Reassessment of Estrogen Receptor Expression in Human Breast Cancer Cell Lines

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Abstract. *Background: Human breast cancer cell (BCC) lines are used extensively in biomedical research and are classified as estrogen receptor (ER)-positive or ER-negative. We used flow cytometry (FCM), reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting (WB) to assess ER expression in human BCC lines reported as being ER-positive (MCF7, T-47D, ZR-75-1) or ER-negative (MDA-MB-231, SK-BR-3, MDA-MB-453, HCC1954) to determine the validity of this classification. Materials and Methods: ER was assessed in permeablized, fixed cells by FCM using two monoclonal anti-ERα antibodies and a polyclonal anti-ERβ antibody, in parallel with RT-PCR and WB. Results: All of the cell lines expressed ERα and ERβ. Indirect immunofluorescence indicated that it was membrane and cytoplasmic ER that was being detected by FCM. Down-regulation by fulvestrant confirmed it was ER. Conclusion: These results demonstrate the importance of reassessing the ER status of human BCC lines that are used widely in biomedical research.*

Breast cancer is a major public health problem worldwide and estrogen and its metabolites are related to both the initiation and promotion of breast cancer (1). Estrogens exert their effects through estrogen receptors (ERs). Two ERs are known to exist, ERα (2) and ERβ (3). More than 70% of primary breast tumors in women are ER-positive (ERα), show estrogen-dependent growth and undergo regression when deprived of supporting hormones. Determination of the ER status (ERα) has, therefore, proved to be a successful therapeutic target for the treatment and prevention of breast cancer (4, 5).

Human breast cancer cell (BCC) lines likely reflect the features of cancer cells *in vivo* (6) and they continue to make contributions to our knowledge of breast cancer *e.g.* the genome-wide analysis of ER-binding sites (7) and in the study of functionally distinct cancer subtypes (8). One of the most defining characteristics of BCC lines is their ER status. Determination of ER status has been defined by nuclear expression of ERα detected by immunohistochemistry (IHC). However, the identification of ERβ (3, 9) and reports of membrane and cytoplasmic expression of ERα (10) have led to this narrow definition of ER status being questioned. We set out to examine whether the traditional classification of BCC lines in the literature as being ER-positive or ER-negative was still valid, using a flow cytometric method reported as being able to detect ER expression in isolated nuclei (11) in parallel with analysis of gene and protein expression.

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Key Words: Estrogen receptor, breast cancer cell lines, flow cytometry, RT-PCR, Western blotting.

Materials and Methods

Cell lines. Seven breast cancer cell lines were used: three reported as ER-positive (MCF7, T-47D and ZR-75-1) and four as ER-negative (MDA-MB-231, MDA-MB-453, SK-BR-3 and HCC1954). BCC lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Media and supplements were obtained from Invitrogen (Paisley, UK). Cell lines were grown as monolayers in the following media: RPMI 1640 (T-47D, ZR-75-1, HCC1954); Eagle's MEM (MDA-MB-231, MCF7); McCoy's 5A (SK-BR-3); Leibovitz's (MDA-MB-453) containing 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml). When required for assays, 5 ml of a 1:10 dilution of trypsin-EDTA in phosphate-buffered saline (PBS) were added to PBS-washed monolayers, followed by incubation at 37˚C for 5-10 min. Cells were centrifuged for 7 min at 130 ×*g*, reconstituted in medium and counted.

Antibodies. Primary: Mouse monoclonal anti-human ERα (SRA-1000, clone H-151, Lot # B210402; Stressgen, Ann Arbor, MI, USA) recognising the hinge region of ER (ERαH). Mouse monoclonal anti-human ERα (SRA-1010, clone C-542, Lot # B112427; Stressgen) recognising the steroid binding domain of ER (ERαS). Rabbit polyclonal anti-human ERβ (Santa Cruz Biotechnology, Inc., CA, USA). For detection of actin, monoclonal IgG1 anti-human actin antibody was used (Lot # J0804; Santa Cruz Biotechnology, Inc.).

Table I. *Summary of ER assessment (% positivity) in breast cancer cell lines by FCM+.*

Cell line	ER (lit)[a]	No. of assays	Isotype control		αS		αH		NRS		β	
			%	MFI	%	MFI	%	MFI	%	MFI	%	MFI
MCF7	+	24	0.71 (0.08)	1.475 (0.34)	44.77 (6.1)	5.25 (0.91)	4.99 (3.05)	0.69 (0.2)	0.515 (0.09)	0.413 (0.05)	61.49 (5.7)	4.91 (0.74)
T-47D	+	9	0.79 (0.2)	0.97 (0.2)	76.84 (7.4)	8.81 (2.03)	1.86 (0.87)	1.69 (0.67)	0.55 (0.07)	0.71 (0.11)	49.45 (11.0)	3.41 (0.63)
ZR-75-1	+	10	0.59 (0.05)	0.7 (0.05)	60.95 (10.7)	3.77 (0.74)	1.39 (0.86)	0.67 (0.2)	0.55 (0.08)	0.58 (0.07)	85.03 (5.62)	4.55 (0.46)
MDA-MB-231	–	7	0.67 (0.1)	0.83 (0.2)	39.94 (14.2)	4.1 (0.9)	5.03 (2.39)	1.34 (0.26)	0.44 (0.09)	0.59 (0.05)	68.3 (7.6)	4.34 (0.87)
SK-BR-3	–	7	0.3 (0.1)	0.36 (0.04)	21.47 (8.8)	1.6 (0.61)	1.36 (0.93)	0.43 (0.1)	0.33 (0.07)	0.36 (0.04)	35.4 (7.4)	1.95 (0.49)
MDA-MB-453	–	7	0.76 (0.2)	0.79 (0.1)	43.35 (13.3)	4.17 (1.4)	1.4 (0.5)	1.1 (0.23)	0.7 (0.19)	0.64 (0.11)	45.96 (10.5)	2.91 (0.44)
HCC1954	–	9	0.68 (0.1)	0.73 (0.15)	46.6 (11.4)	4.12 (1.53)	1.14 (0.65)	0.73 (0.14)	0.46 (0.09)	0.63 (0.09)	71.45 (7.19)	5.26 (1.13)

+Mean + SEM in parentheses; [a]ERα classification from the literature. MFI: Mean fluorescence intensity.

Secondary: Flow cytometry (FCM): Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Immunotech, Beckman Coulter, Marseille, France), 1:50 dilution. FITC conjugated goat anti-rabbit IgG (Sigma, MO, USA), 1:100 dilution. Western blotting: ECL™ sheep anti-mouse IgG horseradish peroxidase (HRP) conjugate (Amersham, Little Chalfont, UK), 1:20,000 in 5% w/v non-fat dry milk (Marvel-Premier International Foods, Spalding, UK) in TBS-T (20 mM Tris, 137 mM NaCl, pH 7.6 and 0.1% v/v Tween 20). HRP-conjugated ECL™ donkey anti-rabbit IgG (Amersham), 1:10,000 dilution in 10% milk in TBS-T.

Flow cytometry. The published protocol was followed (11). Briefly, after trypsinization, cells were permeabilized and fixed at 22˚C: 0.25% paraformaldehyde for 25 min, 1% Triton X-100 (BDH, Poole, UK) for 5 min, 0.6% NP-40 (Sigma) in Hepes buffer for 6 min, followed by incubation at 4˚C overnight in 1% paraformaldehyde in PBS. Cells/nuclei were then incubated with 20% bovine serum albumin (BSA) for 30 min at 37˚C, followed by incubation with primary antibodies at a concentration of 10 μg/ml for 1 h at 37˚C. Labeled cells/nuclei were washed twice and incubated for 30 min on ice with FITC-conjugated secondary antibody and then stained with propidium iodide (PI). Negative controls (P3X63Ag8, a mouse myeloma secreting IgG1 [Ag8] for the monoclonal antibodies, or normal rabbit serum [NRS] for the polyclonal antibody) were used in parallel, both at 10 μg/ml. Samples were analysed using a Coulter Epics XL flow cytometer and Expo™ 32 ADC software (Beckman Coulter Inc, Miami, FL, USA). A total of 10,000 events were measured and the percentage of receptor-positive cells/nuclei was determined by gating to exclude 98% of FITC-positive cells/nuclei in the controls. FCM results were expressed as: the percentage of positive cells/nuclei, calculated from the number of cells/nuclei and mean fluorescence intensity (MFI), calculated from the sum of the log to linear (channel number) × count in the channel/area. A limited number of experiments were also performed using: Intraprep Permeabilization Reagent (Immunotech, Coulter, Miami, FL, USA); 20 μg/ml of primary test antibody or control; secondary antibodies at a 1/20 dilution; overnight (16 h) incubation with the primary antibodies.

Immunofluorescence (IMFL). After overnight incubation of cells/nuclei at 4˚C in 1% paraformaldehyde in PBS, anti-ERαS antibody and control were then applied exactly as for FCM analysis. Cells/nuclei were pelleted at 500 ×*g* for 5 min and washed twice in 3% FBS with 0.1% Triton X-100, resuspended in 1% fetal bovine serum (FBS) in PBS and 40 μl dispensed on to microscope slides and viewed with a fluorescence microscope. Assessment was qualitative. Overnight (16 h) incubation with the primary ERαS, ERαH or ERβ antibodies was also evaluated.

Reverse transcriptase-polymerase chain reaction (RT-PCR). RNA was isolated from a minimum of 5×10^6 cells (12). Samples were then DNAse treated and reverse transcribed using random hexamer primers (13). The PCR reaction was carried out in a programmable thermal cycler (Perkin Elmer, model 9700) using the following primer sets: β-actin (14): forward GTCCTGTGGCATCCACGAAACT, reverse TACTTGCGCTCAGGAGGAGCAA; ERα (14): forward ATGGA ATCTGCCAAGAAGACT, reverse GCGCTTGTGTT TCAAC ATTCT; ERβ (15): forward CGATGCTTTGGTTTGGGT GAT, reverse GCCCTCTTTGCTTTTACTGTC.

Western blotting. Trypsinized cells (~2.5×10^6 cells) were centrifuged at 425 ×*g* for 5 min at 4˚C in homogenization buffer. Total protein was extracted from the cells (20 μg of protein was loaded per lane) and separated using SDS-PAGE. Western blotting analysis was performed as described previously (13). Membranes were probed with the ER antibodies, stripped, and re-probed for actin.

ER down-regulation. Semi-confluent cell cultures of MCF7, MDA-MB-231 and SK-BR-3 were trypsinized and 4.5×10^6 cells were pelleted for 7 min at 130 ×*g*, resuspended in 30 ml of medium

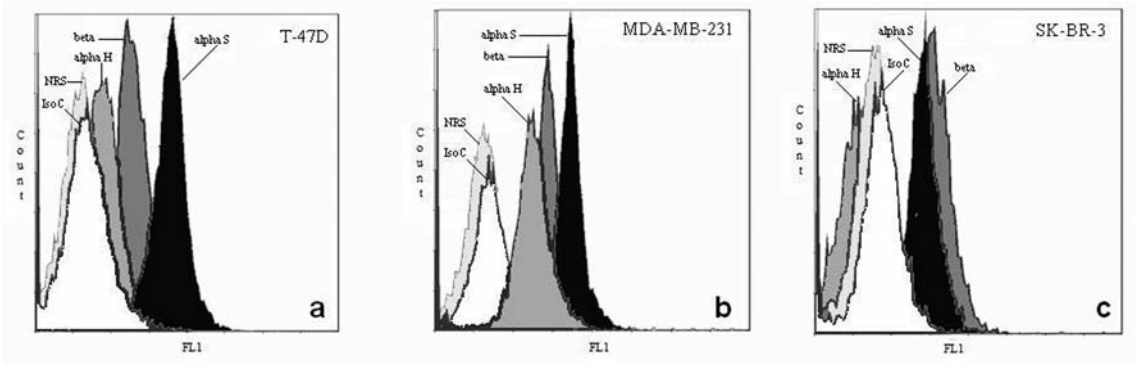

Figure 1. *FCM overlay histograms. Isolated nuclei/cells incubated with anti-ERαH (alpha H), anti-ERαS (alpha S), Ag8 (IsoC), anti-ERβ (beta) and NRS. a: T-47D; b: MDA-MB-231; c: SK-BR-3.*

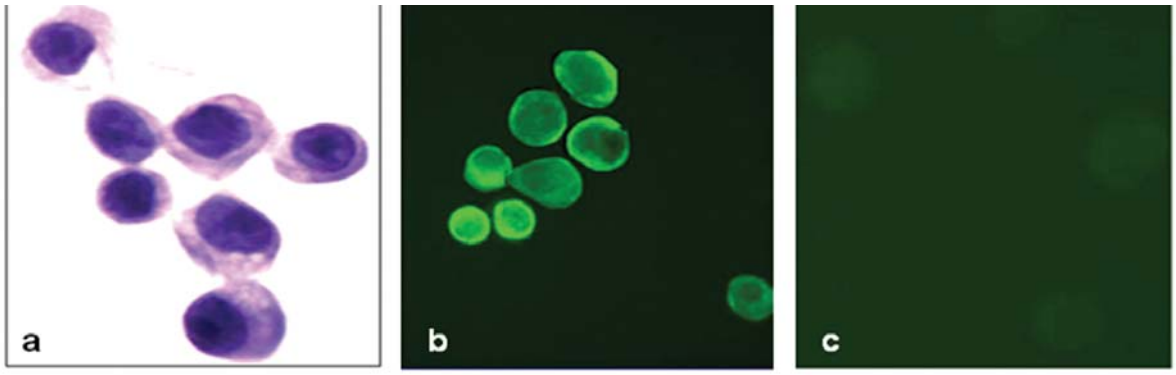

Figure 2. *Representative immunofluorescence results with SK-BR-3. a: H & E stained after nuclear isolation but before incubation with antibodies (original magnification ×40); b: after incubation with anti-ERαS (original magnification ×40); c: after incubation with Ag8 (original magnification ×40).*

(control) or 10 mM fulvestrant (ICI 182780) (Sigma)-containing medium, transferred to tissue culture flasks and incubated at 37˚C for 96 h. Flasks were trypsinized, the cells washed and then processed for Western blotting.

Results

ER expression determined by FCM. Table I summarizes the FCM results with the seven cell lines. For anti-ERαH, only ≤5% of the cells/nuclei were positive, although a comparison of MFI values between the isotype control and test antibody indicates higher values for T-47D, MDA-MB-231, and MDA-MB-453 cells. All of the cell lines were positive with anti-ERαS (range 21.47% for SK-BR-3 to 76.84% with T-47D). The MFI values were also all higher than those for the isotype control (range 1.6 for SK-BR-3 to 8.81 for T-47D). With anti-ERβ, with the exception of SK-BR-3, all the MFIs were greater than 2 (range 2.91-5.26) in comparison to the MFIs for NRS which were all less than 1 (range 0.413-0.71).

Representative overlay plots for three of the cell lines (T-47D, MDA-MB-231, SK-BR-3) are shown in Figure 1. These demonstrate staining of T-47D and MDA-MB-231 for ERαS, ERβ and to a lesser degree ERαH and staining of SKBR-3 for ERαS and ERβ.

Modifications to the method of permeabilization, the concentrations of the primary antibodies and the dilutions of the secondary antibodies did not affect the results significantly in terms of the % positivity or the MFIs. Increasing the incubation time with the primary antibody to overnight (16 h) did result in higher fluorescence intensity and a greater percentage positivity, which was also reflected in higher MF1 values (data not shown).

Immunofluorescence. There was no clear nuclear staining with any of the cell lines in the qualitative anti-ERαS IMFL assessment; however, all of the lines showed evidence of membrane and cytoplasmic staining over repeated assays. An example of this with SK-BR-3 is shown in Figure 2.

ER expression determined by RT-PCR and Western blotting. The expected PCR product (281 bp) for ERα mRNA was detected in five of the cell lines (MCF7, T-47D, ZR-75-1, MDA-MB-231, HCC1954) but not in SK-BR-3 and MDA-MB-453 (Figure 3). The expected 268 bp ERβ PCR product

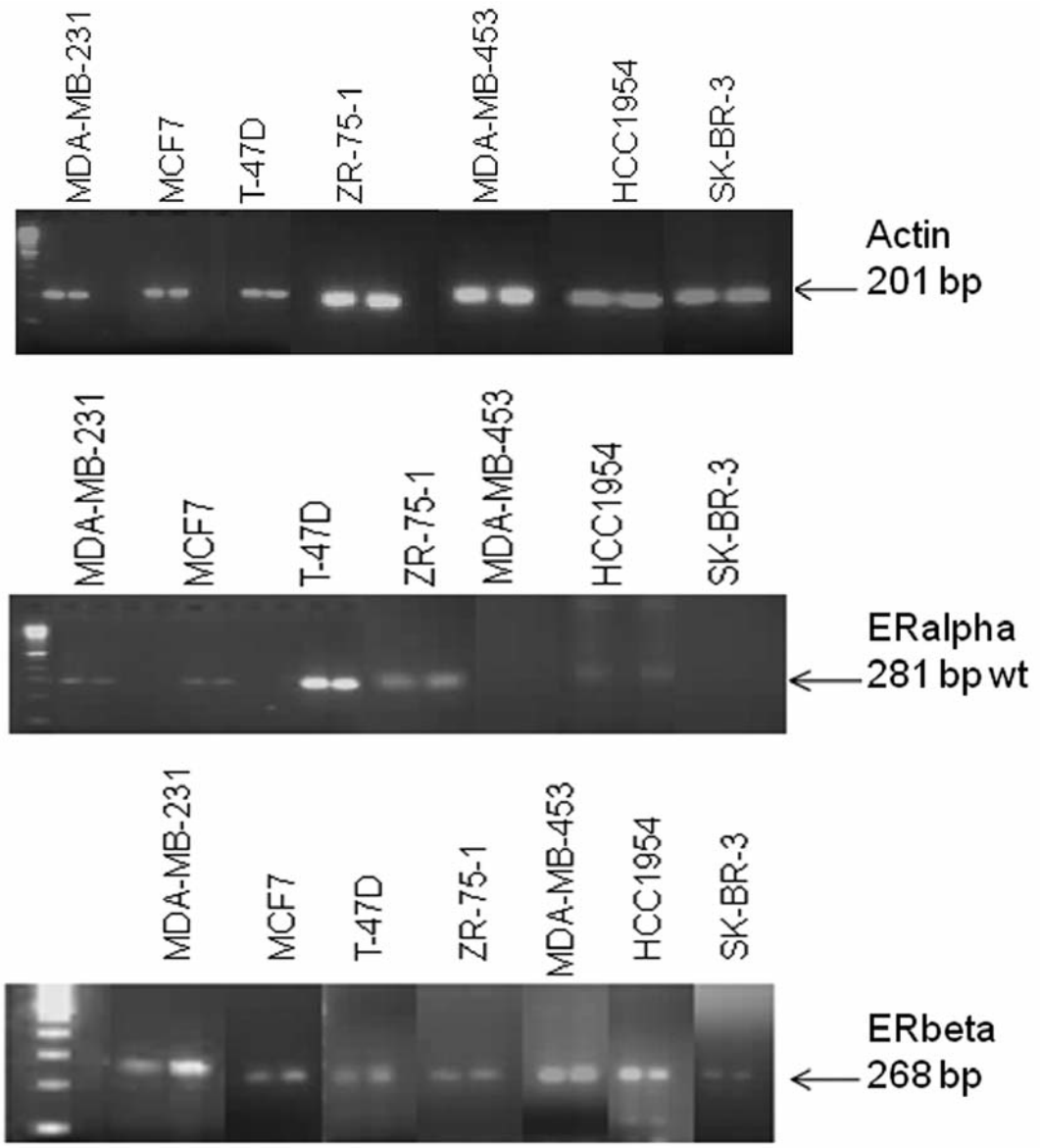

Figure 3. *RT-PCR results: representative ethidium bromide stained gels showing expression of ERα and ERβ. The left-hand lane shows the 100 bp ladder with the size of the products shown on the right-hand side of each gel. Samples for each cell line were run in duplicate. Expression of the 201 bp product for actin, as well as the 268 bp ERβ product, can be seen clearly in all the cell lines. Expression of the 281 bp ERα product can be seen in all the cell lines other than MDA-MB-453 and SK-BR-3.*

was also detected in all of the cell lines (Figure 3). ERα protein and ERβ protein was detected in all of the cell lines by Western blotting (Figure 4).

Down-regulation of ER. To confirm that it was ER that was being detected, one ER-positive (MCF7) and two ER-negative (MDA-MB-231, SK-BR-3) lines were treated with fulvestrant. ER down-regulation was seen with all three cell lines (Figure 5).

Discussion

IHC is the established method for the detection of ER in breast cancer tissue although problems with technical issues, intra- and inter-laboratory validation and reporting of results have been raised (16, 17). FCM has been suggested as a quantifiable method for determining ER in formalin-fixed, paraffin-embedded human breast cancer tissue (11, 18, 19) and in breast cancer cell lines (20-23).

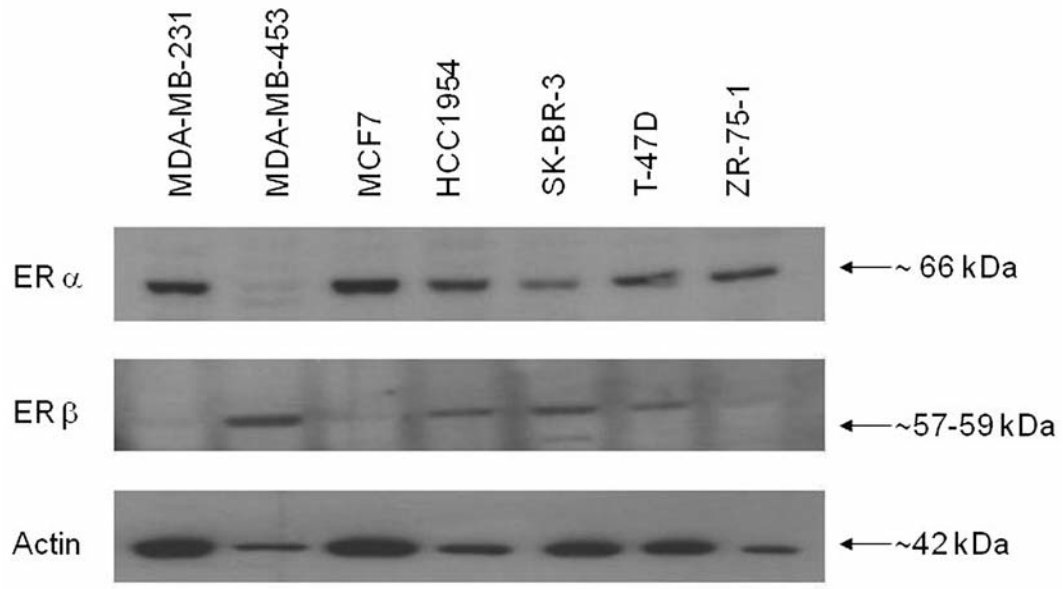

Figure 4. *Western blotting results. Expression of the ~66 kDa ERα product and the ERβ product of ~57-59 kDa can be seen in all of the cell lines.*

We found that BCC lines reported in the literature as being ERα-positive (MCF7, T-47D, ZR-75-1) were also ERβ-positive; cell lines reported as being negative for 'classical' ER (MDA-MB-231, SK-BR-3, MDA-MB-453, HCC1954), which are used extensively in biomedical research as control ER-negative cell lines, were ERα-positive with the FCM technique that we used. ER detection by FCM has been reported for T-47D, MCF7 and ZR-75-1 (20, 22, 23) with MDA-MB-231 showing the lowest MFI (22) and detection of ERβ by FCM has been reported for MCF-7, SK-BR-3 and MDA-MB-231 (21). Our results confirm and extend these observations to seven BCC lines.

When permeabilized cells are incubated with anti-ER antibodies, it is assumed that nuclear staining is what is being detected in FCM. However, our qualitative immunofluorescence microscopy results with anti-ERαS antibody clearly demonstrated cell membrane and cytoplasmic, but no nuclear, staining (Figure 2) with any of the cell lines, including those cell lines traditionally considered as being ER-positive (MCF7, T-47D, ZR-75-1). Overnight incubation (16 h) with the primary antibodies did not alter this.

We were meticulous in our procedures to minimize the likelihood of cross-contamination between cell lines, which has been shown to be a problem when using established cell lines (24). In addition, we used BCC lines that are accepted as being of breast cancer origin and not cell lines misclassified as breast cancer (25). Nevertheless, after our initial results with MDA-MB-231, we obtained fresh stocks of the cell line from the ATCC. Confirmation of our results with MDA-MB-231 with the new ATCC sample gives us confidence that our data are genuine.

We do not think that using over-passaged cell lines (26) can explain our results with the supposedly ER-negative cell lines as the literature suggests that breast cancer cell lines lose, rather than gain, ER expression with increasing passage number. In addition, the majority of assays were performed within a limited range of passages after receiving the lines from the ATCC.

It is now widely accepted that the ER may not be located at all times in a single subcellular compartment and that it may exist in a dynamic equilibrium between the plasma membrane, cytoplasm and nucleus (27, 28). The presence of membrane ER in MCF7 cells has been reported (29, 30) and our results confirm and extend this observation to six additional BCC lines.

ERα nuclear staining using the anti-ERαS (C-542) antibodies used in this study has been reported with rat pituitary tumour (31, 32) and neuronal cells (33) using immunohistochemistry and IMFL. The most notable difference between these studies and ours are (a) that we used human BCC lines and (b) we trypsinized our cell lines prior to processing for FCM analysis. There were also minor differences in the permeabilization and fixation used in the reported studies compared to our study. Our limited experiments with overnight (16 h) incubation of primary antibodies resulted in higher MFIs and intensity of staining, but did not result in nuclear staining (data not shown).

The FCM results were confirmed by the RT-PCR and Western blot analyses. The exceptions were the lack of detection of ERα mRNA in SK-BR-3 and MDA-MB-453. In RT-PCR and RNAse protection assays, T-47D was found to express

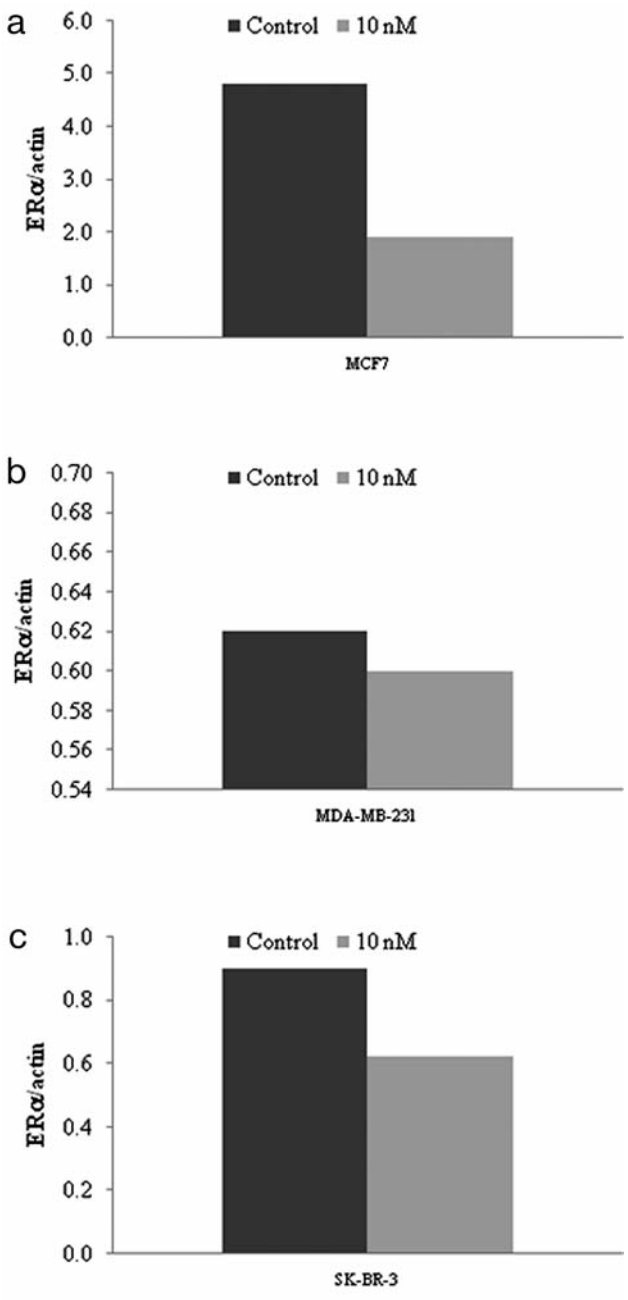

Figure 5. *ER down-regulation. Effects of fulvestrant on a: MCF7; b: MDA-MB-231; and c: SK-BR-3.*

moderate, MDA-MB-231, MDA-MB-453, MCF7 low, and SK-BR-3 undetectable levels of ERβ (34). In our experiments, all of the lines, including SK-BR-3, were found to express ERβ mRNA and this was confirmed by Western blotting.

Fulvestrant is a pure anti-estrogen that induces rapid degradation and loss of ER and it has been used to study down-regulation of ER *in vitro* (35). With the ER-positive (MCF7) and supposedly ER-negative (MDA-MB-231, SK-BR-3) cell lines we found ER down-regulation, confirming that what we were measuring was ER. Whether this is a variant of ER that is expressed on the plasma membrane and in the cytoplasm, for example ER-α36 (36), remains to be determined.

In conclusion, using reported FCM methodology to determine ER expression in isolated nuclei from breast cancer cells, and anti-ERα antibodies that have been reported as being able to detect nuclear ER by immunohistochemistry and immunofluorescence, we found that 7 BCC lines express both ERα and ERβ, including 4 cell lines reported in the literature as being ERα-negative (MDA-MB-231, MDA-MB-453, HCC1954 and SK-BR-3), with SK-BR-3 showing the least expression of both ERα and ERβ. It is probable that the FCM results reflect the membrane and cytoplasmic staining seen in the indirect immunofluorescence assays. RT-PCR and Western blotting corroborated the FCM findings and ER down-regulation confirmed that it was ER that had been measured. These results emphasize the importance of continuing the characterization of BCC lines used in research, particularly with reference to the property for which they are most widely used as models of breast cancer, their ER expression.

Acknowledgements

We thank the following: Mrs. Ani Mathew, Dr. Beryl Rego, Ms. Sheeba Varghese - cell culture and flow cytometry; Dr. Sureikah S. Mohan, Mrs. Lizamma Jacob and Ms. Jocelin Jacob - RT-PCR and protein experiments; Dr. Fahad Al-Mulla for the immunofluorescence images; Mr. K.I. Mathew for facilitating all aspects of the project. Grant sponsor: Kuwait University Research Administration, grant number MY01/02.

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Received November 29, 2010
Revised January 18, 2011
Accepted January 19, 2011