



Published in final edited form as:

J Cell Biochem. 2003 January 1; 88(1): 181–190. doi:10.1002/jcb.10353.

Increased Proteasome-Dependent Degradation of Estrogen Receptor-Alpha by TGF- β 1 in Breast Cancer Cell Lines

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Abstract

Normal mammary epithelial cells are rapidly induced to G₁ arrest by the widely expressed cytokine, transforming growth factor beta (TGF- β 1). Studies in established breast cancer cell lines that express the estrogen receptor alpha (ER α) have demonstrated loss of this responsiveness. This inverse correlation suggests interpathway signaling important to cell growth and regulation. The adenocarcinoma breast cell line BT474, which was not growth arrested by TGF- β 1, was used as a model of estrogen-inducible growth to explore interpathway crosstalk. Although BT474 cells were not growth-arrested by TGF- β 1 as determined by flow cytometry analysis and 5'-bromo-3'-deoxyuridine incorporation into DNA, estrogen receptor protein levels were attenuated by 100 pM TGF- β 1 after 6 h. This decrease in ER α reached 50% of untreated control levels by 24 h of treatment and was further supported by a 50% decrease in estrogen-inducible DNA synthesis. Inspection of ER α transcripts suggested that this decrease was primarily the result of altered ER α protein stability or availability. Use of the proteasome inhibitor, MG132, abolished all effects on ER α by TGF- β 1. Collectively, this data supports a role for TGF- β 1 in regulating the growth of otherwise insensitive breast cancer cells through modulation of ER α stability.

Keywords

breast cancer; estrogen receptor alpha; transforming growth factor beta; ubiquitin; proteasome; cell proliferation

Breast cancer is a leading cause of death among women worldwide and an estimated 203,500 new cases will be diagnosed in the US alone in 2002 [American Cancer Society, 2001]. Systemic and locally produced estrogens are intimately associated with increased risk for breast cancer development. Early menarche and late menopause increase the lifetime exposure to estrogens and as much as a 20% decrease in breast cancer risk is observed for each year this exposure is shortened [Kelsey, 1979]. By the same token, increased and sustained physical activity can reduce the total lifetime ovulatory period and has been shown to confer a significant decrease in breast cancer risk [Bernstein et al., 1994]. Numerous epidemiological studies of breast cancer risk in postmenopausal women have shown higher mean serum estrogen concentrations than disease-free control subjects [Thomas et al., 1997]. These and additional risk factors share a common theme, i.e., increased lifetime tissue exposure to estrogens. Thus, estrogen exposure is a central and reoccurring phenomenon in breast cancer etiology.

Although many risk factors have been identified, the molecular mechanisms underlying the initiation and progression of breast cancer are still elusive. Estrogens such as 17 β -estradiol

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(E₂) play a key role in the development and maintenance of the mature mammary gland. The most well characterized cellular receptor for this steroid hormone is the estrogen receptor alpha (ER α) [Moggs and Orphanides, 2001]. This receptor is expressed in a tissue specific manner and regulates growth and differentiation in response to hormonal stimulation [Dickson, 1995]. Additional co-regulators of ER α action have also been identified and studied in detail providing a foundation for the molecular mechanisms underlying the ligand-induced estrogenic response [Klinge, 2000]. Although the fundamental mode of action to elicit a response through this receptor has been characterized, little is known concerning the endogenous regulation of ER α expression.

On an average, only 7–30% of normal breast epithelial cells show expression of ER α by immunohistochemistry [Ricketts et al., 1991; Ronnov-Jessen et al., 1996]. Moreover, 60% of transformed breast epithelial cells express ER α supporting a role for hormone receptors in the selective growth of cellular subpopulations that may be important for tumor progression [Edwards et al., 1979]. Receptor availability is dictated by mechanisms regulating activity and levels in normal and in transformed breast cells. Methylation of the ER α gene promoter region may play a significant role in this process [Ferguson and Davidson, 1997]. Additionally, signals that regulate receptor protein levels also contribute to estrogen effects in the target tissue. Recent evidence supports the ubiquitin–proteasome pathway in ER α turnover [Nawaz et al., 1999]. It is not known if this pathway is utilized in generalized ER α turnover or if this mechanism is specific for malignancy and thus may contribute to tumor progression. The ubiquitin–proteasome pathway has additionally been associated with growth factor signaling in normal cellular processes [Kavsak et al., 2000; Wojcik et al., 2000].

In established breast cancer cell lines, a correlation has been observed between estrogen receptor content and sensitivity to transforming growth factor beta (TGF- β 1) [Arteaga et al., 1988]. TGF- β is a cytokine growth factor secreted by many cell types that potently suppresses cell growth through autocrine and paracrine interactions [Brandt and Ebert, 1998; Roberts, 1998]. Of the three identified TGF- β isoforms, TGF- β 1 has shown the most activity in epithelial cells [Silberstein and Daniel, 1987]. The primary mechanism of cell growth control by TGF- β 1 is mediated by the induction of selected cyclin dependent kinase inhibitors (cdkis), thus impairing basic cell cycle machinery [Ravitz and Wenner, 1997]. In general, cells expressing ER α are unresponsive to growth-arrest signaling by TGF- β 1, relative to effects in normal breast epithelial cells, while ER (-) cells are responsive [Lynch et al., 2001]. This suggests an estrogenic link to TGF- β signaling which may be involved in tumorigenesis.

Here we describe a model cellular system that implicates TGF- β 1 as being instrumental in regulating growth of hormonally responsive breast cancer cells. TGF- β 1 showed no effect on basal cell growth but instead appeared to suppress hormonally driven growth exclusively through ER α modulation. The thrust of this research was to uncover new signaling pathway interactions underlying cancer cell growth regulation.

MATERIALS AND METHODS

Cell Culture

Breast cell lines BT474, MDA-MB-231, T47D, and ZR75-1 were obtained from the ATCC (Rockville, MD). All cells, except MCF-10F cells, were passaged bi-weekly in B-media custom formula (MEM, Earle's salts, 1.5 \times amino acids, 2 \times non-essential amino acids, L-glutamine, 1.5 \times vitamins). This basal medium was supplemented with 10% FBS (Life Technologies, Brl., Rockville, MD), 2 mM L-glutamine, and 0.4% gentamycin. The immortalized MCF-10F cells [Soule et al., 1990] were cultured in DMEM: F12 supplemented with 5% horse serum (Life Technologies) treated with chelex-100 resin (BioRad, Hercules, CA) to remove calcium, 0.04 mM CaCl₂, 20 ng/ml EGF (Life Technologies), 100 ng/ml cholera toxin (Life Technologies),

10 µg/ml bovine insulin (Life Technologies), and 50 ng/ml hydrocortisone. Experiments were carried out in a defined media system consisting of B-media supplemented with 1% insulin–transferrin–selenium pre-mix (Collaborative Biomedical Products, Bedford, MA) and 2 mM L-glutamine. A 10 ng/ml dose of EGF was supplemented in defined media for MCF-10F cells. No cells were used beyond passage ten.

Proliferation Assay

Cells were plated in 100 µl volumes at densities of 1×10^4 cells per well in 96-well microtiter plates using 10% FBS supplemented growth medium. After a 24 h incubation, cells were replenished with defined medium containing the necessary experimental component and incubated an additional 24 h. After 18 h, cells were dosed with 5-bromo-3'-deoxyuridine (BrdU, Boehringer Mannheim, Germany) according to manufacturers instructions to allow for incorporation into DNA. Assessment of growth was determined using a Spectramax 340 microplate reader (Molecular Devices, Sunnyvale, CA) set at 370 nm.

Western Analysis

Cells were grown to sub-confluence on 100mm culture plates. The plates were rinsed and replenished with defined media in the presence or absence of experimental agent. Each plate was scraped into 1 ml of cold detachment buffer [0.25 M sucrose, 10 mM Tris (7.4), 1 mM EDTA, 0.3 mM PMSF] and pelleted at 2,000g for 10 min. The cells were then lysed by three cycles of freeze/thaw in cold lysis buffer [2 mM NaMO₄, 2 mM NaVO₃, 1 mM DTT, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml PMSF, 1% Triton X-100, 20 mM Tris (7.4), 2 mM EDTA, 25 mM NaF]. Cell debris was pelleted at 12,000g for 15 min and total proteins were resolved by 8% SDS–PAGE. Polyclonal antibodies to ERα (HC-20, Santa Cruz) were used for probing corresponding PVDF blots for 90 min followed by secondary antibody and ECL development. The proteasome inhibitor MG132 (Sigma-Aldrich, St. Louis, Mo) was dissolved and diluted in methanol and used at a concentration of 300 nM.

Cytotoxicity Assay

Cells were incubated in 96-well plates with or without experimental agent. Twenty-four hours following dosage, 50 µl of conditioned media was removed from each well and tested for the presence of lactate dehydrogenase (LDH) using the CytoTox 96 Assay (Promega, Madison, WI). A 100% LDH release control was achieved by three cycles of freeze–thaw on the cells to disrupt cell membrane integrity.

Flow Cytometry

Subconfluent cells were dosed with 10 nM E₂, 100 pM TGF-β, or E₂ + TGF-β on defined media. Cells were harvested at 24 h following dosing and fixed in 70% EtOH overnight at –20° C. Fixed cells were rinsed two times with HBSS (PBS, 1% BSA, 1 mM EDTA) and incubated with 10 µg/ml propidium iodide and 1 mg/ml RNase A at 37°C for 30 min. Samples (2×10^6 cells/ml) were refrigerated in the dark for 24 h followed by Epics Elite Scan analysis (Beckman Coulter, Palo Alto, CA). The immortalized, non-transformed breast epithelial cell line MCF-10F and the tumorigenic MDA-MB-231 cell line were used as positive controls for TGF-β1 induced G₁ arrest.

RT-PCR

RT-PCR analysis was used to evaluate total isolated RNA following TGF-β1 treatment. Primers for the 650 bp ERα product were used as previously described: sense 5'-TAC TGC ATC AGA TCC AAG GG-3'; antisense 5'-ATC AAT GGT GCA CTG GTT GG-3' [Lau et al., 1999]. Internal control gene 36B4, which is unaffected by estradiol, was used to normalize for dosing and consistent gel loading [Laborda, 1991]. Primers for the 550 bp product are:

sense 5'-TTT CAG CAA GTG GGA AGG TG-3'; antisense 5'-AAA CTG CTG CCT CAT ATC CG-3'. Samples were optimized for linearity and Mg^{2+} concentration. RNA was isolated from treated cell cultures using the Trizol reagent (Life Technologies) and quantified by UV absorbance. A 1 μ g sample of RNA was used in subsequent reverse transcription assays using superscript II reverse transcriptase (Life Technologies). A 1 μ l product from each sample was then used in PCR reactions catalyzed by Platinum Taq DNA polymerase (Life Technologies). PCR was performed for ER α (26 cycles) and 36B4 (22 cycles) on an MJ Research PTC-100 Thermal Cycler under the following conditions: ER α [95°C, 1 min; 58°C, 1 min; 72°C, 1 min] and 36B4 [95°C, 45 s; 64°C, 1 min; 72°C, 1 min]. PCR products were mixed at 2:1 and were resolved by 0.9% agarose gel electrophoresis in 1 \times TAE at 90 V for 3 h. The resulting gel was stained with ethidium bromide and fluorescent images were analyzed by Kodak digital science system (Eastman Kodak Company, Rochester, NY) and were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Northern Analysis

Total cellular RNA was isolated by the Trizol method (Invitrogen, Life Technologies, Carlsbad, CA). Probes to ER α and 36B4 were generated by klenow incorporation of 50 μ Ci of 32 P-dCTP in 25 ng of DNA for 4 h at 25°C. Probes were precipitated with 95% ethanol, 3 M NaOAc, and 40 μ g of glycogen at -20°C for 1 h. Pellets were collected by centrifugation and resuspended in molecular biology grade water. Probe specific activity was then determined by scintillation counting. A total of 12×10^6 cpm was used for abundant RNA species and 25×10^6 cpm was used for low level species. Following electrophoresis of RNA samples, resolved lanes were transferred to nitrocellulose membranes using the turbo blotter transfer system. Probing conditions were carried out as previously described, blocking with salmon sperm DNA. Bands were detected by Phosphor-Imager analysis.

Statistics

Statistical and graphical information was determined using GraphPad Prism software (GraphPad Software Inc., San Diego, CA) and Microsoft Excel (Microsoft Corporation, Redmond, WA). *P* values were calculated with the two-tailed unpaired *t*-test at 95% confidence.

RESULTS

E₂-Induced DNA Synthesis is Negated by TGF- β 1 in an ER-Dependent Manner

Three ER (+) and three ER (-) human breast cancer cell lines were evaluated for their growth responsiveness to TGF- β 1. When grown in the presence of serum-free media, the ER (+) cells were unresponsive or slightly stimulated to grow by TGF- β 1, while ER (-) cell growth was reduced to at least 50% of untreated samples (Table I). This data confirmed a positive correlation of ER α status to lack of TGF- β 1 responsiveness. To explore possible links between TGF- β and E₂ signaling, the ER (+) breast cancer cells were also initially screened for E₂-inducible DNA synthesis. The cell line BT474 produced the largest induction of proliferation with E₂ treatment (Fig. 1). Thus, BT474 cells were selected to dissect important interactions between TGF- β 1 and E₂-mediated effects on cell growth.

BT474 cell proliferation was determined at various TGF- β 1 concentrations in the presence and absence of 10 nM E₂ (Fig. 2). As expected for an ER (+) cell line, no growth inhibition was observed with increasing TGF- β 1 concentration. Addition of E₂ resulted in a 75% induction of DNA synthesis at 24 h, which was attenuated by a concentration gradient of TGF- β 1. Addition of tamoxifen, a competitive antagonist of ER α , inhibited E₂-stimulated growth, and TGF- β 1 had no effect under these conditions. Collectively, these results suggested either an induction of TGF- β 1 mediated growth-arrest in the presence of E₂ or a reduction in E₂-

stimulated growth in the presence of TGF- β 1. ER α appeared to underlie this effect since no change was observed in the presence of tamoxifen. These effects were observed in other ER (+) cell lines to a lesser extent (Table II).

Estradiol Does Not Restore TGF- β 1-Induced Growth Arrest

To better understand if TGF- β 1 was active on BT474 cells only in the presence of E₂ a more detailed examination of the effects on the cell cycle was warranted. TGF- β 1 controls epithelial cell proliferation by inducing factors that result in G₁ arrest or attenuation. Flow cytometry was used to distinguish effects of TGF- β 1 on G₁ phase and E₂ on S phase, in the BT474 cell model. Cell lines were cultured in growth medium and treated at sub-confluence in defined media. In the immortalized and transformed ER (-) cells MCF-10F and MDA-MB-231, 100 pM TGF- β 1 altered cell cycle distribution by increased percent G₁ and decreased S phase (Fig. 3A, B). E₂ treatment of BT474 cells resulted in nearly a 100% increase of cells in S phase as predicted, however, TGF- β 1 alone had no effect on cell cycle distribution. In the presence of TGF- β 1, E₂-induced S phase was attenuated nearly 50% that of E₂ treated samples alone (Fig. 3C). These results were consistent with BrdU incorporation studies and suggested that TGF- β 1 could not control basal cell growth in BT474 cells, but instead must regulate E₂-inducible growth exclusively.

Because TGF- β 1 has been reported to induce apoptosis in some cell types, LDH was measured in the conditioned medium [Haufel et al., 1999; Rosfjord and Dickson, 1999]. No increase in released cellular LDH was detected at any concentration of TGF- β 1 or TGF- β 1 plus E₂ (data not shown). Further, TGF- β 1 did not induce genomic DNA laddering as determined by ethidium bromide staining (data not shown). Thus, TGF- β 1 did not induce BT474 cell death or cell cycle arrest in the presence or absence of E₂, implicating a role for TGF- β 1 in estrogen receptor modulation as a novel mechanism of proliferative control.

TGF- β 1 Regulates ER α Protein Levels

To investigate the role of ER α in this proliferative response, Western analysis was performed on BT474 whole cell extracts. Cells were grown to sub-confluence and dosed with varying concentrations of TGF- β 1 in defined media for 24 h. These cells, which exhibited no growth response to TGF- β 1 alone, appeared to respond with a 50% reduction in ER α protein levels at 100 pM TGF- β 1 (Fig. 4A). As expected, cells treated with 10 nM E₂ exhibited a 70% reduction in ER α . The combination of TGF- β 1 and E₂ did not result in additive reduction of ER α levels (Fig. 4B).

A closer inspection of ER α protein levels over a time-course of 40 h suggested an effect occurring by 6 h of treatment with TGF- β 1 and maintaining suppressed ER α levels relative to untreated controls (Fig. 5). Furthermore, suppressed ER α levels were replenished following removal of the TGF- β 1 and replacement with fresh defined media for an additional 24 and 48 h (data not shown). These results suggested that even though BT474 cells lack any basal growth response to TGF- β 1, TGF- β 1 could attenuate ER α levels thereby modulating hormonally induced growth.

TGF- β 1 Shows Slight Effects on ER α Transcript Levels

ER α transcript levels were likewise monitored since TGF- β 1 appeared to modulate ER α protein levels. Cells were cultured identically to those used in the protein level studies and total RNA was isolated at 24 h. PCR primers to ER α and internal control gene 36B4 were then used to amplify ER α transcripts following TGF- β 1 treatment. Samples treated with 100 pM TGF- β 1 exhibited only a slight decrease (~10%) in the levels of the 650 bp PCR product relative to defined media controls (Fig. 6A). This decrease did not occur in cells treated with 10 pM TGF- β 1 at 24 h. The phorbol ester, TPA, which has been shown to reduce ER α RNA levels in cell

cultures, was used in parallel experiments at 100 nM as a positive control. These results were further validated by Northern analysis and yielded similar results (Fig. 6B).

Proteasome Inhibitor MG132 Blocks ER α Modulation by TGF- β 1

The inconsistency of ER α RNA and protein levels suggested that additional mechanisms might be involved in the regulation of ER α . The ubiquitin–proteasome pathway has been previously shown to play an important role in estrogen receptor turnover [Nawaz et al., 1999]. Since TGF- β 1 failed to show effects on ER α transcript levels, the possibility of increased proteasomal degradation was explored. The competitive inhibitor MG132 was used to block proteasome function in BT474 cell cultures. Cells treated with TGF- β 1 for 20 h showed a 50% decrease in ER α protein levels relative to untreated controls (Fig. 7A). The addition of 300 nM MG132 appeared to completely block this effect (Fig. 7B). Moreover, lanes representing cells treated with TGF- β 1 had increased amounts of low molecular weight immunoreactive bands, likely indicative of proteolytic cleavage. This banding pattern was consistent with previously published reports of ER α proteolysis using anti-ER antibody, H222, and may or may not be the result of proteasome-dependent degradation [Nawaz et al., 1999].

DISCUSSION

Although breast cancer appears to be a multifactorial disease, estrogens have been strongly associated with the initiation and progression of this disease. Experimental evidence supports a role for estrogens in DNA synthesis and in general cellular growth-stimulatory pathways [Darbre et al., 1983; Lykkesfeldt and Briand, 1986; Mustafa and Bland, 1998; Castoria et al., 1999]. However, a full understanding of what distinctions define normal hormonally induced growth and differentiation and hormonally induced transformation will require a better understanding of interpathway regulation following hormonal stimulation. An estrogenic response requires estrogen binding to and activation of the estrogen receptor [Nilsson et al., 2001]. Therefore, control of ER α expression levels would offer a direct means of modulating E₂-mediated cellular effects such as growth.

TGF- β 1 is clearly instrumental to this process at the particular stage of cancer depicted in BT474 cells. TGF- β has been implicated in a wide range of effects in normal and disease processes many of which overlap or antagonize effects of estrogen action. It is important in such cellular processes as motility, growth regulation, development, and apoptosis. Loss of cell growth control by TGF- β is a common observed event in malignancy and important regulators of cell growth including p15, p21, and cdc25A are known to be transcriptionally controlled by TGF- β [Lawrence, 1996; Rooke and Crosier, 2001]. Although there have been previous reports of TGF- β 1 modulating ER α levels in a breast cancer cell line [Stoica et al., 1997], to our knowledge this is the first time it has been shown in a classically TGF- β 1 non-responsive cell line. We have shown a multileveled regulation, which mainly involves protein stability, but may include a transcriptional component. Screening of other hormonally responsive breast cell lines suggests that this same interpathway mechanism may exist.

While E₂ has been reported to induce degradation of the ER α through the ubiquitin–proteasome pathway, this auto-regulation likely requires realized molecular end-points of E₂ signaling. Additional factors that regulate the endogenous levels of ER α and thus bypass E₂-mediated effects should be considered key to the growth of normal and cancerous cell types. Here we demonstrate antagonistic growth factor pathways mediating estrogen responsiveness through ER α modulation. This finding is consistent with other published reports of TGF- β 1 effects on downstream estrogenic targets. Growth promoting hormonal targets such as cyclin D1 and c-myc, both highly associated with tumor promotion, have been shown to be negatively regulated by TGF- β via non-ER α mechanisms [Martinez et al., 2000; Seoane et al., 2001].

An important finding from this work is that lack of “growth responsiveness” to TGF- β is not synonymous with lack of “responsiveness” to TGF- β . The apparent “uncoupled” TGF- β signaling, as has been reported in other cell types [Calonge and Massague, 1999; Lu et al., 1999; Yakicier et al., 1999] warrants further examination. Regulation of selected gene expression in the absence of cell cycle control could be a result of mutation or alternative pathways activated only under select conditions. For example, specific regions of T β RII are responsible for a selected set of TGF- β responses [Chen et al., 1993] and inactivation of pRb may be important to this activity, as reported by Massague et al. [Zentella et al., 1991]. Some data on aberrant SMAD signaling coupled to a divergent response has also been reported [Liu et al., 1997]. Another likely explanation, based on reduced surface T β RII levels specific to ER (+) cell lines, could be a resultant sub-critical cdk1 stoichiometry sufficient to induce G₁ arrest [Lynch et al., 2001].

Additional studies should address the molecular aspects of this TGF- β induced ER α degradation. Recent elegant studies by several groups have shed new light on TGF- β induction of the ubiquitin–proteasome cascade. Ubiquitin ligase components such as Smurf1/2 and the anaphase-promoting complex (APC) are activated and/or interact directly with Smad proteins leading to proteasomal degradation of target proteins [Lin et al., 2000; Stroschein et al., 2001]. These targets include Smad components, thus conferring an additional layer of auto-regulation. More importantly, Smad-induced ubiquitin targets include factors that repress or override TGF- β signaling such as the transcriptional co-repressor SnoN [Bonni et al., 2001].

The ER α has been shown here and in other reports to be a target of the Ub–proteasome pathway. A TGF- β mediated pathway to this degradation may be the result of a direct Smad2/Smurf2/ER α complex as ER α has been found directly associated with activated Smads [Yamamoto et al., 2002]. Additionally, the Ski-interacting protein (SKIP) has been shown to interact with both nuclear hormone receptors and TGF- β signaling components potentially serving an adapter role in this regulation [Baudino et al., 1998; Leong et al., 2001].

Key protein stabilities in malignancy and their destructive destination by the ubiquitin–proteasome or related pathways will no doubt be an important focus of future endeavors. The necessity of proteasomal targeting in the delicate control of cellular growth is only beginning to unfold. This research should provide a foundation to further explore new regulatory mechanisms in developmental and tumor cell biology offering new therapeutic targets. Understanding of these basic interactions may also provide a more visible picture of uncoupled TGF- β signaling exhibited by some cell types and the importance of this to hormonally responsive breast cancers.

Acknowledgements

The authors thank Dr. Melanie A. Lynch for her assistance in the cell proliferation assays.

Grant sponsor: NIH; Grant numbers: RO1 CA73698, P30 CA16058; Grant sponsor: Breast Cancer Research Fund (OSUCCC).

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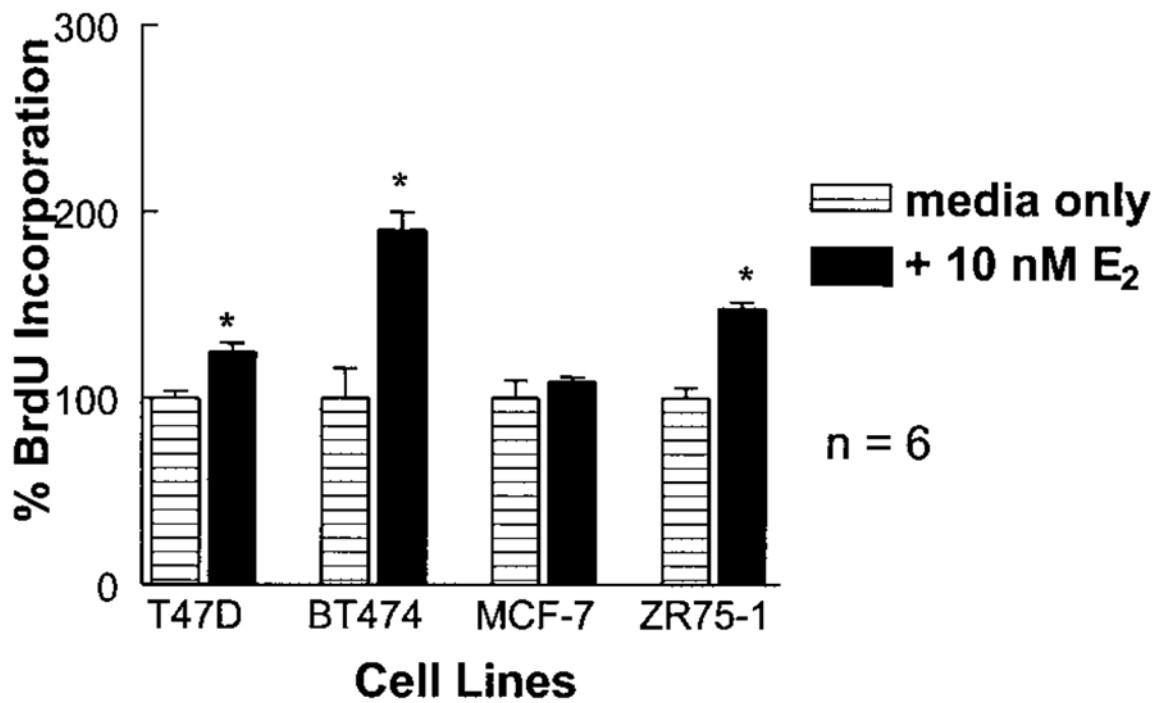


Fig. 1. Inducible cell growth by E₂ in breast cancer cell lines. Breast cancer cell lines were cultured in the presence or absence of 10 nM E₂ for 24 h on defined media. At 18 h, BrdU was added to the media and at 24 h absorbencies were taken to assess DNA synthesis. All tested cell lines exhibited increased DNA synthesis in the presence of E₂; however, those effects were most dramatic in cell line BT474. This data is the average of six independent treatments (**P* < 0.05).

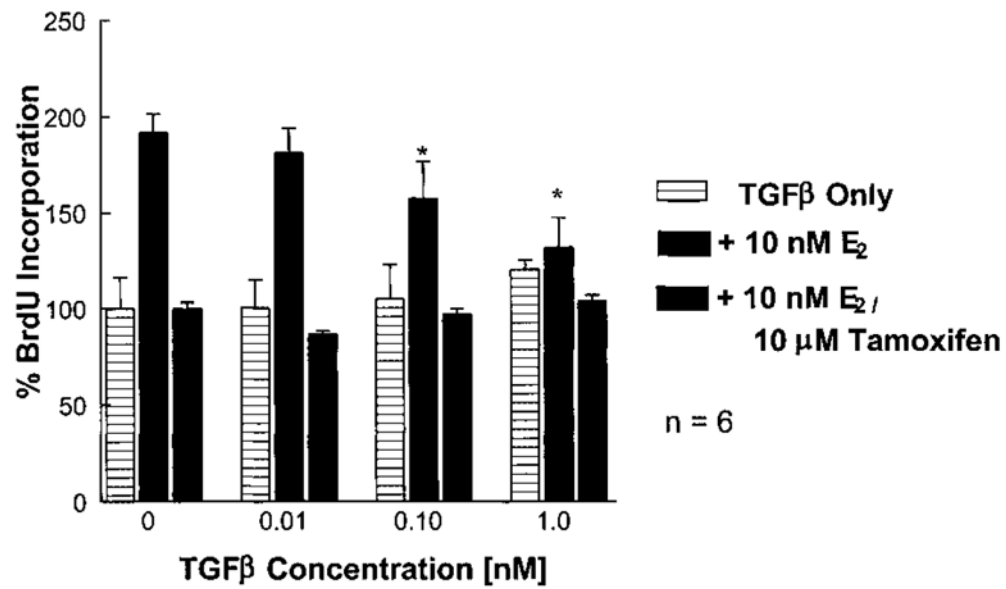


Fig. 2. Effects of TGF- β 1 on E₂-induced cell proliferation. BT474 cells were cultured in the presence or absence of indicated dose for 24 h in defined media. At 18 h, BrdU was added to the conditioned media and at 24 h absorbencies were taken to assess DNA synthesis. The presence of TGF- β 1 appeared to abrogate E₂-induced DNA synthesis. Addition of 10 μ M tamoxifen blocked E₂-induced DNA synthesis and all observed effects by TGF- β 1. This data is the average of six independent treatments (* $P < 0.05$).

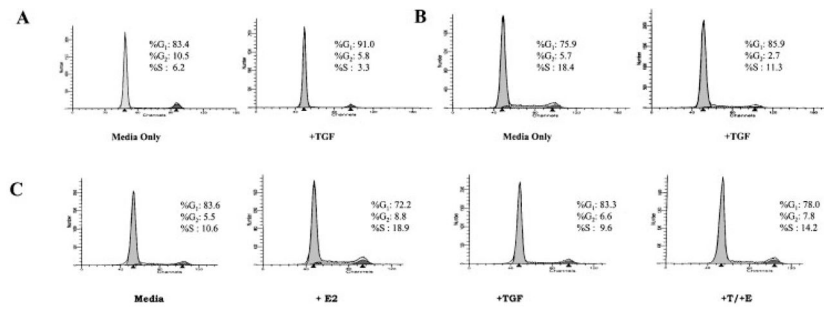


Fig. 3. TGF β modulation of cell cycle. Standard propidium iodide uptake was used to characterize TGF- β 1 induced G₁ phase. **A:** Control non-transformed MCF-10F cells treated with or without 100 pM TGF- β for 18 h show 50% reduction in S phase and 10% induction into G₁ phase. **B:** Example of a TGF- β 1 sensitive, tumorigenic cell line, MDA-MB231, treated with or without 100 pM TGF- β for 18 h also resulting in a 10% induction into G₁ phase. **C:** BT474 breast epithelial cell line treated with either 10 nM E₂, 100 pM TGF- β , or a combination of both for 18 h. The results indicate no increase of cells in G₁ phase following TGF- β 1 treatment. Furthermore, a 50% reduction in E₂-stimulated S phase was observed, consistent with BrdU studies.

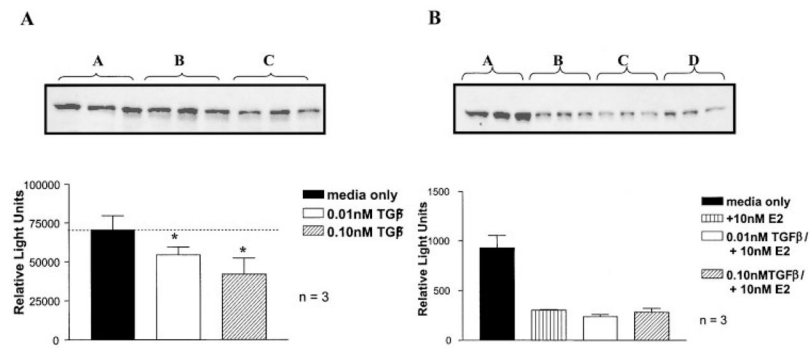


Fig. 4. ER α protein levels in BT474 cells. Protein from BT474 cells treated with or without TGF- β 1 in defined media for 24 h was isolated and probed for relative ER α expression. Total protein was isolated from BT474 cells treated for 24 h with experimental agent. Blots were probed for ER α using standard ECL detection. **A:** Up to a 50% reduction in ER α levels was observed at a concentration of 100 pM TGF- β 1. **B:** An expected 70% reduction in ER α was observed in E $_2$ only treated samples. ER α levels were not further reduced in TGF- β 1 + E $_2$ samples indicating that TGF- β 1 does not act in a concerted additive fashion with E $_2$ (* $P < 0.05$).

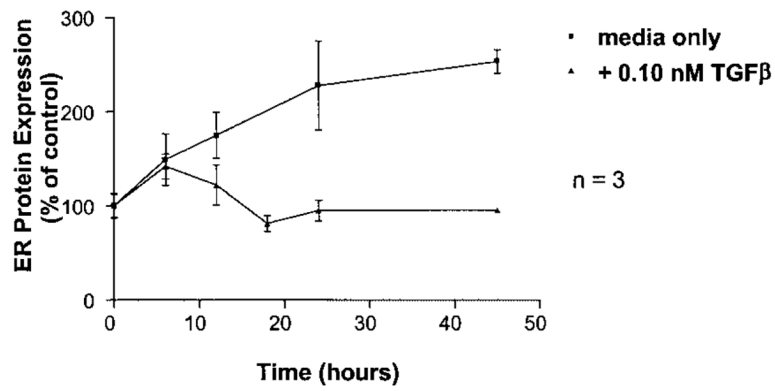


Fig. 5.

Time-course effects of TGF- β 1 on ER α levels. Total protein was isolated from TGF- β 1 treated and untreated BT474 cell cultures at multiple time points spanning 40 h in defined media. A 50 μ g quantity of total protein was resolved by 8% SDS-PAGE and the resulting PVDF membrane probed with antibody to ER α . Untreated samples showed increase in ER α levels with time. TGF- β 1 treated samples showed effects occurring between 6 and 12 h that maintain suppressed ER α levels.

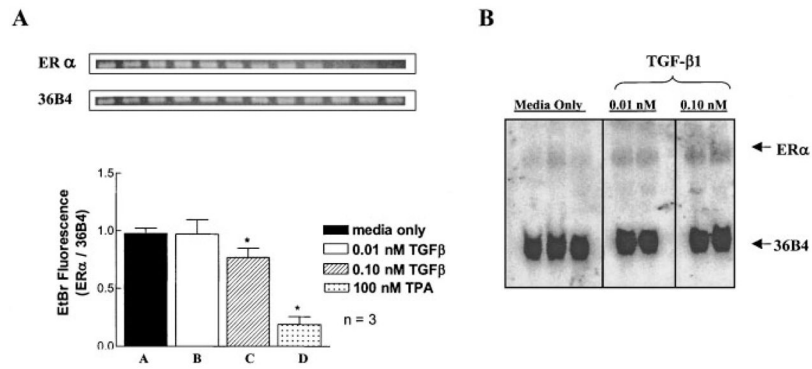
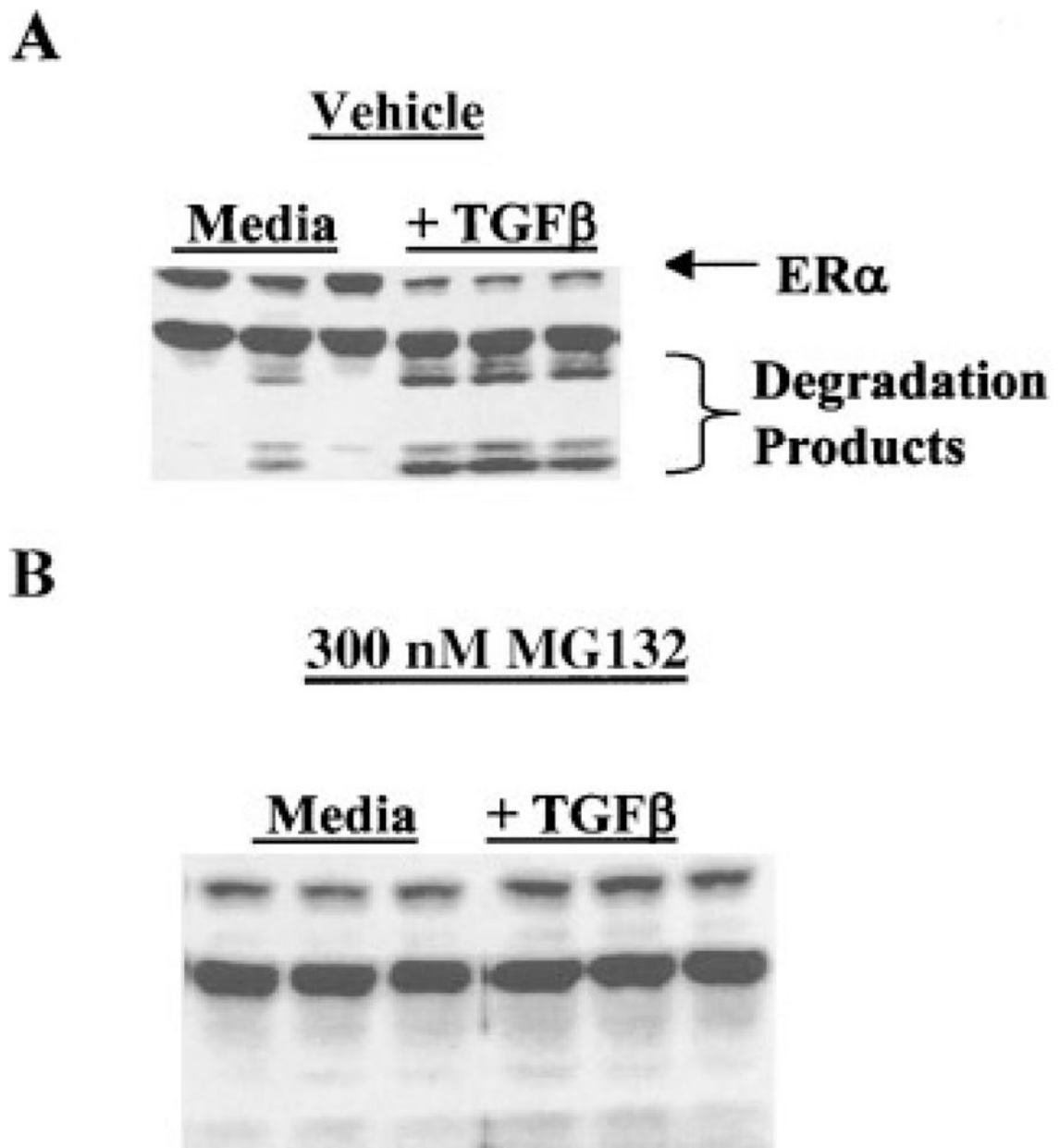


Fig. 6. Regulation of ER α transcript levels by TGF- β 1. **A:** BT474 cells were treated for 24 h with 0.01 or 0.10 nM TGF- β 1. Total RNA was isolated and RT-PCR was performed as described in Materials and Methods for ER α and 36B4. Combined PCR products were resolved by 0.9% agarose in $1 \times$ TAE. Bands were visualized following ethidium bromide staining and were quantified using ImageQuant software ($*P < 0.05$). **B:** Northern analysis was carried out using a cloned fragment of human ER α labeled with α - 32 P-dCTP as a probe. Low endogenous levels of ER α in the BT474 cell line exceeded the level of sensitivity of this assay; however, slight increases in ER α mRNA levels over untreated samples were apparent. The ER α signal was normalized to the internal control gene 36B4, which remained constant throughout. Qualitative binding was detected by phosphorimager analysis (n = 3 for media; n = 2 for TGF- β 1).

**Fig. 7.**

Abrogation of TGF-β1 mediated effects on ERα. Sub-confluent cultures of BT474 cells were treated with or without 100 pM TGF-β1 in the presence or absence of 300 nM MG132 for 24 h. **A:** A 50% suppression in ERα levels was observed in TGF-β1 treated samples compared with media alone (n = 3). **B:** No suppression in ERα levels by TGF-β1 was observed relative to control samples in the presence of the proteasome inhibitor MG132 (n = 3).

TABLE IGrowth Responsiveness to TGF- β in Various Breast Cancer Cell Lines

	TGF- β 1 concentration			
	ER	0.01 nM	0.10 nM	1.0 nM
BT20	-	73.7 \pm 3.7*	71.3 \pm 4.8*	48.7 \pm 3.4*
MDA-MB231	-	78.9 \pm 7.4*	52.7 \pm 3.5*	48.7 \pm 4.1*
SkBr3	-	70.4 \pm 7.7*	49.9 \pm 8.0*	36.2 \pm 5.2*
T47D	+	103.1 \pm 5.3	93.2 \pm 7.2	92.9 \pm 3.8
BT474	+	95.7 \pm 4.7	97.9 \pm 3.3	98.1 \pm 4.0
ZR75-1	+	97.6 \pm 9.4	105.6 \pm 6.5	107.6 \pm 5.6

A panel of breast cancer cell lines was grown with increasing titer of TGF- β 1 for 24 h in defined media. During the final 6 h, 5-bromo-2'-deoxyuridine (BrdU) was added to the media to allow for cellular uptake and DNA incorporation. At 24 h, percent of incorporated BrdU was determined at 370 nm. Untreated control absorbencies were taken as 100% growth. Values shown are percent of untreated controls and are the average of six independent treatments.

* $P < 0.05$.

TABLE II
Additional ER (+) Cell Lines Showing TGF- β Regulation of E₂-Induced Growth

	TGF- β 1 concentration					
	0 nM	0.01 nM		0.10 nM		1.0 nM
E ₂ (10 nM)	-	-	-	-	-	-
T47D	100 \pm 3.8	124.8 \pm 4.9*	113.8 \pm 5.8	112.5 \pm 3.8	96.9 \pm 3.0	104.8 \pm 1.7*
ZR75-1	100 \pm 5.6	148.3 \pm 3.2*	127.6 \pm 3.8	138.9 \pm 2.6*	137.5 \pm 2.2	114.1 \pm 5.2*

Cell lines T47D and ZR75-1 also demonstrated some signaling cross talk when initially screened in BrdU proliferation assays following a 24 h E₂ treatment. Values shown are percent of untreated control BrdU incorporation into the DNA of proliferating cell cultures. Data is the average of six independent treatments at each concentration.

* Asterisks denote significant differences between E₂ treated and untreated samples at each TGF- β concentration. $P < 0.05$.