized epithelial cell normally interacts with the basement membrane via its basal surface, and undergoes a series of morphological and biochemical changes that allow it to adopt a mesenchymal phenotype (21,22). This phenotype increases migratory capacity, invasiveness, resistance to apoptosis and the production of extracellular matrix components (21). EMT is one of the multiple cellular processes that are regulated by NF-κB, since it also regulates the expression of SNAIL and Twist in breast, renal and colon cancer (23-25). EMT is an early step of cancer metastasis. During EMT, cell morphology and shape are modified to gain motility/invasive features, epithelial markers such as E-cadherin are downregulated and cells acquire mesenchymal markers such as N-cadherin and vimentin; these changes are controlled by the transcription factors SNAIL, Twist and Slug (21,26-28).

Paclitaxel is widely used in breast cancer treatment. Resistance is the main cause of treatment failure. The present study aimed to examine the association between paclitaxel resistance and mesenchymal phenotype, and its putative mechanism, in a model of primary breast cancer cells. Induction of cell death, apoptosis and NF-κB activation by paclitaxel were evaluated. To probe the importance of the NF-κB signaling pathway in paclitaxel resistance, a proteasome inhibitor (ALLN) was employed to interfere with the paclitaxel-induced cell death, the activation of NF-κB and induction of apoptosis. The association of paclitaxel resistance with the NF-κB signaling pathway in mesenchymal breast cancer cells will provide a potential target to overcome paclitaxel resistance in patients with breast cancer.

Materials and methods

Reagents and antibodies. Crystal violet and ALLN were purchased from MilliporeSigma, and paclitaxel from Intas Pharmaceuticals Ltd. Antibodies against E-cadherin (cat. no. 3195; clone 24E10; 1:1,000), N-cadherin (cat. no. 13116; clone D4R1H; 1:1,000), SNAIL (cat. no. 3879; clone C15D3; 1:1,000), vimentin (cat. no. 5741; clone D21H3; 1:1,000), pp65-Ser536 (cat. no. 3033; clone 93H1; 1:1,000), GAPDH (cat. no. 2118; clone 14C10; 1:2,000), Bcl-2 (cat. no. 4223; clone D55G8; 1:1,000), Bcl-xL (cat. no. 2764; clone 54H6; 1:1,000) and Bax (cat. no. 5023; clone D2E11; 1:1,000) were acquired from Cell Signaling Technology, Inc. Anti-Twist (cat. no. GTX-127310; 1:1,000) was purchased from GeneTex, Inc. Anti-IκBα (cat. no. SC-371; clone C-21; 1:1,000), anti-IKKα/β (cat. no. SC-7607; clone H-470; 1:1,000), anti-NF-κB p65 (cat. no. SC-8008; clone F-6; 1:1,000), anti-NF-κB p50 (cat. no. SC-114; 1:1,000), anti-β-actin (cat. no. SC-47778; clone C4; 1:2,000), and anti-caspase-3 (cat. no. 271028; clone B-4; 1:1,000) were obtained from Santa Cruz Biotechnology, Inc.

Primary breast cancer cell cultures. The primary breast cancer cell cultures MBCDF, MBCD17, MBCD3 and MBCDF-D5 were originated from a biopsy obtained from a radical mastectomy in a patient diagnosed with ductal infiltrating carcinoma as described previously (29,30) (protocol approved by the Research Ethics Committee from the National Institute for Medical Sciences and Nutrition 'Salvador Zubirán' (INCMNSZ); Ref 1549, BQO-008-06/9-1). Biopsies were minced and seeded as explants in RPMI-1640 (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.), and 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific, Inc.). Cells that grew from the explants were left to fill the plate, and then trypsinized and treated as a regular cell line and grown over 6 months in RPMI-1640 with 10% FBS, and 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C (5% CO_2). All primary cell cultures were tested for mycoplasma contamination. Mycoplasma-positive breast cancer cells were treated with 250 μg/ml tylosin (Merck & Co, Inc.) in RPMI-1640 with 10% FBS, and 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C (5% CO_2) for 12 days before performing any experiments. Experiments were performed after passage 10, when the phenotype observed was homogeneous. After tylosin treatment, primary breast cancer cells were maintained in RPMI-1640 with 10% FBS, and 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C (5% CO_2) until experimentation. MBA-MB-231 (ATCC® HTB-26™) and HCC1937 (ATCC® CRL-2336™) triple negative breast cancer cell lines were purchased from American Type Culture Collection. MDA-MB-231 and HCC1937 were cultured in RPMI-1640 with 10% of FBS, and 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C and 5% CO_2.

Western blot analysis. MBCDF, MBCD17, MBCD3 and MBCDF-D5 breast cancer cells were lysed in a buffer containing 50 mM HEPES (pH 7.4), 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 10 mM NaF, 50 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1X protease inhibitor cocktail (Complete EDTA-free; Roche Diagnostics). Protein was quantified using a Bradford assay (Bio-Rad Laboratories, Inc.). Whole protein extract (20 μg) was separated by 9 and 12% SDS-PAGE then transferred to Immobilon-P PVDF membranes (MilliporeSigma). The membranes were blocked with 5% skim milk in PBS-Tween (0.1%) for 1 h at room temperature. After this time, primary antibodies were added and incubated overnight at 4˚C. Membranes were washed and then incubated with anti-mouse horseradish peroxidase-conjugated secondary antibodies (cat. no. 115-035-003; 1:10,000; Jackson ImmunoResearch Laboratories, Inc.) or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (cat. no. 111-035-003; 1:10,000; Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature. Proteins were visualized using the ECL plus western blotting detection

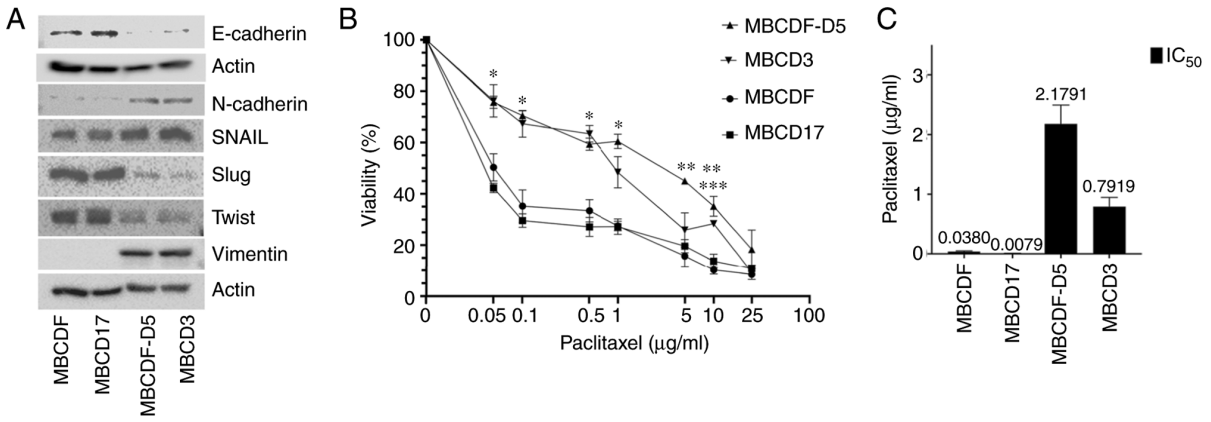

Figure 1. Mesenchymal phenotype is associated with paclitaxel resistance in primary breast cancer cells. (A) Expression of epithelial-mesenchymal transition markers (E-cadherin, N-cadherin, SNAIL, Slug, Twist and vimentin) was measured in the primary breast cancer MBCDF, MBCD17, MBCDF-D5 and MBCD3 cells by western blotting. (B) MBCDF, MBCD17, MBCDF-D5 and MBCD3 breast cancer cells were treated with increasing doses of paclitaxel (0, 0.05, 0.1, 0.5. 1, 5, 10 and 25 μg/ml). Cell viability was evaluated by crystal violet assay 48 h after treatment. Data represent the mean ± SEM of three independent experiments performed in triplicate *P<0.05 vs. control (0 μg/ml paclitaxel). **P<0.05 MBCDF-D5 (5 and 10 μg/ml paclitaxel) vs. control (0 μg/ml paclitaxel). ***P<0.05 MBCD3 vs. control (0 μg/ml paclitaxel). (C) IC_{50} was calculated using non-linear regression.

system (GE Healthcare) and blot images were digitized using Chemidoc (Bio-Rad Laboratories, Inc.). Densitometry of the bands was measured using ImageLab software v6 (Bio-Rad Laboratories, Inc.).

Viability assays. MBCD3, MBCDF-5, MBCD17 and MBCDF breast cancer cells were plated at 10,000 cells/cm^2 in a 48-well plate. On the following day, paclitaxel was added at increasing doses (0, 0.05, 0.1, 0.5, 1, 5, 10 and 25 μg/ml) and cells were cultured for 48 h at 37˚C (5% CO_2). Cell viability was evaluated by staining with crystal violet for 20 min at room temperature. The dye was dissolved in 400 μl of 10% acetic acid and the absorbance was read at 595 nm in a microplate reader (Opsys MR; Dynex Technologies). The percentage of cell viability was calculated after normalizing the absorbance of paclitaxel treated cells to the absorbance of non-treated cells. The plates were seeded in triplicate in at least three independent experiments. The data are presented as the mean ± SEM. In the cell viability assays using ALLN, MBCDF-D5 and MBCD3 cells were seeded at 10,000 cell/cm^2 in a 48-well plate. The next day ALLN (10 μM) was added for 2 h at 37˚C (5% CO_2), after this time breast cancer cells were treated with paclitaxel (0, 0.1, 1 and 10 μg/ml). Cells were cultured for a further 48 h at 37˚C (5% CO_2). Cell viability was evaluated using the crystal violet assay as aforementioned.

Statistical analysis. The results are presented as the mean ± SEM of three independent repeats. Paclitaxel dose-response curves were analyzed by two-way ANOVA and multiple comparisons were then performed employing Tukey's Honestly Significant Difference post-hoc test. Half maximal inhibitory concentration (IC_{50}) was calculated using a non-linear regression with 95% CI. In ALLN-paclitaxel combination assays, two-way ANOVA with Bonferroni's correction for multiple comparisons was used to analyze the statistical differences in cell viability between ALLN and ALLN plus paclitaxel. Data were analyzed using GraphPad PRISM v6.01 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Resistance to paclitaxel is associated with a mesenchymal phenotype. We have demonstrated previously that primary breast cancer cells with a specific pattern of receptor tyrosine kinase present with resistance to paclitaxel (30); moreover, we have also shown that cells with a mesenchymal phenotype are resistant to metformin (29). The present study investigated whether the mesenchymal phenotype is associated with paclitaxel resistance, and the putative mechanism. Four different primary breast cancer cell cultures, two of epithelial phenotype and two of mesenchymal phenotype, were used. MBCDF and MBCD17 cells expressed E-cadherin, SNAIL, Slug and Twist, and low levels of N-cadherin, and no vimentin expression was observed. MBCDF-D5 and MBCD3 cells expressed low levels of E-cadherin, Slug and Twist, but expressed N-cadherin and vimentin, and higher levels of SNAIL (Fig. 1A). These four different primary breast cancer cells were treated with increasing doses of paclitaxel (0, 0.05, 0.1, 0.5. 1, 5, 10 and 25 μg/ml) (Fig. 1B). It was found that paclitaxel induced cell death in a dose-dependent manner, with different slopes when comparing epithelial and mesenchymal cells. MBCDF-D5 and MBCD3 cells were more resistant to paclitaxel compared with MBCDF and MBCD17 cells. In the mesenchymal breast cancer cells, cell viability decreased to ~75% at 0.05 μg/ml of paclitaxel, and at the highest dose of 25 μg/ml, cell viability fell to 9-18%. By contrast, MBCDF and MBCD17 epithelial cells were more sensitive to paclitaxel; viability decreased to 50 and 42%, respectively, at the lower dose of 0.05 μg/ml, with a downward trend that dropped to 10% for MBCDF cells and 8% for MBCD17 cells at 25 μg/ml (Fig. 1B). Furthermore, the IC_{50} for epithelial breast cancer cells varied from 0.01 to 0.046 μg/ml for the MBCD17 and MBCDF cells, respectively. In the case of mesenchymal breast cancer cells, the IC_{50} was higher; MBCDF-D5 cells exhibited an IC_{50} of 2.57 μg/ml and MBCD3 cells had an IC_{50} of 0.93 μg/ml (Fig. 1C). To confirm that the effect of paclitaxel on primary breast cancer cells was similar to that on established cell lines, a cytotoxicity assay was performed. Two breast cancer cell lines (MDA-MB-231

and HCC1937) were selected. MDA-MB-231 cells resemble a mesenchymal phenotype similar to MBCDF-D5 cells by expressing vimentin, and HCC1937 cells express E-cadherin similar to MBCDF cells (31). MDA-MB-231, HCC-1937, MBCDF and MBCDF-D5 cells were treated with increasing doses of paclitaxel (0, 0.05, 0.1, 0.5, 1, 5, 10 and 25 μg/ml; Fig. S1). MBCDF primary breast cancer cells were more sensitive to paclitaxel than MBCDF-D5 cells, which were the most resistant. HCC-1937 and MDA-MB-231 cell lines showed an intermediate sensitivity compared with the primary breast cancer cell cultures, with HCC-1937 cells being slightly more resistant to paclitaxel than MDA-MB-231 cells (Fig. S1).

Increased expression of NF-κB signaling molecules. Since NF-κB has been shown to be involved with EMT as well as chemoresistance (23), the putative role of NF-κB in paclitaxel resistance was investigated in the present study. The expression of certain members of the NF-κB signaling pathway were evaluated in both epithelial and mesenchymal primary breast cancer cells. The results showed that the NF-κB p65 subunit, as well as IKKα/β were upregulated in the MBCDF-D5 and MBCD3 cells (mesenchymal phenotype). In contrast to the results observed for NF-κB p65, the p50 subunit was downregulated in the mesenchymal cells. IκBα did not show marked changes in its expression levels in both phenotypes compared with the actin loading control (Fig. 2).

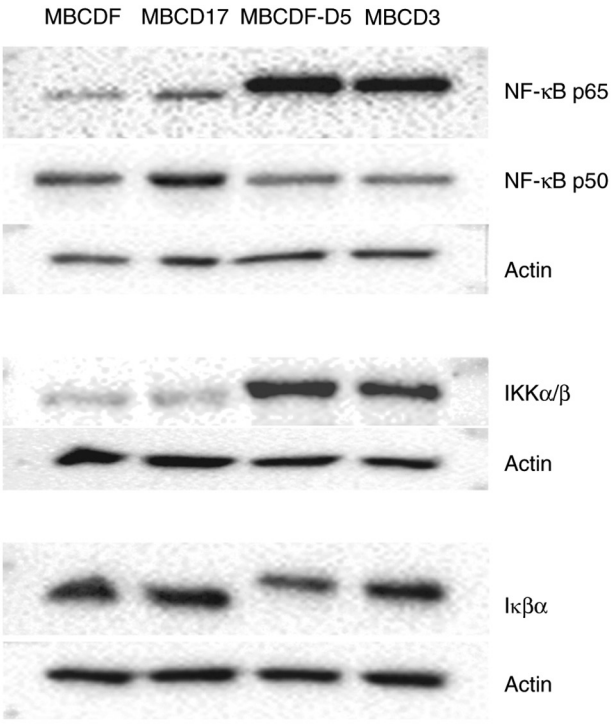

Figure 2. Expression analysis of NF-κB signaling molecules. NF-κB p65, NF-κB p50, IκBα and IKKα/β expression in MBCDF, MBCD17, MBCDF-D5 and MBCD3 breast cancer cells was evaluated by western blotting. Actin was used as a loading control. NF-κB, nuclear factor-κB; IKK, IκB kinase; IκB, inhibitor of NF-κB.

NF-κB activation of mesenchymal primary breast cells. Given the aforementioned observations, MBCDF-D5 and MBCD3 were first treated with increasing doses of paclitaxel (0, 1, 5, 10, and 50 μg/ml), and the activation of NF-κB by phosphorylation at S536 was evaluated. Phosphorylation of p65 at S536 increased in a dose-dependent manner in response to paclitaxel. The NF-κB p65 total levels were not changed with paclitaxel treatment (Fig. 3A). Secondly, investigations were performed to determine whether there was IκBα degradation upon different doses of paclitaxel. MBCDF-D5 and MBCD3 cells were treated with 0, 1, 5, 10 and 50 μg/ml paclitaxel (Fig. 3B). IκBα was degraded in a paclitaxel dose-dependent manner (Fig. 3B). Next, the effect of paclitaxel on NF-κB activation over time was evaluated. It was observed that phosphorylation of NF-κB at Ser536 reached its maximum levels at 1 h after treatment with paclitaxel (5 μg/ml) in MBCDF-D5 cells. NF-κB activation had a slight decrease at 2 h. In the case of MBCD3 cells, NF-κB phosphorylation at Ser536 increased in an oscillatory manner (Fig. 3C). Finally, MBCDF-D5 and MBCD3 were treated at different time points to evaluate IκBα degradation. Paclitaxel provoked IκBα degradation in a time-dependent manner. MBCDF-D5 cells exhibited evident IκBα degradation after 1 h of paclitaxel treatment. Meanwhile, MBCD3 cells showed marked IκBα degradation after 2 h of paclitaxel addition (Fig. 3D).

Bcl-2, Bax, Bcl-xL and procaspase-3 expression in response to paclitaxel in paclitaxel-resistant primary breast cancer cells. To evaluate the role of apoptosis in the paclitaxel-resistant primary breast cancer cells, the expression of Bcl-2, Bax, Bcl-xL and procaspase-3 was examined by western blotting. First, changes in these markers in response to increasing doses of paclitaxel were measured. In the MBCDF-D5 cells, Bcl-2 expression was elevated by paclitaxel at the concentrations of 1 and 5 μg/ml, and was downregulated at 10 and 50 μg/ml. Bax expression had a slight fluctuation with concentrations varying from 1 to 2 fold-change difference. Bcl-xL expression increased in response to paclitaxel in a dose-dependent manner and procaspase-3 levels decreased in a dose-dependent manner (Fig. 4A; left panel). In the MBCD3 cells, Bcl-2 expression was elevated by paclitaxel in an increasing trend from 1 to 10 μg/ml, and at the highest dose (50 μg/ml), dropped to 1.7 fold-change difference. Bax expression was augmented in a dose-dependent manner, increasing to an 11 fold-change difference at 5 μg/ml and with the highest level at 50 μg/ml. Bcl-xL was upregulated, procaspase-3 was downregulated, and both respond in a dose-dependent manner (Fig. 4A; right panel). Secondly, Bcl-2, Bax, Bcl-xL, and procaspase-3 were assessed over a time-course of 3 h. The results showed that Bcl-2 reached its peak at 3 h of paclitaxel treatment in MBCDF-D5 cells. By contrast, Bax levels were decreased to 0.5-fold at 0.5 h, then increased up to basal levels at 3 h. Paclitaxel augmented Bcl-xL expression 3.1-fold at 3 h and procaspase-3 had small fluctuations during the 3 h period (Fig. 4B). In the MBCD3 cells, paclitaxel increased Bcl-2 expression levels to a maximum of 1.9-fold at 0.5 h, which began to diminish by 1 h, reaching the lowest levels at 3 h. Bax and procaspase-3 expression exhibited small variations during the 3-h period. Bcl-xL expression increased up to 1.5-fold at 1 h and then the expression decreased (Fig. 4B). Since changes were not observe in some of the markers over this short interval of time, MBCF-D5 and MBCD3 cell were next treated with paclitaxel for 24 h. Paclitaxel induced downregulation of Bax and procaspase-3

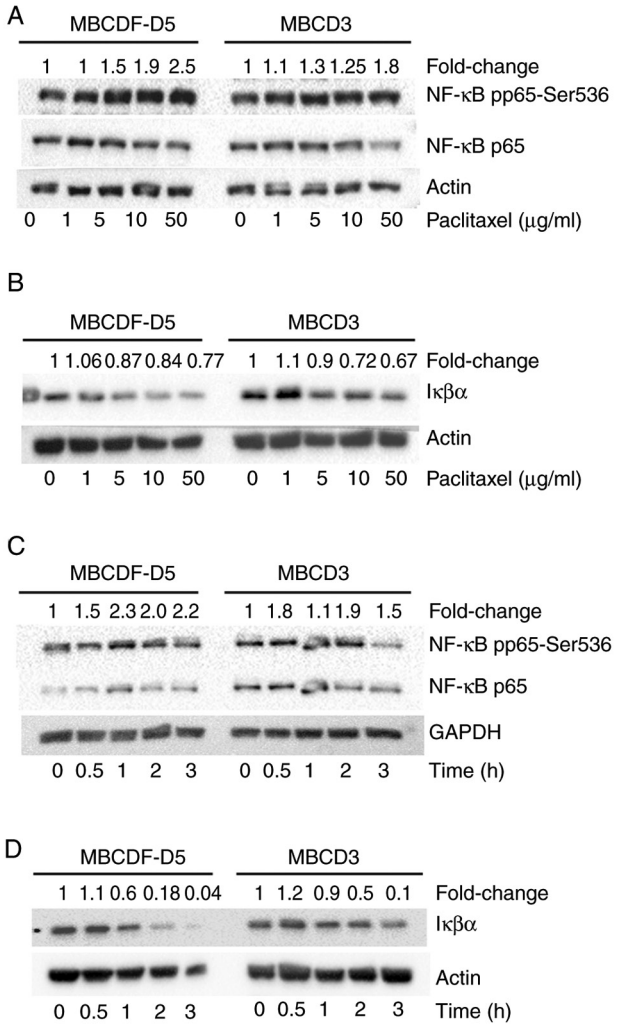

Figure 3. Paclitaxel induces phosphorylation of NF-κB at Ser536 and degradation of IκBα. (A) Expression of NF-κB pp65-Ser536, and NF-κB p65 in MBCDF-D5 and MBCD3 cells treated with 0, 1, 5, 10 and 50 μg/ml paclitaxel. (B) Degradation of IκBα in MBCDF-D5 and MBCD3 cells treated with 0, 1, 5, 10 and 50 μg/ml paclitaxel. (C) Expression of NF-κB pp65-Ser536, and NF-κB p65 in MBCDF-D5 and MBCD3 cells treated with 5 μg/ml paclitaxel for 0, 0.5, 1, 2 and 3 h. (D) Degradation of IκBα in MBCDF-D5 and MBCD3 cells treated with 5 μg/ml paclitaxel for 0, 0.5, 1, 2 and 3 h. Actin and GAPDH were used as loading controls. Densitometry of the bands was quantified by ImageLab software. The ratio between NF-κB pp65-Ser536 and NF-κB p65 was normalized against the loading control. The fold change of IκBα was calculated by normalizing IκBα against actin. NF-κB, nuclear factor-κB; IκB, inhibitor of NF-κB.

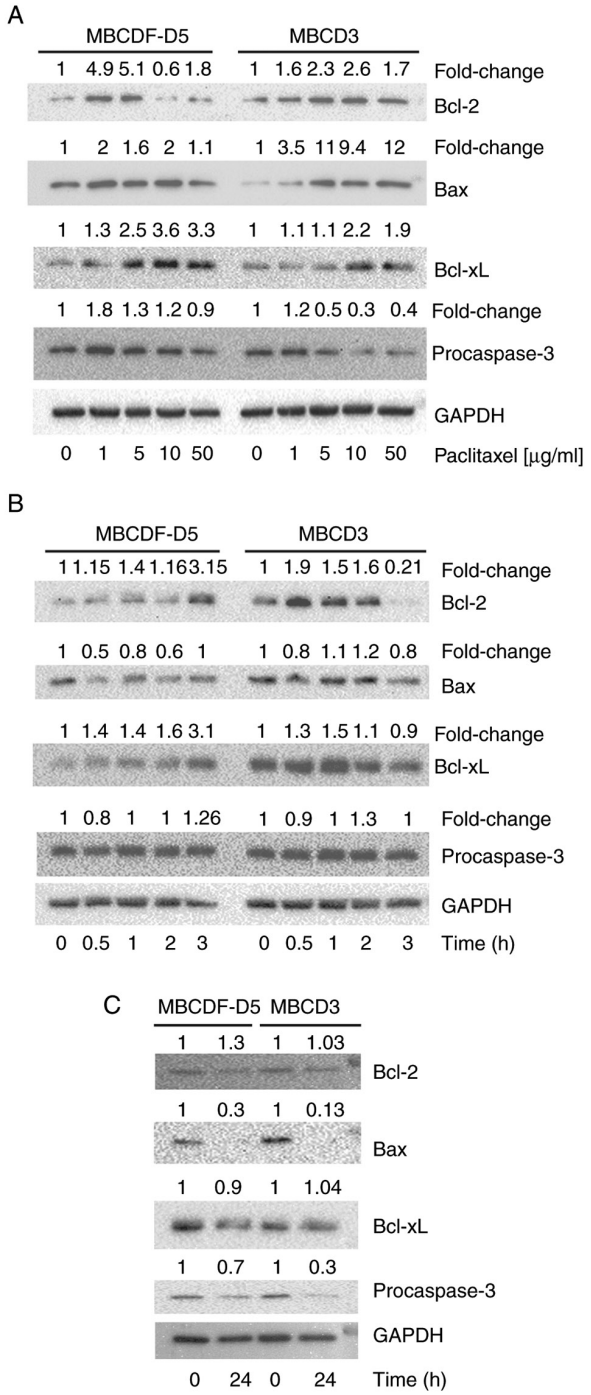

Figure 4. Expression of Bcl-2, Bax, Bcl-xL and procaspase-3 in breast cancer cells treated with paclitaxel. (A) Bcl-2, Bax, Bcl-xL and procaspase-3 were evaluated by western blotting in MBCDF-D5 and MBCD3 cells treated with 0, 1, 5, 10 and 50 μg/ml paclitaxel. (B) Bcl-2, Bax, Bcl-xL and procaspase-3 expression was assessed by western blotting in MBCDF-D5 and MBCD3 cells as in (A) following treatment with paclitaxel for 0, 0.5, 1, 2 and 3 h. (C) MBCDF-D5 and MBCD3 cells were treated with 5 μg/ml for 24 h. Bcl-2, Bax, Bcl-xL and procaspase-3 expression was evaluated by western blotting following treatment with paclitaxel for 0 or 24 h. GAPDH was used as a loading control. Densitometry of the bands was quantified by ImageLab software. Fold change of Bcl-2, Bax, Bcl-xL and procaspase-3 expression was calculated as the ratio of paclitaxel-treated cells over untreated cells and normalized against the loading control.

after 24 h of stimulation (Fig. 4C). In addition, it was observed that although Bcl-2 and Bcl-xL were upregulated over a short period of time (Fig. 4B), they returned to their basal levels after 24 h (Fig. 4C).

ALLN enhances phosphorylation of NF-κB at Ser536 and cell death in paclitaxel-resistant primary breast cancer cells. ALLN is a proteasome inhibitor that functions by stabilizing the degradation of IκBα and then producing inhibition of NF-κB (32,33). To investigate whether ALLN could interfere with paclitaxel-induced NF-κB and, for instance, overcoming the resistance to paclitaxel, MBCDF-D5 and MBCD3 cells were pre-treated with 10 μM ALLN 2 h before paclitaxel addition. Proteasome inhibition with ALLN and ALLN plus paclitaxel provoked a 7.4- and 8.4-fold increase, respectively, in NF-κB pp65 expression, with no changes in total NF-κB p65 in the MBCDF-D5 cells (Fig. 5A). In the MBCD3 cells,

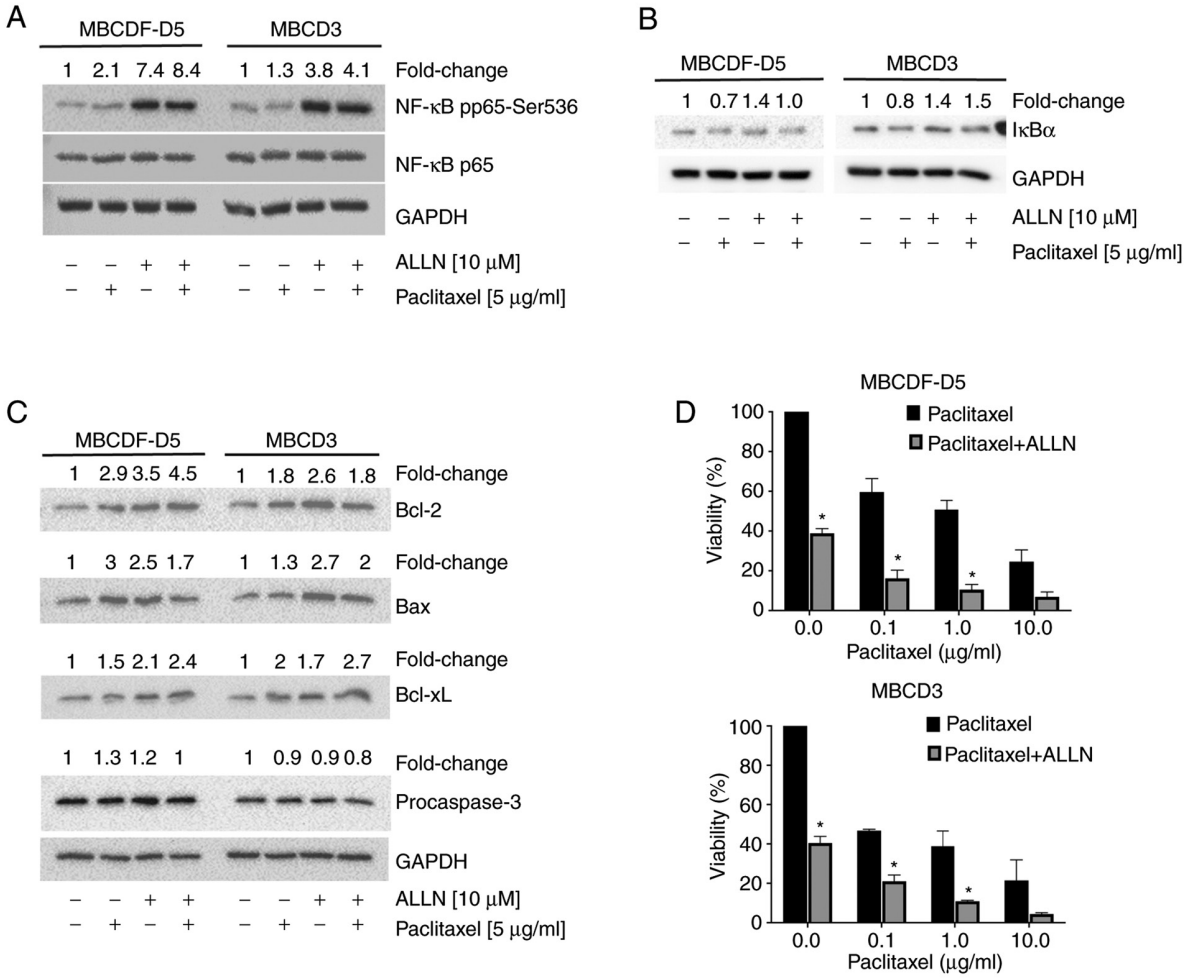

Figure 5. Proteasome inhibition with ALLN provokes IκBα degradation and accumulation of NF-κB pp65-Ser536, and increases paclitaxel-induced cell death. (A) MBCDF-D5 and MBCD3 primary breast cancer cells were treated with 10 μM ALLN for 2 h, and after this, 5 μg/ml paclitaxel was added, and cells were incubated for 1 h. Western blotting was performed to evaluate the phosphorylation of NF-κB p65 and total NF-κB-p65. GAPDH was used as a loading control. (B) MBCDF-D5 and MBCD3 cells were treated as in (A). Western blotting was performed to assess IκBα degradation. GAPDH was used as a loading control. (C) MBCDF-D5 and MBCD3 cells were treated as in (A). Apoptosis markers Bcl-2, Bax, Bcl-xL and procaspase-3 were evaluated by western blotting. GAPDH was used as a loading control. (D) MBCDF-D5 and MBCD3 cells were pre-treated with 10 μM ALLN for 2 h before the addition of 0, 0.1, 1 and 10 μM paclitaxel. Cell viability was evaluated 48 h after treatment with crystal violet. Data represent the mean ± SEM of three independent experiments performed in triplicate. Two-way ANOVA with Bonferroni's correction for multiple comparisons was used to analyze the statistical differences in cell viability between ALLN and ALLN plus paclitaxel. *P<0.05 vs. paclitaxel.

ALLN produced similar results, with a 3.8- and 4.1-fold increase in NF-κB pp65 expression for ALLN and ALLN plus paclitaxel, respectively, while the amount of NF-κB p65 was not affected by treatment with ALLN, paclitaxel or ALLN plus paclitaxel (Fig. 5A). Second, the study examined whether ALLN prevented IκBα degradation. MBCDF-D5 and MBCD3 cells were stimulated with 10 μM ALLN for 2 h before the addition of 5 μg/ml of paclitaxel (Fig. 5B). The results showed that ALLN blocked IκBα degradation and produced its accumulation due to proteasome inhibition in MBCD3 breast cancer cells. In the case of MBCDF-D5 cells, ALLN plus paclitaxel prevented degradation of IκBα by only returning to basal levels but was not associated with its accumulation (Fig. 5B). Next, Bcl-2, Bax, Bcl-xL, and procaspase-3 expression were evaluated under the aforementioned conditions in both MBCDF-D5 and MBCD3 cells. ALLN alone increased Bcl-2 expression, 3.5-fold in MBCDF-D5 cells and 2.6-fold in MBCD3 cells (Fig. 5C). The combination of ALLN plus paclitaxel resulted in a 4.5-fold increase in expression in MBCDF-D5 cells; meanwhile, expression in MBCD3 cells decreased to 1.8-fold. Bcl-xL expression was also increased following treatment with ALLN alone in both primary breast cancer cell cultures, and ALLN plus paclitaxel further raised Bcl-xL expression. ALLN and the combination of ALLN plus paclitaxel had few effects on procaspase-3 expression (Fig. 5C). Finally, the study evaluated whether ALLN could increase paclitaxel-induced cell death. Cytotoxicity assays were performed by adding 10 μM ALLN at 2 h before the addition of increasing doses of paclitaxel. Cell viability was evaluated at 48 h after paclitaxel stimulation using a crystal violet assay. It was found that ALLN potentiates paclitaxel-induced cell death in both MBCDF-D5 and MBCD3 breast cancer cells in a dose-dependent manner (Fig. 5D).

Discussion

Paclitaxel is employed for breast cancer treatment; however, chemoresistance is often a challenge to its use (34). This

chemoresistance has been attributed to several mechanisms, including the upregulation of P-glycoprotein (35) and mutations in β-tubulin that affect the binding of paclitaxel (36,37). However, the exact mechanism of paclitaxel resistance is still elusive. The goal of the present study was to examine the association of paclitaxel resistance with mesenchymal phenotype, and its putative mechanism in primary breast cancer cells. Primary breast cancer cells with mesenchymal phenotype showed resistance to paclitaxel, and this was associated with increased levels of NF-κB p65 and IKKα/β. Moreover, paclitaxel induced NF-κB phosphorylation of NF-κB p65 at Ser536 and degradation of IκBα. It was observed that upon paclitaxel-induced NF-κB activation, there was upregulated expression of Bcl-2 and Bcl-xL, which are NF-κB target genes. Bax and procaspase-3 expression was negatively regulated after paclitaxel treatment. Furthermore, NF-κB inhibition using the proteasome inhibitor ALLN provoked accumulation of phosphorylated NF-κB and IκBα, and increased susceptibility to paclitaxel.

EMT is considered the first step for metastasis and is characterized by dissolution of tight junctions, loss of cell polarity and epithelial markers such as E-cadherin, and gain in expression of mesenchymal markers such as N-cadherin and vimentin (21,22). Furthermore, EMT has been associated with resistance to conventional therapies (38,39). Our previous study have shown that primary breast cancer cells with expression of HER1, HER3, c-Met and VEGFR2 are resistant to paclitaxel (30). The data from the present study indicated that primary breast cancer cells with expression of the aforementioned receptor tyrosine kinases presented mesenchymal features and were resistant to paclitaxel compared with breast cancer cells with an epithelial phenotype. These data correlate with previous investigations that have suggested that EMT produces chemotherapy resistance (38-40).

The EMT process is controlled by several transcription factors, including SNAIL, Slug and Twist. NF-κB is another transcription factor that is important for EMT via the regulation of SNAIL and Twist expression in breast, renal and colon cancer (23-25,41). Several reports have associated NF-κB with certain pathologies such as inflammation, cancer and chemotherapy resistance (20,23). The elevated expression of NF-κB p65 has been observed in ovarian and breast cancer, and has been associated with the clinical stage and the histological subtype (42,43). The present study found increased levels of IKKs and NF-κB p65 in mesenchymal primary breast cancer cells. These results were consistent with a previous study where NF-κB p65 has been shown to be required in the late stages of tumorigenesis, to mediate and maintain the EMT phenotype (28); these authors also showed that inhibition of IKKβ/IκBα/NF-κB axes using the super-repressor of IκBα, which contains mutations in Ser32/36Ala, inhibited NF-κB DNA-binding activity and TGF-β-induced EMT (28). By contrast, expression of constitutively active IKKβ increased NF-κB DNA-binding activity and EMT markers independently of TGFβ stimulation (28). Other investigations demonstrated that IKKβ is an upstream regulator of NF-κB activation that has been involved in the regulation of proliferation and migration of cisplatin-resistant head and neck squamous carcinoma cells (23,44). Expression of IKKβ has also been associated with higher levels of N-cadherin and cisplatin resistance in head and neck squamous carcinoma cells (23). In accordance with the aforementioned results, the present study observed elevated levels of IKKβ and N-cadherin in mesenchymal breast cancer cells and paclitaxel resistance. In melanoma, NF-κB provokes tumor aggressiveness by activating MMP9 (45). Moreover, integrin β-like 1 induces metastasis, invasion and EMT in prostate cancer through activation of NF-κB (46). In the breast cancer MDA-MD-231 and HCC-1937 cell lines, inhibition of NF-κB decreased levels of EMT markers such as Slug, Sip1, Twist and MMP11, and decreased aggressiveness measured by migration and invasion assays (47). These results suggest a correlation between the high expression of NF-κB/IKK, mesenchymal phenotype and paclitaxel resistance in primary breast cancer cells. In addition, paclitaxel induced activation of NF-κB in mesenchymal primary breast cancer cells that agreed with previous reports showing paclitaxel-induced NF-κB activation in breast and ovarian cancer cells (48,49). Furthermore, NF-κB has been implicated in the regulation of other mechanisms of paclitaxel resistance, such as the induction of P-glycoprotein expression. In accordance with these findings, we have observed high expression of MDR in MBCF-D5 and MBCD3 mesenchymal breast cancer cells that have elevated levels of NF-κB p65 concomitant with paclitaxel resistance (data not shown). In this context, Abdin *et al* (50) demonstrated that breast cancer MCF-7 and MDA-MB-231 cell lines resistant to doxorubicin exhibited increased levels of NF-κB p65, which is linked to high expression of MDR. The study showed that the combination of doxorubicin and NF-κB inhibitors sensitized breast cancer cell lines to chemotherapy and decreased expression of MDR (50).

By contrast, the present study found that levels of NF-κB p50 were higher in epithelial cells compared with those in mesenchymal cells. NF-κB p50 or NF-κB1 is a product of proteasomal processing of p105. Structurally, NF-κB p50 lacks the transcriptional domain, and thus its function as a transcription factor depends on its heterodimerization with other members of the family. Functionally, NF-κB p50 participates in the reprogramming of genes involved in inflammation, the inhibition of apoptosis, angiogenesis, cell survival and cell proliferation (51). Overexpression of NF-κB p50 induces tumor growth with an increase in the formation of p50-p50 homodimers, while p50-p65 heterodimers are diminished (52). Normal expression levels of NF-κB p50 have been found in breast cancer, melanoma and lung cancer (51). By contrast, high expression of NF-κB p50 in gastric cancer has been shown to correlate with tumor size and metastasis (53). According to the aforementioned findings, the role of NF-κB p50 seems contradictory in different types of cancer. The reason why less expression of NF-κB p50 was observed in the present study is not clear, and further studies must be developed to understand the role of NF-κB p50 during EMT in breast cancer cells.

NF-κB resides inactive in the cytoplasm; its activation requires phosphorylation of IκBα by IKKs targeting IκBα for degradation by the proteasome. Proteasome inhibitor ALLN is a potent inhibitor of NF-κB activation that functions by preventing IκBα degradation (32,33). In the present study, ALLN pre-treatment before paclitaxel treatment induced degradation of IκBα and accumulation of phosphorylated NF-κB p65 in mesenchymal breast cancer cells, and increased paclitaxel-induced cell death. These results suggest that

inhibition of NF-κB activation reverses paclitaxel resistance in mesenchymal breast cancer cells.

NF-κB has been associated with the transcription of anti-apoptotic genes such as Bcl-2 and Bcl-xL (54). The present results showed that paclitaxel increased the expression of these antiapoptotic markers, most likely through NF-κB activation. These results agreed with a previous study where paclitaxel upregulated NF-κB (55). In the present study, the proapoptotic marker Bax was downregulated by paclitaxel and procaspase-3 was not cleaved, suggesting that apoptosis was inhibited in these cells. Notably, ALLN did not abrogate the high levels of Bcl-2 and Bcl-xL induced by paclitaxel, and when used alone actually induced the expression of both antiapoptotic markers. Bcl-2 is known to be regulated by the proteasome and this could explain the accumulation of Bcl-2 in the presence of ALLN (56). The fact that ALLN sensitized resistant breast cancer cells to paclitaxel suggested that the cell death caused by ALLN plus paclitaxel was through a mechanism independent of Bcl-2 family members.

Although other breast cancer cell lines had similar behavior in their sensitivity to paclitaxel (Fig. S1), the present primary breast cancer cell cultures model retains some features of the original tumor, such as intratumor heterogeneity, as previously demonstrated (57). The primary breast cancer cells have less accumulated mutations due to continuing passaging *in vitro* compared with established cell lines such as MDA-MB-231 and HCC1937 (58). Even though this system turns out to be extraordinarily reproducible and permitted the exploration of different aspects of cell signaling and the mechanisms for drug resistance, the translation from an *in vitro* model to humans has required further research. Two models have been used to closer approach what occurs in humans. One is the use of 3-dimensional (3D) cultures and tumor xenografts in an immunosuppressed mouse model. Organoids are produced by culturing embryonic stem cells, tissues, or induced pluripotent stem cells in a 3D extracellular matrix, typically using Matrigel; this method has received special attention since it recreates the spatial conformation of a tumor mass trying to overcome the limitations of monolayer cell culture (59,60). The use of embryonic or adult stem cells is crucial for self-organization, self-renewal and differentiation. Organoids provide a better representation of solid tumors when compared with monolayers, since they can resemble a tumor mass where the exterior cells will be better exposed to nutrients, oxygen and, importantly, to chemotherapy agents, and by contrast, cells in the interior of the organoid will have little exposure. When investigating paclitaxel resistance in organoids originating from primary breast cancer cells, the organoid will represent a tumor mass in which the paclitaxel will have little access to the cells in the center. Organoids are not as complex as tumors, since they lack the components of the tumor microenvironment. By contrast, a tumor xenograft is a piece of tumor tissue, a cell suspension from a disaggregated tumor or a cell line that is injected into a subcutaneous position or an orthotopic site (61,62). The tumor xenograft is subjected to a selective pressure in its new niche, and its successful growth will depend on different factors, including the mouse strain, the aggressiveness of the tumor and the interactions that are established within the new microenvironment (63,64). The use of a tumor xenografted model with primary breast cancer cells will closely resemble the original tumor. An orthotopic xenograft model will provide relevant information on the *in vivo* efficacy of the combination of paclitaxel and a proteasome inhibitor such as bortezomib (Velcade) to inhibit NF-κB.

The present study demonstrated that primary breast cancer cells with mesenchymal phenotype have high levels of IKK/IκBα/NF-κB p65 and that this is associated with the resistance to paclitaxel. We previously demonstrated that these paclitaxel-resistant breast cancer cells present a specific pattern of receptor tyrosine kinase and the present study showed that these cells had a mesenchymal phenotype and elevated expression of the NF-κB/IKK axis. Together, this research highlights the importance of targeting the IKK/IκBα/NF-κB p65 axes in mesenchymal breast cancer cells resistant to paclitaxel and the putative use of a proteasome inhibitor as a regimen to overcome paclitaxel resistance.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Design and coordination of the study: MDJIS, JEL and ELR. Generation of primary breast cancer cell cultures: MDJIS and JEL. Paclitaxel cytotoxicity assays: OL and JCGD. Western blot assays: JEL and ENDLCE. ALLN assays: ENDLCE. Statistical analysis: MDJIS and OL. Data analysis and discussion: MDJIS, JEL and ELR. Writing of the manuscript: MDJIS and JEL. MDJIS, JEL and OL confirm the authenticity of all raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Primary breast cancer cells were generated from a tissue biopsy obtained during surgery in patients with breast cancer. All patients provided written informed consent for a protocol

approved by the Ethics and Research Committee of the National Medical Sciences and Nutrition Institute Salvador Zubirán (approval no. 1549, BQO-008-06/9-1).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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