Breast Cancer Res Treat
DOI 10.1007/s10549-008-0231-y

BRIEF REPORT

Tamoxifen and ICI 182,780 increase Bcl-2 levels and inhibit growth of breast carcinoma cells by modulating PI3K/AKT, ERK and IGF-1R pathways independent of ERα

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Received: 15 August 2008 / Accepted: 17 October 2008

Abstract We recently showed that estrogen withdrawal from the ERα$^+$, high Bcl-2-expressing breast carcinoma cells (MCF-7B) reduced Bcl-2 protein levels while increasing cell–cell adhesion, and junction formation. Here we compared these cells with the ERα$^+$ and low Bcl-2-expressing MCF-7 cells and with the normal mammary epithelial cell line MCF-10-2A not expressing ERα or Bcl-2. All cell lines expressed normal HER2. Antiestrogen (Tamoxifen and ICI 182,780) treatment increased Bcl-2 levels in both MCF-7 and -7B cells and led to the formation of acinar structures. This treatment led to the dissociation of junctions and redistribution of junctional components to the cytoplasm in MCF-10-2A and -7 cells, while in MCF-7B cells junctional proteins redistributed to membranes. Antiestrogen treatment decreased PI3K/Akt activation and increased ERK activation regardless of ERα status. IGF-1R was inactivated in the antiestrogen-treated MCF-7 cells while it was activated in MCF-7B cells. Our data show that Tamoxifen and ICI 182,780 can induce growth inhibitory effects via the sustained activation/inactivation of signaling pathways that regulate cell survival, cell death and differentiation in the absence of ERα. Furthermore, Bcl-2 overexpression may alter the functional interactions among these pathways in response to antiestrogens, which also may provide a potential explanation for the observation that Bcl-2 overexpressing tumors have a better prognosis.

Keywords Cadherin · Estrogen receptor · Bcl-2 · PI3K/AKT · ERK · IGF-1R · Tamoxifen · ICI

Introduction

Disruption of cell–cell adhesion is essential for cell proliferation and migration during tumorigenesis and metastasis. As in other epithelia, cells in mammary glands are held together by cadherin-mediated adhesion. E-cadherin is a calcium-dependent transmembrane glycoprotein and the central component of the adherens junction [1]. The extracellular domains of E-cadherin molecules on adjacent cells interact in a homotypic fashion to bridge cells together. Intracellularly, E-cadherin interacts with several cytoplasmic proteins, mainly catenins. Catenins are cytoplasmic proteins with dual adhesive and signaling functions [2]. β-Catenin or γ-catenin (plakoglobin, Pg) interacts directly, and in a mutually exclusive manner, with the C-terminal domain of E-cadherin. The E-cadherin-β-catenin or -Pg complexes in turn associate with α-catenin, which connects the cadherin–catenin complex directly or indirectly to the actin cytoskeleton. p120 Catenin binds to the juxtamembrane domain of E-cadherin, and while it does not interact with the actin cytoskeleton, it plays an important role in regulating the adhesive strength of the cadherin–catenin complex. Thus, E-cadherin interactions with catenins lead to the reorganization of the actin cytoskeleton, stabilization of adhesion complexes at the membrane and activation and/or modulation of a number of intracellular signaling

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Electronic supplementary material The online version of this article (doi:10.1007/s10549-008-0231-y) contains supplementary material, which is available to authorized users.

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pathways that regulate cell proliferation, differentiation, motility, migration and programmed cell death [1–4].

The balance between cell proliferation and cell death is essential for the maintenance of cell number and tissue homeostasis. Physiological/programmed cell death (apoptosis) is necessary for proper embryonic development, tissue homeostasis and host-defense mechanisms [5 and references therein]. The Bcl-2 family of proteins, with pro- and anti-apoptotic members, regulates apoptosis during mammary gland development and mammary tumorigenesis [6, 7]. Bcl-2 protein, the prototypic member of the Bcl-2 family, has anti-apoptotic activities and a newly defined role in the regulation of the cell cycle [8 and references therein].

Bcl-2 is expressed in 40–80% of breast tumors and while it is not an independent marker of prognosis, its expression has been associated with favorable prognosis in estrogen receptor alpha positive ($ER\alpha^+$) breast tumors [9 and references therein]. About 70% of breast tumors are $ER\alpha^+$ and over 50% of them respond favorably to antiestrogen therapy, a response that generally coincides with decreased Bcl-2 levels [10, 11].

Estrogen regulates both E-cadherin and Bcl-2 expression, although in opposite ways. Estrogen directly upregulates the expression of the *Bcl-2* gene [12] and decreases the expression of the E-cadherin gene (*CDH1*) by either binding directly to the *CDH1* promoter or by increasing the expression of *snail*, a repressor of *CDH1* [13]. Previously, we showed that overexpressing Bcl-2 in MCF-7 breast carcinoma cells (hereafter MCF-7B) decreased cadherin-mediated adhesion. Furthermore, when MCF-7B cells were cultured in estrogen-depleted media, Bcl-2 levels were decreased in a subset of cells where cadherin-mediated adhesion was restored [14].

In the present study, we have used 2- and 3-D cultures of mammary carcinoma cell lines, with different ERα status and Bcl-2 protein levels in conjunction with the antiestrogens Tam and ICI to assess the relationship between Bcl-2 expression and cadherin-mediated adhesion. We show that Bcl-2 overexpression in MCF-7B cells interferes with contact inhibition of growth but invasiveness remains the same as in MCF-7 cells. Tam increases and ICI drastically decreases ERα levels, yet both treatments increase Bcl-2 levels. Both antiestrogens lead to the membrane redistribution of junctional proteins in MCF-7B cells. In spite of increased Bcl-2 levels, both antiestrogens have growth inhibitory effects in 2-D cultures and induce acini formation in 3-D cultures. The growth inhibitory effect of both antiestrogens involves the PI3K/Akt, ERK and IGF-1R pathways. Regardless of ERα and Bcl-2 expression, Tam and ICI both decrease PI3K/Akt phosphorylation and increase ERK1/2 phosphorylation. The antiestrogen-treated MCF-10-2A and -7 cells, with no and low Bcl-2 expression, respectively, show decreased IGF-1R phosphorylation, while the Bcl-2 overexpressing MCF-7B cells show increased IGF-1R phosphorylation. These changes in signaling pathways coincide with cell cycle arrest, cell death and/or cell differentiation, depending on the Bcl-2 levels.

Materials and methods

Cells, culture conditions, drug treatment and antibodies

All chemical and reagents were purchased from Sigma–Aldrich (Canada) unless stated otherwise. MCF-10-2A, a non-tumorigenic mammary epithelial cell line was obtained from ATCC (Manassas, VA) and cultured in DMEM/F12 (Gibco BRL) media supplemented with 5% donor horse serum (Gibco BRL), 20 ng/ml EGF, 10 ng/ml insulin, 100 ng/ml cholera toxin, 500 ng/ml hydrocortisone, 50 U/ml penicillin (Gibco BRL), 50 mg/ml streptomycin and 50 mg/ml Kanamycin (Gibco BRL). MCF-10A-NeoST, a Ras-transformed MCF-10A cell line [15] was kindly provided by Dr. Mina Bissell (Lawrence Berkeley National Laboratory, Berkeley, CA). MCF-7 cells and their Bcl-2 overexpressing transfectants (MCF-7B) have been described [14]. MCF-7, -7B and -10A-NeoST were maintained in MEM (Gibco BRL] supplemented with 10% fetal bovine serum (FBS) and antibiotics. The phenol red-free alpha-MEM used in antiestrogen experiments was purchased from Gibco BRL.

For 2-D cultures, cells were trypsinized, resuspended in the appropriate media and plated at 5×10^4 cells per well on 12 mm (diameter) glass coverslips or at 5×10^5 cells/cm^2 in tissue culture dishes. Media were replaced every 3 days. Three-D cultures were established in Matrigel (BD Biosciences, San Jose, CA, USA) and prepared according to the manufacturer's instructions. Eight-chambered RS glass slides (Fisher, Canada) were coated evenly with 45 µl Matrigel per well and allowed to solidify for 15 min. Cells from various cell lines were trypsinized and resuspended in the corresponding medium at a concentration of $(1–5) \times 10^4$ cells/ml. The cells were mixed 1:1 with medium containing 4% Matrigel and 400 µl of the cell suspension was added to each chamber.

ICI and Tam were purchased from Tocris (Missouri, USA) and Sigma–Aldrich (Canada), respectively. Stock solutions of 1 mM (ICI) and 10 mM (Tam) were prepared in DMSO. ICI and Tam were added to the culture media at final concentrations of 10^{-7} and 10^{-6} M, respectively.

For antiestrogen treatment, cultures were established in normal media for 24 h and then transferred into phenol red-free, alpha MEM media containing charcoal-stripped serum (CS-FBS) for 48 h before antiestrogens were added.

Antiestrogen treated cultures were maintained under these conditions for an additional 6–8 days with daily media replacement before they were processed for immunofluorescence staining or preparation of total cell extracts.

The PI3K (LY-294002) and ERK1/2 (UO126) inhibitors were purchased from Sigma–Aldrich and Promega, respectively. Control and antiestrogen-treated cultures were incubated in media containing 50 mM of each inhibitor for 24 h before processing for Western blot.

Antibodies used in various assays are listed in Table 1. The adherens junction markers, E-cadherin, α-catenin, β-catenin and plakoglobin and the tight junction marker, ZO-1 were used to assess junction formation.

Growth assays

Two-D cultures of MCF-10-2A, -7, -7B or ICI and Tam treated MCF-7 and -7B cells were plated in triplicate wells of 24-well plates at single cell density (10^4 cells/cm^2). At 1, 4, 7, 10, and 14 days after plating, cultures were trypsinized and cells were counted. Each time point represents the average of three independent experiments.

Matrigel invasion assays

Assays were performed according to the manufacturer's protocol (BD Bioscience, San Jose, CA, USA). Briefly, MCF-10-2A, -10A-NeoST, -7 and -7B cells were maintained in the corresponding serum-free media overnight before they were trypsinized and resuspended (10^6 cells/ml) in the same media. For each cell line, 5×10^5 cells in 0.5 ml serum-free media were plated in the top compartment of Matrigel-coated invasion chambers (0.8 μm pore membrane). Fibroblast conditioned media (0.75 ml) was added to the bottom chambers and plates were incubated overnight at 37°C in 5% CO_2 atmosphere. Twenty-four hours later, the membranes were recovered, fixed, and stained with DiffQuick (IMEB, San Marcos, CA, USA).

Table 1 Antibodies, fluorescent probes and their respective dilutions in specific assays

	Assay		Source
	IF	WB	
1^0 Antibodies			
AKT	–	1:1000	Santa Cruz (SC-8312)
pAKT	–	1:1000	Cell signaling (9271)
β-Actin (M[a])	–	1:5000	Sigma (A-4700)
α-Catenin (R[b])	1:100	1:1000	Sigma (C-2081)
β-Catenin (R)	1:100	1:1000	Sigma (C-2206)
β-Catenin (M)	1:100	1:1000	Sigma (C-7207)
Bcl-2 (M)	1:100	1:1000	Upstate (05-341)
Bcl-2 (Sheep)	1:100	–	Andrews, D[c]
E-cadherin (M)	1:200	1:1000	Transduction lab (610182)
ERα (R)	1:100	–	Santa Cruz (SC-543)
ERα (R)	1:100	1:1000	Santa Cruz (SC-H-20)
ERK1/2 (p42)	–	1:1000	Cell signaling (9107)
pERK1/2 (p44/42)	–	1:1000	Cell signaling (4377)
IGF-1R	–	1:500	Santa Cruz (C-20)
pIGF-1R	–	1:500	Calbiochem (PS1009)
PI3K P85 (19H8)	–	1:1000	Cell signaling (4257)
pPI3K p85	–	1:1000	Cell signaling (4228)
Plakoglobin (M)	1:100	1:1000	Transduction lab (C26220)
β-Tubulin (M)	–	1:200	DSHB (E7)[d]
ZO-1 (R)	1:150	1:500	Zymed (61-7300)
2^0 Antibodies			
Alexa Fluor 546 (R)	1:250	–	Molecular probes (A11035)
Alexa Fluor 488 (M)	1:250	–	Molecular probes (A11029)
Anti-M-FITC	1:100	–	Sigma (821462)
Anti-R-Rhodamine	1:100	–	Sigma (1238841)
Anti-M-HRP	–	1:2000–1:10000	Sigma (054H-8914)
Anti-R-HRP	–	1:2000–1:10000	Sigma (054H-8918)

[a] *R* rabbit

[b] *M* mouse

[c] Dlugoz et al. [80]

[d] *DSHB* Developmental studies hybridoma bank

Cells that had invaded through Matrigel were counted at the underside of the membrane using the 20× objective of an inverted microscope. Duplicates of each cell line were plated for each invasion assay and three independent experiments were performed. The results represent average numbers of three experiments.

Immunofluorescence analysis

For 2-D cultures, coverslips were rinsed with PBS, fixed with 3.7% (v/v) paraformaldehyde for 15 min at room temperature (RT) and permeabilized with 0.5% (v/v) Triton X-100 for 5 min on ice. Coverslips were then blocked for 1 h with 4.0% goat serum and 50 mM NH_4Cl_4 in PBS containing 0.2% BSA (PBS-BSA) and processed for indirect immunofluorescence (IF). All antibodies were diluted in PBS-BSA and used at concentrations detailed in Table 1. Primary antibody incubation was for 1 h at RT followed by fluorochrome-conjugated species-specific secondary antibody incubation for 20 min. Nuclei were stained by 5 min incubation in 0.5 ng/ml DAPI at RT. Coverslips were mounted in elvenol containing 0.2% (w/v) paraphenylene diamine (PPD) and viewed using a BX50 Olympus microscope or processed for confocal analyses using LSM510 META (Zeiss) laser scanning confocal microscope.

The 3-D acinar structures were fixed in freshly prepared 2% paraformaldehyde in PBS for 20 min at RT. Fixed structures were then rinsed in PBS-glycine (PBSG; 130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 100 mM glycine) three times for 10 min each. The washed structures were permeabilized with 0.5% Triton X-100 in PBS for 10 min at 4°C and blocked in IF buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 7.7 mM NaN_3, 0.1% BSA, 0.2% Triton X-100, 0.05% Tween-20) containing 10% goat serum for 1 h at RT. Following three (20 min each) PBSG washes, acinar structures were incubated overnight in the primary antibodies (diluted in IF buffer containing 10% goat serum) at concentrations listed in Table 1. After three PBSG washes (20 min each), acini were incubated with Alexa Fluor-conjugated species specific secondary antibodies (Molecular Probes) for 45 min. Unbound antibodies were washed in PBSG and nuclei counterstained by 15 min incubation in 0.5 ng/ml DAPI at RT. Acinar structures were mounted with the anti-fade agent Prolong (Molecular Probes), allowed to dry and processed for confocal analysis.

Preparation of cell extracts and Western blotting

Extracts from confluent 2-D cultures of control and drug-treated cells were prepared by direct solubilization in hot SDS sample buffer (10 mM Tris–HCl pH 6.8, 2% (w/v) SDS, 50 mM dithiothreitol, 2 mM EDTA, 0.5 mM PMSF) followed by boiling for 10 min. Protein concentrations were determined by Bradford (Pierce) assays according to the manufacturer's instructions. Twenty to 25 μg samples of total cell extracts from each cell line were resolved by SDS–PAGE, transferred to nitrocellulose membranes and immunoblotted. Membranes were incubated with primary antibodies overnight followed by the species-specific HRP-conjugated secondary antibodies for 1 h and developed by standard ECL (Perkin Elmer LAS, Inc. Boston, MA, USA) procedures. Primary and secondary antibodies concentrations used for Western blotting are listed in Table 1.

ECL results were scanned and the amount of each protein band was quantified using Image J analysis software. Unless stated otherwise, histograms were generated by normalizing the amount of each protein to the amount of tubulin or actin detected in the same extract sample. Each experiment was repeated at least three times and data is presented for one typical experiment.

Results

Antiestrogen treatment induces formation of acinar structures in MCF-7 and -7B cells without reducing Bcl-2 levels

The 3-D cultures of MCF cell lines were established in control media and allowed to grow for 3–5 days. Cultures remained untreated or were transferred into media containing Tam or ICI for an additional 6 days and processed for phase (Fig. 1a) and confocal fluorescence microscopy (Figs. 1b, 2). The Bcl-2 negative MCF-10-2A cells formed single-layered acinar structures with distinct central lumens (Fig. 1a, b). MCF-7 cells, which express low levels of Bcl-2, formed aggregates with small off-centered lumenal structures, whereas MCF-7B cells expressing high levels of Bcl-2 formed large aggregates without any lumenal structures (Fig. 1a, b). Tam and ICI treatment had little apparent effect on the size of the MCF-10-2A acinar structures. In contrast, treatment with estrogen antagonists not only reduced the number of cells within the aggregates in both MCF-7 and -7B cells; it also induced the formation of acinar like structures (Fig. 1a, b).

Immunofluorescent staining of cells grown in 3-D cultures with E-cadherin and ZO-1 antibodies showed overlapping and, primarily, membrane localization for both proteins in all cell lines (Fig. 2a, Control). This was in contrast to our previous observation in 2-D cultures of MCF-7B cells in which E-cadherin distribution was both membrane and cytoplasmic and ZO-1 distribution was primarily cytoplasmic and nuclear [14].

Our previous studies with MCF-7B cells grown in 2D cultures showed both the disruption of tight and adherens

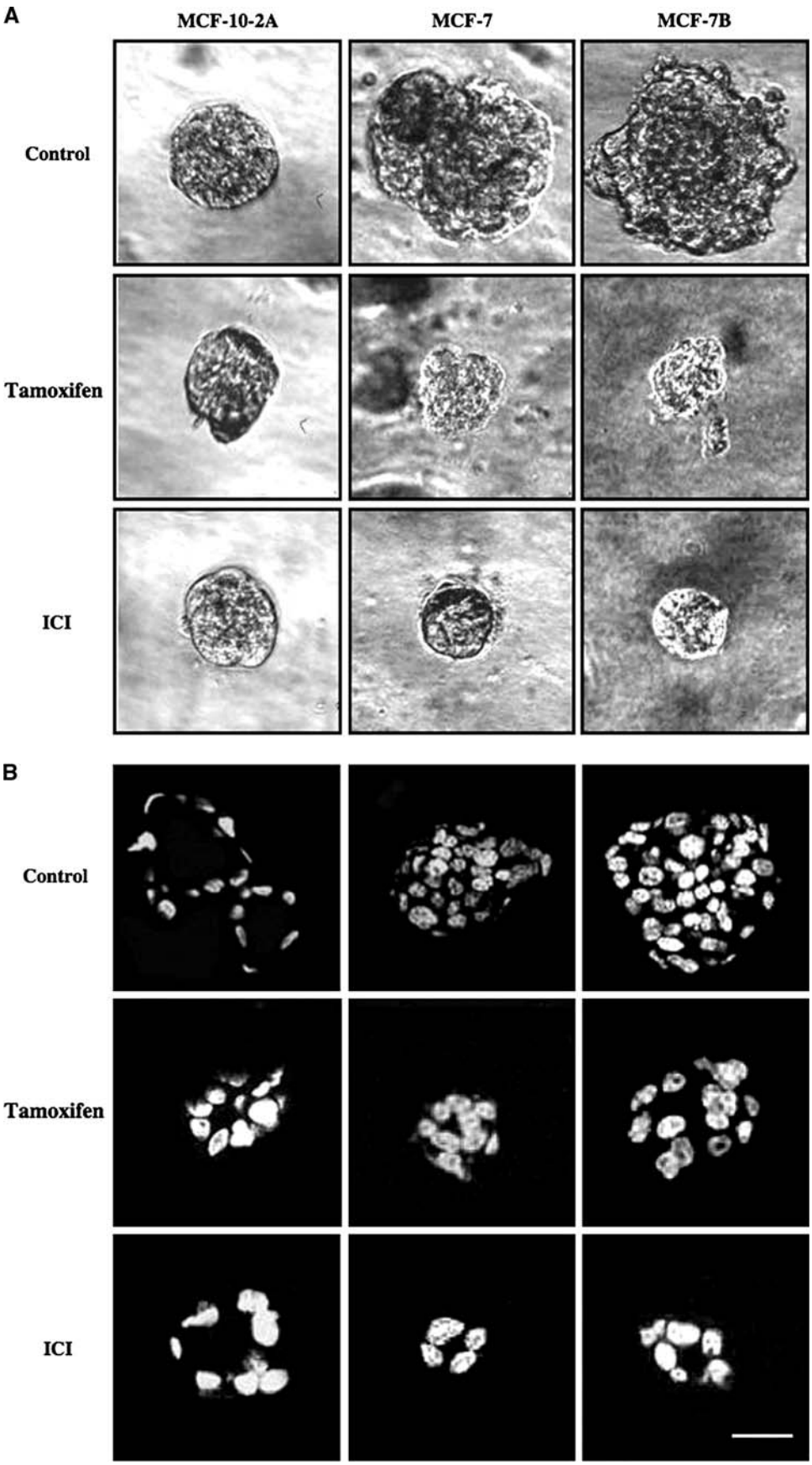


Fig. 1 Morphology of acinar structures in control and antiestrogen-treated MCF cell lines. **a** Cells were grown in Matrigel matrix for 5–7 days. Duplicate cultures were treated with Tam or ICI as described in Materials and methods. Control and drug treated cultures were processed for phase microscopy using 20× objectives. **b** Control cultures were grown in Matrigel for 6 days and then fixed, permeabilized and processed for nuclei staining with DAPI. *Bar* 20 μm

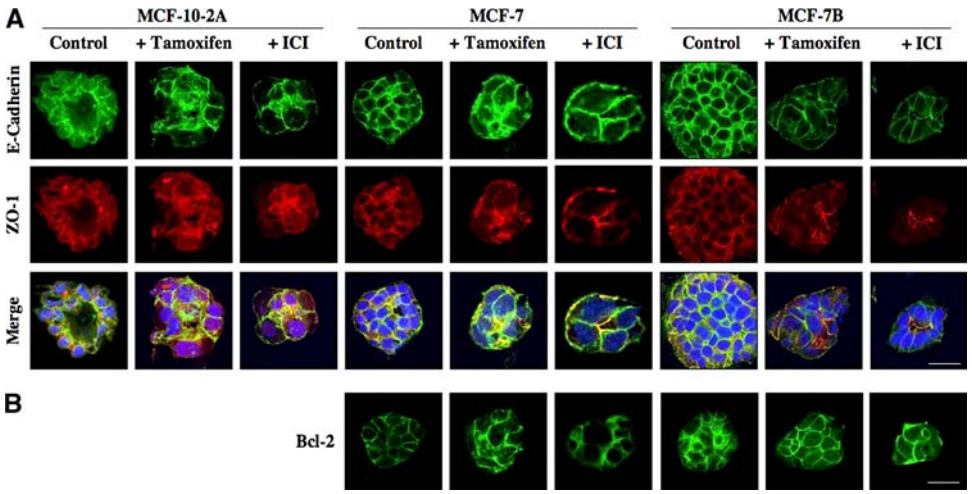


Fig. 2 Formation of acinar structures and localization of E-cadherin, ZO-1 and Bcl-2 in control and antiestrogen-treated 3-D cultures of MCF cells. Replicate cultures were established in Matrigel and grown in control media for 3–5 days. Cultures were then remained untreated or transferred into media containing Tam or ICI for another 6 days as described in Materials and methods. **a** Matrigel grown structures were fixed, permeabilized, stained with E-cadherin and ZO-1 antibodies and processed for confocal microscopy. Nuclei were counterstained with DAPI and images were observed using a 100× objective. *Bar* 10 mm. **b** Control and antiestrogen-treated 3-D cultures of MCF-7 and -7B cells were processed for staining with Bcl-2 antibodies and confocal analysis. Images were observed using a 40× objective and enlarged 2×. *Bar* 20 μm

junctions and intracellular redistribution of E-cadherin and ZO-1. Furthermore, we showed that estrogen withdrawal in these cultures reduced Bcl-2 protein levels while increasing cell–cell adhesion, and junction formation [14]. To further examine the consequences of estrogen withdrawal, we treated the 3D cultures of MCF-10-2A, -7 and -7B with pure antiestrogens and assessed the effects on Bcl-2 expression and distribution of junctional proteins. Acinar structures from the drug-treated MCF cultures were fixed and stained with E-cadherin, ZO-1 and Bcl-2 antibodies and examined by confocal microcopy as described in Materials and methods (Fig. 2).

In MCF-10-2A cultures, Tam and ICI treatments caused cytoplasmic redistribution of E-cadherin and ZO-1. In addition, some ZO-1 staining was apparent in the nuclei of the antiestrogen-treated MCF-10-2A cells (Fig. 2a). Small, round and single cell-layered acinar structures with central lumen were formed in MCF-7 cells treated with Tam or ICI. In these structures, E-cadherin and ZO-1 were co-distributed and primarily localized to the membrane (Fig. 2a). Tam-treated MCF-7B cells formed single layered irregular aggregates with off-center lumenal structures, whereas the ICI-treated cultures formed well-organized acinar structures with distinct central lumens. E-cadherin and ZO-1 were codistributed and mainly localized to the membrane in these cells. Very little, if any, nuclear ZO-1 staining was detected in the antiestrogen-treated MCF-7 and -7B cells (Fig. 2a).

Examination of the Bcl-2 levels in the acinar structures of the drug-treated MCF-7 and -7B cells showed no apparent changes in Bcl-2 levels in these cultures (Fig. 2b). These results suggest that antiestrogens decrease the growth of MCF-7 and -7B cells in 3D cultures and induce acinar formation. The effect of the antiestrogen drugs on cadherin-mediated adhesion in MCF-7B is similar in monolayer/2-D [14] (Fig. 5) and 3-D cultures. However, while in 2D cultures, serum withdrawal induced membrane redistribution of junction proteins, the formation of acinar structures in the estrogen antagonist-treated MCF-7B cells occurred in spite of high Bcl-2 levels. Therefore, we examined the effect of these antiestrogens in 2D cultures and performed detailed growth and biochemical studies to investigate the way in which antiestrogens reduce the growth of MCF-7 and -7B cells and induce differentiation/acinar formation.

Bcl-2 expression impedes contact inhibition of growth but has no effect on the invasiveness of MCF-7 cells in vitro

Bcl-2 protein expression was examined by Western blot of total cell extracts with anti-Bcl-2 antibodies. MCF-10-2A cells had no detectable Bcl-2 protein, whereas very low and high levels of this protein were expressed in MCF-7 and -7B cells, respectively (Fig. 3a). Immunofluorescent staining with anti-Bcl-2 antibodies further confirmed the results of the Western blot (Fig. 3b).

Comparison of the growth properties of MCF-10-2A, -7 and -7B cell lines showed notable differences in their growth rates (Fig. 3c). In MCF-10-2A and -7 cultures, cell proliferation reached a plateau by day 7 and 10, respectively, the time at which cultures became confluent. MCF-7B cultures also became confluent by 10 days but these

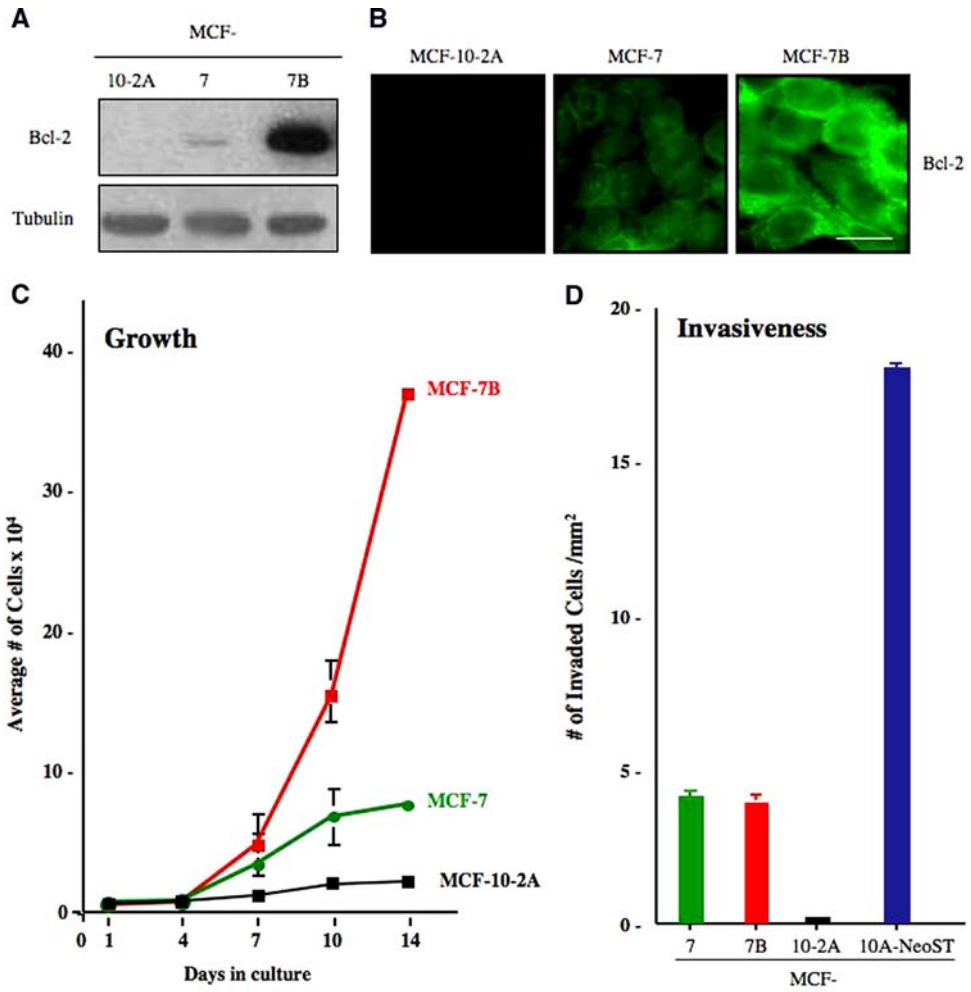


Fig. 3 Bcl-2 levels and localization in MCF-7 and MCF-7B breast carcinoma cell lines and growth and invasive properties. The "normal" mammary epithelial cell line MCF-10-2A and the Ras-transformed MCF-10A-NeoST were included as controls. **a** Equal amounts of total cellular protein from MCF-10-2A, -7 and -7B cells were resolved by SDS–PAGE and processed for immunoblotting with Bcl-2 and tubulin antibodies. **b** MCF-10-2A, MCF-7 and -7B cells were grown on glass coverslips and processed for immunofluorescence microscopy using Bcl-2 antibodies. Coverslips were viewed with a 63× objective. *Bar* 20 μm. **c** Growth properties of MCF-10-2A, -7 and -7B cells. Replicate cultures were established for each cell line at single cell density and cells counted at 4, 7, 10 and 14 days. Each time point represents the average of three independent experiments. The absence of error bars at some time points is due to the small differences among the experiments. **d** The in vitro invasiveness of MCF-10-2A, -7 and -7B cells was assessed by measuring invasion through Matrigel matrix as described in Materials and methods. Duplicates of each cell line were plated for each invasion experiment, and three independent experiments were performed. The results represent the average of three experiments

cells remained in an exponential growth phase and continued to proliferate.

Matrigel invasion assays were used to examine the effect of Bcl-2 expression on the invasive properties of MCF-7 cells. MCF10-2A and MCF-10A-NeoST (a Ras-transformed MCF-10A cell line) cells were used as the non-invasive and invasive control lines, respectively (Fig. 3d). In this assay, we seldom detected any cells on the underside of the transwell membranes in MCF-10-2A cultures. In contrast, MCF-10A-NeoST cells were highly invasive, and an average of 18 cells/mm^2 crossed the membrane in three independent assays. The average number of cells invaded was similar in MCF-7 and -7B cultures ($\sim$4 cells/mm^2) and was significantly lower than that of MCF-10A-NeoST cultures. The results of the growth and invasion assays indicate that Bcl-2 overexpression leads to loss of contact inhibition of growth, but has no effect on the invasiveness of MCF-7 cells.

Antiestrogen treatment increases Bcl-2 protein levels in MCF-7 and -7B cells and only ICI decreases ERα levels

Replicate cultures of MCF-10-2A, -7 and -7B cells were grown in control media for 3–5 days. After this initial period, one set of cultures remained in control media and

the other two sets were transferred into media containing Tam or ICI. Cultures were allowed to grow for an additional 6–8 days and then processed for preparation of total cell lysates and Western blotting with antibodies to ERα, Bcl-2 and tubulin. No ERα or Bcl-2 protein was detected in MCF-10-2A cells (Fig. 4a). Bcl-2 was expressed at very low and high levels in MCF-7 and -7B cells, respectively, and both cell lines had substantial amount of ERα (Fig. 4a, b). Treatment with both antiestrogens increased Bcl-2 levels in MCF-7 and MCF-7B cells and only ICI was effective in reducing ERα levels in both cell lines (Fig. 4a, b). Immunofluorescent staining of control and antiestrogen-treated cultures with Bcl-2 antibodies also showed increased staining in antiestrogen-treated cultures (Fig. 4c). ERα staining was primarily nuclear in control MCF-7 cells whereas it was distributed both in the cytoplasm and nuclei of MCF-7B cells. ERα distribution remained unchanged in Tam treated MCF-7 cells whereas in MCF-7B cells it became exclusively nuclear (Fig. 4c). In contrast to Tam treatment, ERα was barely detectable when MCF-7 and -7B cultures were treated with ICI (Fig. 4c). These results show that both antiestrogen drugs increased Bcl-2 levels and only ICI effectively reduced ERα levels in MCF-7 and -7B cells.

Antiestrogen treatment increases the levels of cell adhesion proteins

Total cellular proteins from untreated and antiestrogen-treated MCF cell lines were processed for Western blotting with E-cadherin, ZO-1 and tubulin antibodies (Fig. 4d). Compared to MCF-10-2A cells, MCF-7 and -7B had lower levels of both E-cadherin and ZO-1. Antiestrogen treatment increased the levels of both proteins in all cell lines and the largest increase was detected in MCF-7B cells (Fig. 4d, e). We also noted a faster migrating band for E-cadherin in all cultures treated with ICI (Fig. 4d). The faster migrating E-cadherin band was particularly pronounced in ICI-treated MCF-7 and -7B cells (Fig. 4d). Treatment of E-cadherin immunoprecipitates from control and antiestrogen-treated cultures with endo-*β*-*N*-acetylglucosaminidase H (endoH) showed endoH sensitivity for the slower migrating band suggesting that the fast migrating band is primarily the unprocessed E-cadherin localized to ER and ER-Golgi compartments (data not shown).

Effect of antiestrogen treatment on subcellular localization of E-cadherin and ZO-1

In MCF-10-2A and -7 control cultures, the staining of E-cadherin and ZO-1 was mainly peripheral and localized to areas of cell–cell contact (Fig. 5a). In MCF-7B cultures, E-cadherin staining appeared both membrane-associated and cytoplasmic whereas ZO-1 distribution was primarily intracellular and nuclear, as we have reported previously (Fig. 5a) [14].

MCF-10-2A cells treated with Tam or ICI showed cytoplasmic redistribution of E-cadherin and cytoplasmic and nuclear redistribution of ZO-1. In fact, the majority of ZO-1 staining in the antiestrogen-treated MCF-10-2A cells appeared to be nuclear (Fig. 5b). A similar cytoplasmic redistribution of E-cadherin and ZO-1 was observed in MCF-7 cells, which also showed some nuclear staining for ZO-1 (Fig. 5b). When MCF-7B cells were treated with Tam or ICI, two discrete staining patterns were observed for E-cadherin: at the membrane and in a highly distinct perinuclear vesicular pattern in the cytoplasm (Fig. 5b). In these cultures, most of the ZO-1 staining was redistributed from the cytosol and nuclei to the membrane, where it codistributed with E-cadherin (Fig. 5b). The cytoplasmic redistribution of E-cadherin is consistent with the unprocessed E-cadherin band observed in Western blots in the drug-treated cultures. These results show that antiestrogen treatments affect cadherin-mediated adhesion differently in the high Bcl-2-expressing mammary epithelial cells relative to those cells with no or low levels of Bcl-2.

These results show that the effect of the antiestrogen drugs on cadherin-mediated adhesion is similar in monolayer and 3-D cultures of MCF-7B cells and suggest that membrane redistribution of junctional proteins and formation of acinar structures occur in spite of increased Bcl-2 levels.

We then sought to determine whether the decreased cell numbers in both 2- and 3-D cultures of MCF-7 and -7B are correlated with increased levels of proapoptotic proteins (Fig. S1). Western blot analysis of the total cell lysates with Bad, Bax and Bim antibodies detected increased levels of all three proteins in the Tam-treated MCF-7 cells whereas, in MCF-7B cells only Bax was notably increased (Fig. S1). It is noteworthy that this increase in the levels of proapoptotic proteins is concurrent with increased Bcl-2 levels in the antiestrogen-treated cultures. In ICI-treated cultures, levels of proapoptotic proteins remained unchanged. Together, these results suggest that treatment with estrogen antagonists increases the levels of both anti- and proapoptotic proteins and it is not likely that the formation of lumenal acinar structures in the drug treated 3-D cultures of MCF-7 and -7B is mediated by increased apoptosis.

Growth inhibition of antiestrogen-treated cultures is mediated by growth factor signaling pathways involving PI3K/Akt, ERK and IGF-1R pathways

We showed that while lumenal acinar structures were formed in both Tam and ICI treated cultures of MCF-7 and -7B cells, Bcl-2 levels were increased and ERα levels were reduced significantly only in ICI treated cells. These

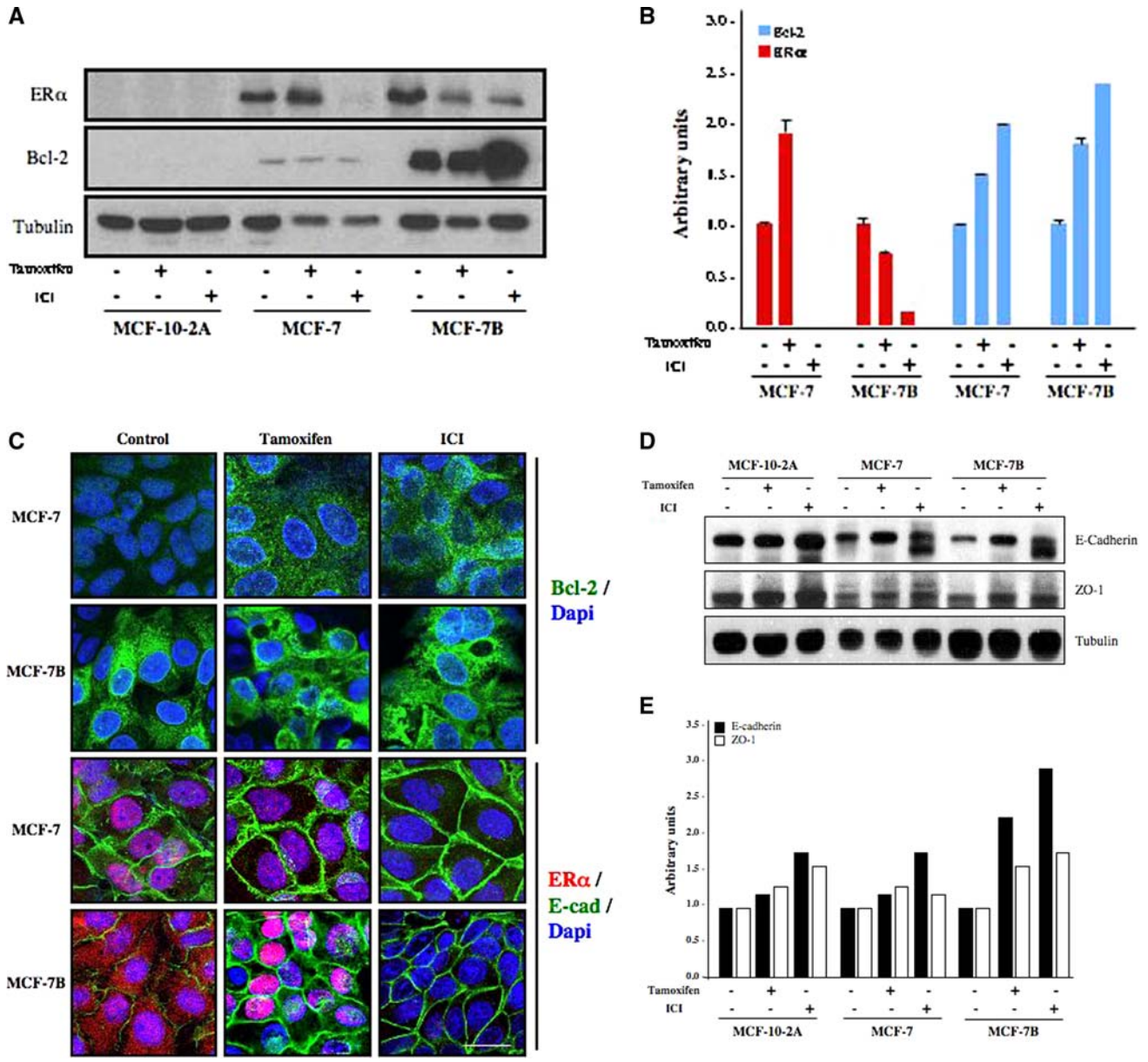


Fig. 4 Levels and localization of ERα, Bcl-2, E-cadherin and ZO-1 in control and antiestrogen-treated MCF cells. Triplicate cultures of MCF-10-2A, -7 and -7B were established and maintained in control media for 3–5 days. After this period, one set of cultures remained in control media and the other two sets were transferred into media containing Tam or ICI as described in Materials and methods. Control and drug-treated culture were allowed to grow for another 6–8 days. **a** Total cells lysates from control and drug-treated cultures were processed for immunoblotting with antibodies to ERα and Bcl-2. Membranes from each experiment were blotted with tubulin antibodies to verify protein loading. **b** The levels of ERα and Bcl-2 in MCF-7 and -7B were quantitated from gels in (**a**) using Image J software. Histograms for each cell line represent the amount of each protein relative to the amount of tubulin in the same lysate normalized to the untreated lysates. **c** Triplicate cultures from MCF-7 and -7B cell line were established on glass coverslips and grown in control media for 3–5 days. At this time, one set of cultures remained in normal media (*Control*) and the other two sets were transferred into media containing Tam or ICI as described in Materials and methods. Cultures were grown for additional 6 days and then processed for immunofluorescent staining with antibodies to Bcl-2, ERα and E-cadherin. Nuclei were counterstained with DAPI. Coverslips were viewed with a 63× objective. *Bar* 10 μm. **d** Total cells lysates from control and drug-treated cultures were processed for immunoblotting with antibodies to E-cadherin and ZO-1. Duplicate membranes from each experiment were blotted with tubulin antibodies to verify protein loading. **e** Protein bands were quantified from the blots in (**d**) using Image J software. Histograms were generated by normalizing the amount of each protein to the amount of tubulin detected in the same extract sample relative to the untreated lysates

observations suggest that the growth inhibitory effect of these antiestrogens is not due to apoptosis and furthermore, may not be mediated by ERα. Estrogen regulation of cell proliferation involves both direct and indirect interactions between ERα and components of growth regulating signaling pathways including PI3K/AKT and MAPK; and ICI

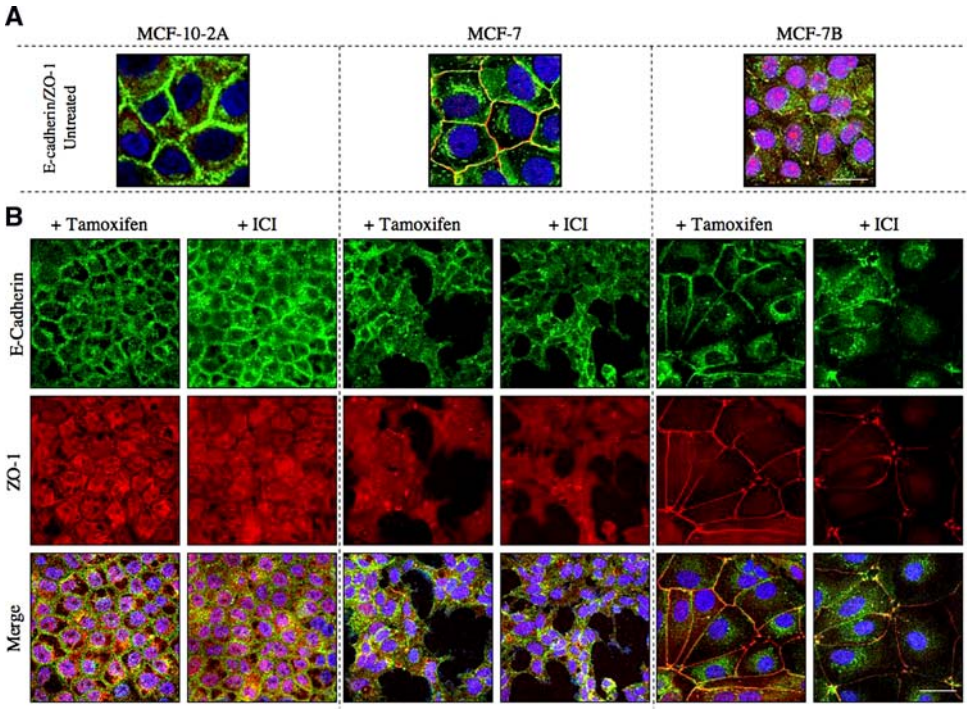


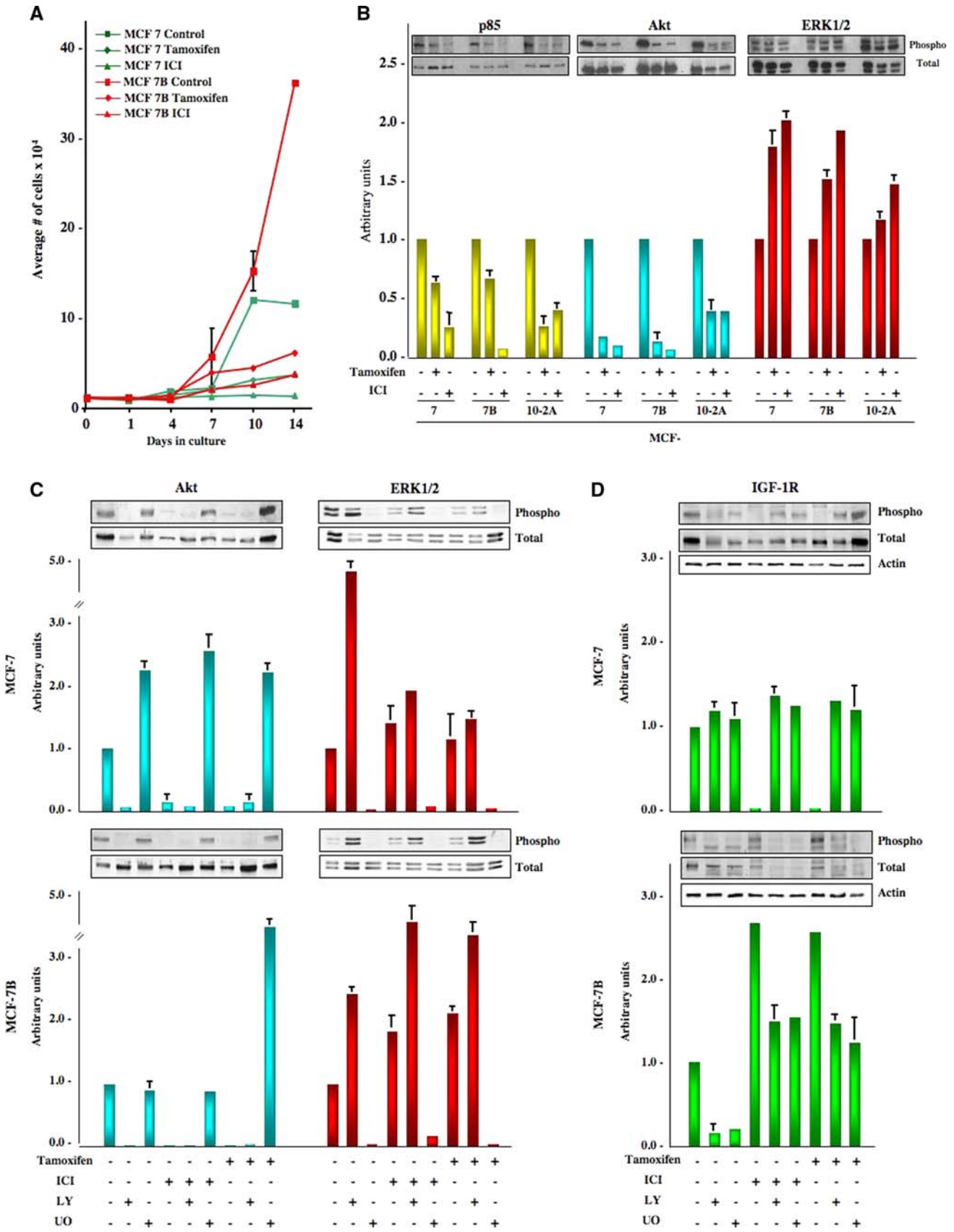
Fig. 5 Localization of E-cadherin and ZO-1 in untreated control and antiestrogen-treated cultures of MCF-10-2A, -7 and -7B cells. Triplicate cultures from each cell line were established on glass coverslips and maintained in normal media (**a**) or transferred into media containing Tam or ICI (**b**) as described in Materials and methods and then processed for immunofluorescence with antibodies to E-cadherin and ZO-1. Primary antibodies were detected by species specific Alexa Flour conjugated secondary antibodies and processed for confocal analyses using a 63× objective. *Bars* **a** 10 μm and **b** 20 μm

and Tam can exert their growth inhibitory effects via modulation of growth promoting pathways [16–18]. Therefore, we examined the growth properties, as well as the protein phosphorylation and activation of the PI3K/AKT, ERK1/2 and IGF-1R signaling pathways in the antiestrogen-treated cultures of MCF-7 and -7B cells.

MCF-7 and -7B cells grown in media containing Tam and ICI showed significantly reduced growth rate (Fig. 6a). Notably, antiestrogen treatment did not induce a rapid decrease in cell number in either cell line. Both cell lines reached a plateau within 4 days of drug treatment, indicating an induction of growth arrest as opposed to cell death in these cultures.

Examination of the PI3K regulatory subunit (p85) phosphorylation showed ∼40% reduction in phosphorylated p85 (pp85) in MCF-7 and -7B and 70% reduction in MCF-10-2A cells treated with Tam. In ICI treated cultures, pp85 was reduced by ∼70, 90 and 60% in MCF-7, -7B and 10-2A, respectively (Fig. 6b). A corresponding decrease in the levels of pAkt, a downstream effector of PI3K, was detected for all of the antiestrogen-treated cultures. The levels of phosphorylated Akt (pAkt) were reduced by >80% in antiestrogen-treated MCF-7 and -7B cells while MCF-10-2A showed a reduction of ∼60% (Fig. 6b). In contrast to pp85 and pAkt, the levels of phosphorylated ERK1/2 (pERK1/2) were increased in all antiestrogen-treated cultures and this increase was particularly pronounced for MCF-7 and -7B cells (Fig. 6b). These results suggest that the anti-tumor activities of Tam and ICI may be due to their functional interactions with growth factor-mediated signaling pathways and not their antiestrogenic properties, and furthermore, the effect of these interactions are more pronounced in Bcl-2 overexpressing cells. To

Fig. 6 Antitumor activity of Tam and ICI is mediated by the PI3K-Akt and ERK pathways. Cultures of MCF-7, -7B and 10-2A were established in control media for 3–5 days, at which time replicate cultures were transferred into media containing Tam or ICI. **a** Replicate cultures were established for each cell line at single cell density and cells were counted at 4, 7, 10 and 14 days. Each time point represents the average of three independent experiments. The absence of error bars at some time points indicate small differences among the experiments. **b** MCF cultures were grown in the absence or presence of antiestrogens as described in Materials and methods. Equal amounts of total proteins from control and drug-treated cultures were processed for Western blot with antibodies to p85, pp85, AKT, pAKT, ERK 1/2 and pERK1/2. For each protein, the same membrane was processed first with the phospho-antibodies followed by the respective total antibodies. Blots were quantitated using Image J software. Histograms represent the ratio of phospho/total protein normalized to the same ratio in the untreated control cultures for each cell line. Histograms represents the average of at least three independent experiments and the absence of error bar is due to the small differences among the experiments. **c** Control and antiestrogen-treated MCF-7 and -7B cultures were treated with LY294002 and UO126 inhibitors of PI3K and MAPK pathways and processed for blotting with total and phospho-specific antibodies to Akt and ERK1/2, and actin. Blots were quantitated using Image J software and histograms were constructed as described in (**b**), which represent the ratio of phospho/total Akt and ERK1/2 normalized to the amount of actin in each cell line. **d** Cultures of MCF-7 and -7B cells were established and treated with antiestrogens with or without inhibitors of PI3K and MAPK pathways as described in (**c**). Western blots of total cell extracts were processed with phospho and total IGF-1R antibodies. Blots were quantitated using Image J software and histograms were constructed as described in (**b**), representing the ratio of phospho/total IGF-1R normalized to the amount of actin in each cell line


A
MCF 7 Control
MCF 7 Tamoxifen
MCF 7 ICI
MCF 7B Control
MCF 7B Tamoxifen
MCF 7B ICI
Average # of cells x 10⁴
Days in culture
B
p85
Akt
ERK1/2
Phospho
Total
Arbitrary units
Tamoxifen
ICI
7
7B
10-2A
MCF-
C
Akt
ERK1/2
Phospho
Total
MCF-7
MCF-7B
Arbitrary units
Tamoxifen
ICI
LY
UO
D
IGF-1R
Phospho
Total
Actin
MCF-7
MCF-7B
Arbitrary units
Tamoxifen
ICI
LY
UO

further confirm this possibility, we treated control and antiestrogen-treated cultures of MCF-7 and -7B cells with PI3K and ERK1/2 inhibitors and examined the levels of Akt and ERK1/2 phosphorylation.

In these experiments, control and antiestrogen-treated MCF-7 and -7B cells remained untreated or were treated with the PI3K inhibitor LY294002 or with the ERK kinase (MEK) inhibitor UO126 and processed for Western blotting with total and phospho-specific antibodies to Akt and ERK1/2. Treatment of control cultures with LY294002 and UO126 reduced Akt and ERK1/2 phosphorylation by >90%, respectively (Fig. 6c). Interestingly, inhibition of the PI3K pathway led to increased ERK1/2 phosphorylation and inhibition of the ERK pathway increased Akt phosphorylation in both control and antiestrogen-treated cultures of MCF-7 and -7B cells.

In the antiestrogen-treated MCF-7 and -7B cultures, Akt phosphorylation was barely detectable with or without LY294002. ERK1/2 phosphorylation, which was significantly increased by antiestrogen treatment in both cell lines, was completely inhibited by UO126, suggesting that the antiestrogens' effects must occur upstream of MEK activation (Fig. 6c).

PI3K and ERK pathways are known to be involved in multiple molecular cross talks between ERα and IGF-1R. Therefore, we sought to examine IGF-1R phosphorylation and activation in Tam and ICI treated MCF-7 and -7B cells with or without PI3K and ERK pathway inhibitors. In Fig. 6d, histograms represent the pIGF-1R/IGF-1R ratios in control and antiestrogen-treated cultures with or without kinase inhibitors. pIGF-1R was detected in control cultures of both MCF-7 and -7B cells. Inhibition of PI3K and ERK pathways did not change the pIGF-1R/IGF-1R ratio in control MCF-7 cells. In MCF-7 cells treated with antiestrogens, pIGF-1R was barely detectable. The addition of PI3K and MAPK inhibitors to these cells increased the level of pIGF-1R to that of control cultures (Fig. 6d).

In contrast to MCF-7, both inhibitors drastically reduced pIGF-1R levels in control MCF-7B cells (Fig. 6d). In these cells, antiestrogen treatment increased IGF-1R phosphorylation and pIGF-1R/IGF-1R ratio by more than 150%. The further addition of kinase inhibitors to the antiestrogen-treated MCF-7B cells lowered this ratio to levels similar to that of control cultures. Together, these results suggest that Bcl-2 overexpression may modify the cross talk among signaling pathways that regulate cell proliferation, survival and differentiation.

Discussion

MCF-10-2A, an ERα$^-$/Bcl-2$^-$ normal mammary epithelial cell line, and two ERα$^+$ mammary carcinoma cell lines MCF-7 (low Bcl-2) and -7B (Bcl-2 overexpressing) with normal HER2 expression, were used in 2- and 3-D culture studies to examine the effects of selective antiestrogens Tam and ICI on Bcl-2 levels and cadherin-mediated adhesion. In 3-D cultures of all cell lines, both E-cadherin and ZO-1 were membrane localized. However, while untreated MCF-7 and -7B cells formed aggregates in 3-D matrices, the antiestrogen-treated cells formed acinar structures without any decrease in Bcl-2 levels. The antiestrogen-treated 2-D cultures of MCF-10-2A and -7 cells showed junction dissociation and cytoplasmic redistribution of junctional components whereas MCF-7B cells showed membrane redistribution of junctional proteins. These morphological changes in MCF-7 and -7B cells coincided with increased Bcl-2 levels and decreased growth. Levels of protein phosphorylation and activation/inactivation of the PI3K/Akt, ERK and IGF-1 pathways in control and antiestrogen-treated cultures suggested that the anti-tumor activity of Tam and ICI was unlikely to be mediated by ERα. Antiestrogen treatment decreased PI3K/Akt and increased ERK activation, irrespective of ERα expression. Interestingly, IGF-1R was inactivated in the antiestrogen-treated MCF-7 cells while it was activated in MCF-7B cells.

Bcl-2 overexpression interfered with contact inhibition of growth, in keeping with its anti-apoptotic and tumorigenic function [19–21], but did not change the invasive properties of MCF-7 and MCF-7B cells. Very few in vitro studies have assessed the role of Bcl-2 overexpression in invasiveness. Our results, however, are supported by numerous in vivo studies in which Bcl-2 expression is associated with good prognosis and longer overall and disease-free survival of breast cancer patients, and its decreased expression is associated with the progression from adenomas to invasive carcinomas [22–26].

The growth-stimulatory effect of estrogens in breast cancer is mediated by ERα [27]. Activated ERs can act via both genomic and non-genomic mechanisms. In the genomic pathway, ERs regulate target gene expression by direct or indirect binding to DNA [28, 29]. In the non-genomic or membrane-initiated pathway, ER activation leads to its interactions with signaling complexes, including kinases and phosphatases, thereby modulating cytoplasmic signal transduction pathways and regulating transcription indirectly [30, 31]. ERα activates gene expression via two transcriptional activation function domains (AF1 and AF2). AF1 activation is ligand independent and can be mediated by growth factors, whereas AF2 overlaps with the ligand binding domain and becomes transcriptionally active upon binding to ligands [31]. Tam and ICI, selective estrogen receptor modulators and downregulators, respectively, are used to block ERα function [32–34]. Tam functions as partial antagonist by

binding to AF2 and inhibiting the AF2-regulated target gene expression, while AF1-regulated transcription remains active [32, 35, 36]. In contrast, ICI has no agonist activities and inhibits ERα by binding to and promoting ERα degradation via the nuclear matrix-associated proteosomes [37–40]. Consistent with the Tam and ICI modes of action, ERα levels were significantly reduced only in the ICI treated cultures of MCF-7 and -7B cells.

Antiestrogens inhibit growth by inducing cell cycle arrest and/or apoptosis [41, 42]. Both Tam and ICI can induce apoptosis [42]. Estrogen regulates the expression of the Bcl-2 family of pro- and anti-apoptotic proteins [6, 7]. Estrogen induces Bcl-2 expression both directly and indirectly, and some studies have reported decreased Bcl-2 protein levels in MCF-7 cultures in response to estrogen withdrawal or antiestrogen treatment [12, 42–44]. Here, we observed increased Bcl-2 levels in both MCF-7 and -7B cells treated with Tam and ICI. This discrepancy may be explained by the differences in the experimental conditions. In previous studies, the decrease in Bcl-2 levels occurred when MCF-7 cells were treated with antiestrogens together with estrogen. In our studies, cultures were treated with antiestrogens without added estrogen. Using experimental conditions similar to ours, Wang et al. [45] recently showed that treatment of $ER\alpha^{+}$ breast carcinoma cells (including MCF-7) with ICI leads to the downregulation of ERα, concurrent with activation of the NFκB signaling pathway and upregulation of Bcl-2. Furthermore, increasing Bcl-2 levels by exogenous expression or ICI treatment decreased E-cadherin-mediated adhesion and induced an epithelial to mesenchymal transformation. Consistent with these observations, we previously reported both the disruption of junctions and intracellular redistribution of E-cadherin and ZO-1 in the Bcl-2 overexpressing MCF-7B cells [14]. Here, the increased Bcl-2 levels in the Tam or ICI-treated MCF-7 cells also associated with the cytoplasmic redistribution of E-cadherin and ZO-1. However, the antiestrogen treatments of MCF-7B cells led to the membrane redistribution of junctional proteins and their increased steady state levels, as was observed previously with estrogen withdrawal [14]. Interestingly, the junction assembly in estrogen-depleted cultures of MCF-7B was concurrent with decreased Bcl-2 expression, whereas upon antiestrogen treatment, Bcl-2 levels were not reduced in either 2- or 3-D cultures. Furthermore, while membrane redistribution of junctional proteins occurred in both Tam and ICI-treated MCF-7B cells, ERα levels were reduced only in ICI treated cells. These results strongly suggest that the effects of antiestrogen treatments on cadherin-mediated adhesion in MCF-7B cells are not mediated solely via ERα.

Tissue culture cell lines grown in 3-D matrices show cytological characteristics that are very similar to cells in vivo and exhibit differential expression of various cell–cell and cell-extracellular matrix adhesion proteins [46–49]. Consistent with these studies, we found that the distribution of E-cadherin and ZO-1 in control 3-D cultures of all cell lines was almost exclusively membranous regardless of the amount of Bcl-2 protein. This was clearly different from the distribution of these proteins in 2-D cultures of MCF-7B cells in which a significant amount of E-cadherin was localized intracellularly, and ZO-1 distribution was primarily cytoplasmic and nuclear. In 3-D matrices, MCF-10-2A cells formed polarized, acinar structures with distinct central lumens [50, 51]. MCF-7 cells grew as aggregates with occasional small and off-centered hollow lumens, while MCF-7B cells formed very large aggregates without any lumenal structures. Tam and ICI treatment led to the formation of smaller, well-formed acinar structures, with central lumens in both MCF-7 and -7B cultures and no reduction in Bcl-2 levels. The reduced size of the aggregates in the drug-treated cultures is consistent with the growth inhibitory properties of these antiestrogens and may be mediated by increased Bcl-2 levels and induction of cell cycle arrest as opposed to apoptosis, as Bcl-2 can induce cell cycle arrest in both normal and carcinoma cell lines [52–55]. Consistent with these observations, the antiestrogen-treated MCF-7 and -7B cells with increased Bcl-2 levels showed a growth plateau as opposed to a reduction indicative of cell death.

In 3-D matrices of mammary epithelial cells, selective apoptotic death of centrally located cells within the aggregates is one mechanism by which cells are removed during lumen formation. The balance between pro- and anti-apoptotic proteins regulates apoptosis. Proapoptotic proteins are involved in acinar formation and mammary morphogenesis both in vitro and in vivo [56–58]. Here, we did not detect any substantial increase in the amounts of several proapoptotic proteins upon antiestrogen treatment, and despite increasing Bcl-2 levels, both Tam and ICI induced lumen formation. Previous studies also have demonstrated that Bcl-2 overexpression and inhibition of apoptosis does not inhibit lumen formation [56, 59]. Paraptosis, an alternative nonapoptotic form of programmed cell death, has been described recently and is involved in the death of the centrally located cells during lumenogenesis [60]. Paraptosis occurs independent of Bcl-2 and caspases, can be induced by IGF-I (and other growth factors) and is mediated by ERKs [61, 62]. Here, we showed that the phenotypic changes induced by antiestrogens are concurrent with changes in signaling pathways that regulate cell growth, survival and differentiation. Regardless of ER status, both antiestrogens down-regulated the PI3K/Akt pathway and upregulated the ERK pathway, which strongly suggest that their anti-tumor activity may be ERα-independent. The use of specific PI3K/Akt and ERK inhibitors further confirmed the effect of the

antiestrogens via these pathways. Estrogen is known to upregulate PI3K/Akt via ERα and constitutive activation of Akt is associated with resistance to Tam and ICI both in vitro and in vivo [63–66]. Inhibitors of PI3K/Akt or its downstream targets suppress the growth of various breast carcinoma cell lines [67, 68]. Previous works on the effect of Tam or ICI on PI3K/Akt activation in culture generally used these drugs at high concentrations for short times and/or in combination with estrogen. At 5 μM, Tam was recently shown to induce ERK1/2 activation within 40 min followed by rapid cell death, independent of ERα [69]. Here, we showed the sustained down regulation of Akt for long periods of time (>8 days) induced in MCF cultures by low/physiological concentrations of antiestrogens, also independent of ERα. The ERK signaling pathway is one of the three major MAPK pathways and the most relevant to breast cancer [70]. The ERK pathway can be activated by the nongenomic activity of ERα by interacting with components of other growth factor pathways and their subsequent activation, ultimately leading to increased cell proliferation and survival [67, 71]. We showed sustained ERK activation in antiestrogen-treated cells independent of ERα and this activation was not associated with apoptosis, consistent with increased levels of Bcl-2 in these cultures. Depending on the duration and cell context, ERK activation can lead to different outcomes such as proliferation versus differentiation, and more recently, cell death [62, 69, 72]. Here, in 2D cultures, antiestrogen-treated MCF-7 cells exhibited cell cycle arrest and cell death, while MCF-7B cells showed cell cycle arrest, cell death and differentiation, as determined by decreased growth and membrane redistribution of junctional proteins. In 3D cultures, both cell lines formed acinar structures, a further indication of their differentiation. Lumen formation may be mediated by paraptosis, which can be induced by IGF-1R activation [60, 62].

ER signaling and the IGF-1R pathway can interact functionally, with ERα activation upregulating IGF-1R and IGF-1R stimulation increasing the phosphorylation and activity of ERα [73–75]. In $ER\alpha^{+}$ cells, IGF-1 is mitogenic by activating several signaling pathways including PI3K/Akt and ERK while in $ER\alpha^{-}$ cells, IGF-1R activation is growth inhibitory and these kinases are not phosphorylated [73]. Here, the substantial amount of pIGF-1R in control MCF-7 cells was significantly decreased and became undetectable in antiestrogen-treated cultures. Previous reports have shown decreased pIGF-1R in mammary glands of rats treated with Tam and ICI, as well as down regulation of IGF signaling and growth inhibition in ICI treated MCF-7 cells [16, 76]. PI3K/Akt and ERK inhibitors did not change the amount of pIGF-1R in control cultures. However, when these inhibitors were applied to the antiestrogen-treated cultures, they reversed the inhibitory effect of the antiestrogens on IGF-1R phosphorylation. These results suggest that IGF-1R phosphorylation occurs independently of the Akt and ERK pathways, whereas its inhibition by Tam and ICI does not. Both Akt and ERK inhibitors inhibited IGF-1R phosphorylation in MCF-7B control cultures, suggesting that in these cells IGF-1R phosphorylation depends on the PI3K/Akt and ERK pathways. Furthermore, in contrast to MCF-7 cells, both antiestrogens increased IGF-1R phosphorylation, which also decreased when the PI3K/Akt and ERK pathways were inhibited. These results suggest that the Bcl-2 overexpression in MCF-7B cells may modify functional interactions among the IGF-1R, PI3K/Akt and ERK pathways. Interactions among these pathways following the non-genomic activation of ERα in breast carcinoma cell lines has been shown previously [71]. However, the way in which Bcl-2 modulates these interactions is not clear. Bcl-2 overexpression sensitizes MCF-7 cells to the anti-tumor activity of genistein by multiple mechanisms involving cell cycle regulators and changing Bcl-2 into a proapoptotic protein, but its effect on signaling pathways has not been investigated and justifies further study [53].

At the outset of these studies, we anticipated that Tam and ICI treatment of Bcl-2 overexpressing cells would downregulate and/or inactivate ERα, decrease Bcl-2 expression and induce junction formation. In contrast, we found out that these antiestrogens can exert their anti-tumor activity independent of ERα, in spite of increasing Bcl-2 levels, by modulating major signaling pathways that regulate cell survival, differentiation and cell death. While further investigation is warranted, our results may provide an explanation for the paradoxical observation that Bcl-2 overexpressing tumors have a better prognosis and respond more favorably to hormone therapy and chemotherapies [22, 77–79]. Our data provide further insight into the interactions among signaling pathways that regulate cell proliferation, survival and differentiation in carcinoma cells. A better understanding of these interactions will be beneficial for devising effective treatment strategies.

Acknowledgments We are grateful to Kim Chapman and Drs. E. Shibuya, J. Mackey and R. Campenot for their critical review of the manuscript. This project has been made possible through grants from the Canadian Breast Cancer Foundation- PRAIRIES/NWT Chapter, and Alberta Cancer Foundation.

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