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Report

GREB1 is a critical regulator of hormone dependent breast cancer growth

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Key words: breast cancer, estrogen induced growth, GREB1, hormonal control

Summary

Background. Estrogen plays a central role in breast cancer pathogenesis and many potent risk factors for the development of the disease can be explained in terms of increased lifetime exposure to estrogen. Although estrogen regulated genes have been identified, those critically involved in growth regulation remain elusive.

Methods. To identify candidate genes involved in estrogen stimulated breast cancer growth, DNA microarray based gene expression profiles were generated from three estrogen receptor α (ERα) positive breast cancer cell lines grown under multiple stimulatory and inhibitory conditions.

Results. Only three genes were significantly induced by 17β-estradiol (E2) relative to control in all three cell lines: GREB1, stromal cell-derived factor 1 (SDF-1) and trefoil factor 1 (pS2). Quantitative real-time PCR assays confirmed that in all three cell lines, GREB1 was induced by E2, but not by the antiestrogens tamoxifen (TAM) or ICI 182,780. GREB1 expression level was strongly correlated with ERα positivity in 39 breast cancer cell lines of known ERα expression status. GREB1 induction by E2 was rapid (7.3 fold by 2 h for MCF-7) and mirrored the fraction of cells entering S-phase when released from an estrogen deprivation induced cell arrest. Suppression of GREB1 using siRNA blocked estrogen induced growth in MCF-7 cells and caused a paradoxical E2 induced growth inhibition.

Conclusion. These data suggest that GREB1 is critically involved in the estrogen induced growth of breast cancer cells and has the potential of being a clinical marker for response to endocrine therapy as well as a potential therapeutic target.

Abbreviations: E2: 17β-*estradiol*; ERα: estrogen receptor alpha; ERβ: estrogen receptor beta; FBS: fetal bovine serum; GREB1: gene regulated in breast cancer 1; pS2: trefoil factor 1; SDF-1: stromal cell-derived factor 1

Introduction

Breast cancer is the most common malignancy in women in the Western world with over 200,000 new cases per year in the United States alone [1]. It has been well established that the hormone estrogen plays a significant role in breast cancer development and progression [2]. Many of the most potent risk factors for the development of breast cancer can be explained in terms of increased lifetime exposure to estrogen, and drugs that block estrogen action have been shown to prevent breast cancer [3]. Estrogen mediates many of its physiological effects by binding to specific steroid receptors, estrogen receptor alpha (ERα) and beta (ERβ). These receptors are the key regulators of estrogen signaling and can mediate a number of effects within the cell mainly by altering the expression of genes via direct interaction with their promoters or through binding to other proteins, which in turn interact with and regulate gene promoters [4]. While the hormonal dependence of some human breast cancer has provided many useful interventions, a failure to fully understand both the exact genes uniquely responsible for hormone stimulation of growth and pathways of growth control in hormone independent states has limited therapy.

Our laboratory is interested in identifying the key genes uniquely responsible for hormone stimulated growth. We used Affymetrix U133A Genechip® Arrays to analyze the gene expression patterns induced in three separate ERα-positive, estrogen dependent breast cancer cell lines (MCF-7, T47D and BT-474) grown in steroid depleted medium or in the presence of E2, 10% fetal bovine serum (FBS), TAM or ICI 182,780. These lines are classic estrogen dependent breast cancer cells whose growth *in vitro* and *in vivo* can be blocked by estrogen depletion and with antiestrogens [5]. Specifically, we

sought genes with expression patterns common to all three cell lines that were correlated with the proliferative behavior of the cells in response to estrogen or antiestrogen treatment. In this study we describe the detailed analysis of one of the genes (GREB1) identified using this method. Our results show that GREB1 is an early response gene regulated by ERα both *in vitro* and *in vivo* and is a key regulator of estrogen induced breast cancer growth.

Methods

Cell lines, culture conditions and growth assays

Tumor cell lines were maintained and growth assays performed as described previously [5]. For defined estrogen culture experiments, cells were washed and grown in steroid depleted media (phenol red-free IMEM supplemented with 10% charcoal stripped calf bovine serum – Valley Biomedical Products, VA) as described previously [5]. For growth assays, cells were plated in steroid depleted media at 2×10^3 cells/well in 96-well plates (Falcon, Lincoln Park, NJ) and cultured for 3 days before treatment with estrogen, antiestrogens or the appropriate vehicle control (ethanol 0.1%). Relative cell number was determined using the crystal violet assayed as described previously [6].

RNA extraction

Total RNA was isolated using TRIzol® Reagent (Invitrogen, CA) according to the manufacture's instructions. Yield and quality were determined by spectrophotometry (Beckman DU® 640, Beckman Coulter, Inc., Fullerton, CA) and using a Bioanalyzer RNA 6000 Nano chip (Agilent Technologies, Palo Alto, CA). The RNA from the panel of breast cancer cell lines grown under standard culture conditions was kindly provided by Dr Joe W. Gray at the University of California Breast SPORE program. All samples were stored at −80 °C. RNA for microarray analysis was labeled and hybridized according the Affymetrix protocol (Affymetrix GeneChip Expression Analysis Technical Manual, Rev. 3) by the University of Michigan Comprehensive Cancer Center Affymetrix and cDNA Microarray Core Facility.

Affymetrix microarray analysis

Gene expression patterns were determined using Affymetrix U133A Genechip® Arrays. Arrays were normalized and compared using DNA-Chip Analyzer software (dChip) [7]. Array samples were then compared to the control sample using a 90% confidence interval for fold change with the cutoffs for fold change and *p*-value being 1.2 and 0.1 respectively. Hierarchical clustering was performed using a subset of genes in which the variation across samples was greatest. All samples with a standard deviation/mean value of greater than 0.375 were included in this subset.

Flow cytometry

Cells ($\sim 7.5 \times 10^5$) were washed once with 1XPBS and re-suspended in 0.5 ml 1XPBS before drop-wise addition of ice cold ethanol (0.5 ml). The samples were incubated at room temperature for 30 min, and then centrifuged at $50 \times g$ for 5 min. The supernatant was removed and the cells stained in 0.5 ml of a solution of 50 ug/ml Propidium Iodide and 100 μg/ml Ribonuclease A (Sigma-Aldrich Co., St. Louis, MO). The samples were incubated in the dark at room temperature for 30 min before analysis using a FACStarPlus™ cell sorter. S-phase fraction was estimated using MultiCycle/MultiPlus software (Phoenix Flow Systems Inc., San Diego, CA).

Real-time PCR

GREB1 has three known alternative splice variants: GREB1a – 8482-bp (accession number = NM_014668), GREB1b – 2347-bp (accession number = NM_033090), and GREB1c – 2341-bp (accession number = NM_148903). Two separate quantitative real-time PCR assays were developed that detect GREB1a or all three transcripts. Primers were designed using the Primer3 program from the Whitehead Institute (www-genome.wi.mit.edu) and purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Total RNA (1 μg) was reverse transcribed using Reverse Transcription System (Promega, Madison, WI) and quantitative real-time PCR was performed using an iCycler Thermal Cycler (Bio-Rad, Hercules, CA). Two and a half % of the cDNA was amplified in 25 μl reactions using Platinum Supermix UDG (Invitrogen Corp., Carlsbad, CA), 250 nM of each primer, 10 nM fluorescein (BioRad Inc., Hercules, CA), and Syber Green. The GREB1a forward primer 5′-AAA TCG AGG ATG TGG AGTG-3′ and GREB1a reverse primer 5′-TCT CAC CAA GCA GGA GGA G-3′ amplify a 158-bp fragment spanning nucleotides 3317–3475 (accession number = NM_033090) a region specific for GREB1a. The GREB1a–c forward primer 5′-CAA AGA ATA ACC TGT TGG CCC TGC-3′ and reverse primer 5′-GAC ATG CCT GCG CTC TCA TAC TTA-3′ amplify a 172-bp fragment spanning nucleotides 1160–1331 a region to common to all three splice variants. Real-time PCR assay for the pS2 gene was adapted from Bosma et al. [8]. To control for RNA quality and quantity we used two separate housekeeping genes, glyceraldehydes-3-phosphate dehydrogenase (GAP DH) and estradiol-independent mRNA 36B4 [9] with the GAPDH forward primer 5′-GAA GGT GAA GGT CGG AGT C-3′ and reverse primer 5′-GAA GAT GGT GAT GGG ATT TC-3′ and 36B4 forward primer 5′-GTG TTC GAC AAT GGC AGC AT-3′ and reverse primer 5′-GAC ACC CTC CAG GAA GCG A-3′. For sequence

verification, PCR products were cloned into the pCR® 2.1 vector using the TA Cloning® Kit (Invitrogen Corp., Carlsbad, CA) according to the manufacture's instruction and representative clones subjected to dideoxy-sequencing.

To compare the relative efficiencies of each PCR reaction, serial dilutions (100, 10, 1, 0.1 and 0.01 pg) of template were prepared using GREB1 or housekeeping gene plasmid DNA. To evaluate the quality of product of real-time PCR assays, melt curve analysis was performed after each assay. Relative expression was determined using the $\Delta\Delta C_T$ method with either GAPDH or 36B4 as the reference gene [10].

GREB1 mRNA suppression using siRNA

Four synthetic small interfering RNA (siRNA) duplexes were designed using standard criteria and purchased from Dharmacon (Lafayette, CO). A non-silencing siRNA duplex, with no homology to any known sequence was used as a control (catalog #D-001206-09, Dharmacon Inc., Lafayette, CO). MCF-7 cells were cultured for four days in hormone-free medium as described above, then transfected with 100 nM siRNA specific to GREB1 or control siRNA using Oligofectamine™ reagent (Invitrogen, Carlsbad, CA) in serum-free OptiMEM-1 medium (Invitrogen) according to the manufacture's instructions for 4 h. One half volume of IMEM containing 30% CCS was added after 4 h. After 24 h, the cells were treated with a dose range of E2 (10^{-12} to 10^{-9} M) or 0.01% ethanol.

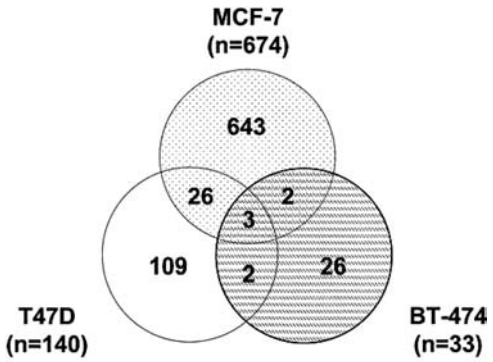
MCF-7 xenograft model

MCF-7 cells ($\sim 10^7$) were inoculated subcutaneously in the mammary fat pad region of 6-week old ovariectomized athymic nude mice containing sustained-released E2 pellets (0.72 mg/pellet, 60-day release, Innovative Research of America, Sarasota, FL) as described previously [11]. After 4 weeks, the mice were randomized into control (continued E2 supplementation) or E2 withdrawal (surgical removal of pellet). Tumors excised 24 and 48 h later, RNA was extracted and assayed from GREB1 mRNA. All animal experiments were performed according to a protocol approved by University of Michigan Guideline for Use and Care of Animals in compliance with US and UKCCR guidelines [12].

Results

We established appropriate culture conditions to develop an initial data set of estrogen regulated genes in MCF-7 cells. After 24 h of hormonal exposure, cellular RNA was extracted and the gene expression patterns determined using the Affymetrix U133A Genechip®. Previous studies in our laboratory have shown that replicate analysis of the same sample yielded 99.9% identical results indicating that the chips produce highly reproducible patterns of expression. The intensity data from the microarrays were normalized and modeled using DNA-Chip Analyzer software (dChip) [7]. Results of cluster analysis (provided as supplemental data 1) show that the pattern of gene expression in the estrogen depleted (vehicle control) cells was most like that of the ICI 182,780 treated cells while the E2 induced a pattern most similar to 10% FBS. The gene expression pattern induced by TAM showed similarities with both groups. Using our statistical criteria, only one gene, KIAA0575 also called GREB1 (Gene Regulated by Estrogen in Breast Cancer 1), was repressed by both TAM and ICI 182,780 while simultaneously induced by E2.

We repeated the array experiments on two additional ERα-positive/estrogen dependent breast cancer cell lines BT-474 and T-47D [5]. We hypothesized that by identifying genes altered by estrogen treatment that were common to three separate cells lines, we might reduce the number of candidate genes involved in estrogen induced growth. We used Venn analysis to generate the diagram shown in Figure 1. In the MCF-7 cells, 674 genes were significantly altered by estrogen while 140 and 33 were altered in the T47D and BT-474, respectively. Of these, only three induced genes were common to all three cell lines and include: pS2, SDF-1 and GREB1. Their fold induction is shown in the table. It has been previously reported that pS2 is not involved in cellular growth so we ruled it out as a critical regulator [13]. In contrast, SDF-1 has been shown in one study [14] to induce growth in breast cancer and its characterization is the topic of a separate study. Since very little is known about the role of GREB1 in estrogen induced breast cancer growth, we chose this gene for further characterization.



		Fold Induction		
Gene	Accession #	MCF-7	T47D	BT-474
GREB1	NM_014668	6.93	4.08	3.63
SDF-1	U19495	3.16	10.63	2.27
pS2	NM_003225	1.79	3.06	2.39

Figure 1. Venn diagram of genes significantly altered by E2 treatment in three estrogen receptor-positive, estrogen dependent breast cancer cell lines (MCF-7, BT-474 and T47D). Cells were grown in steroid depleted medium for 4 days and then treated with E2 (10^{-8} M) for 24 h. RNA was isolated and gene expression profiles measured using the Affymetrix U133A Genechip® Arrays. Results were generated from replicate biological samples. Only three genes were common to each cell line and their fold induction shown in the table.

To validate our microarray results we established two separate a real-time PCR assays. GREB1 has three known splice variants [15], and so one assay was designed to measure the longest transcript (GREB1a = 8482-bp), while the second measures the aggregate level of all three transcripts. Measurement of samples with both assays yielded similar results confirming that the level of all transcripts are regulated coordinately (data not shown). We confirmed that in all three cell lines GREB1 is induced by E2 and FBS but not by the antiestrogens TAM and ICI 182,780. Treatment with E2 and 10% FBS induced GREB1 mRNA levels 23.9 ± 0.01 and 14.1 ± 0.02 fold over control, respectively. In contrast, neither TAM nor ICI 182,780 treatments altered GREB1 levels (0.98 ± 0.05 and 0.94 ± 0.02, respectively). Similar results were observed using the T47D and BT-474 cell lines, validating our microarray results and confirming that GREB1 expression is regulated by estrogen in all three breast cancer cell lines.

We examined the correlation between GREB1 expression and ERα status in a panel of breast cancer cell lines. Shown in Figure 2 are the results for GREB assays in 39 breast cancer cell lines and sublines of known ERα status, plotted as the ratio of GREB1 transcripts/1000 GAPDH transcripts in decreasing order from left to right. The results show that expression of GREB1 correlated with the known estrogen receptor status [16].

We next studied the relationship between GREB1 expression and parameters of cell growth. As shown in Figure 3a, the proliferation over 4 days (plotted as % change in cell number over vehicle control) induced by E2 (304 ± 11%) was suppressed by ICI 182,780 (120 ± 2%) while ICI 182,780 alone had no effect (102 ± 7%). Changes in proliferation were correlated with the changes in GREB1 mRNA induction (shown in Figure 3b). Similar data were shown *in vivo*. MCF-7 cells were grown as xenografts in ovariectomized athymic nude mice implanted with sustained–release E2 pellets. After measurable tumors were established (~4 weeks), the mice were randomized into control (continued E2 supplementation) or E2 withdrawal (surgical removal of pellet) groups, and tumors assayed for GREB1 expression 24 or 48 h later. GREB1 mRNA levels were lower in all four tumors after E2 pellet removal compared to control tumors (Figure 3c), an effect that was not dependent on tumor size (expressed in mm^3 under each bar) showing that GREB1 is regulated by E2 *in vivo*.

The dose response relationship for GREB1 induction by E2 matches that for proliferation. GREB1 was induced by E2 in all three cell lines in a dose dependant manner (Figure 4). The greatest induction was seen in MCF-7 cells with 10^{-8} M E2 inducing GREB1 by 40 ± 5 fold in 24 h. These results are consistent with the published estrogen proliferative responses of these cells [5].

The time course of E2 induced GREB1 mRNA expression and induction of growth were measured in MCF-7 cells arrested by culture in estrogen-free conditions for 4 days. The cells were treated with E2 (10^{-9} M), total RNA and cell samples for flow cytometry were collected, and results are shown in Figure 5. GREB1 is an early response gene induced 7.26 ± 0.44 fold by 2 h reaching 29.86 ± 5.35 fold by 24 h. These changes mirrored the increase in the percentage of cells in S-phase with increases observed at 2 h reaching 37 ± 0.99% by 24 h. This experiment was repeated using the BT-474 and T47D cell lines with similar results (data not shown).

To test whether inhibition of GREB1 expression would affect growth, four synthetic small interfering RNA (siRNA) duplexes were designed to target regions common to all three GREB1 transcript variants. The

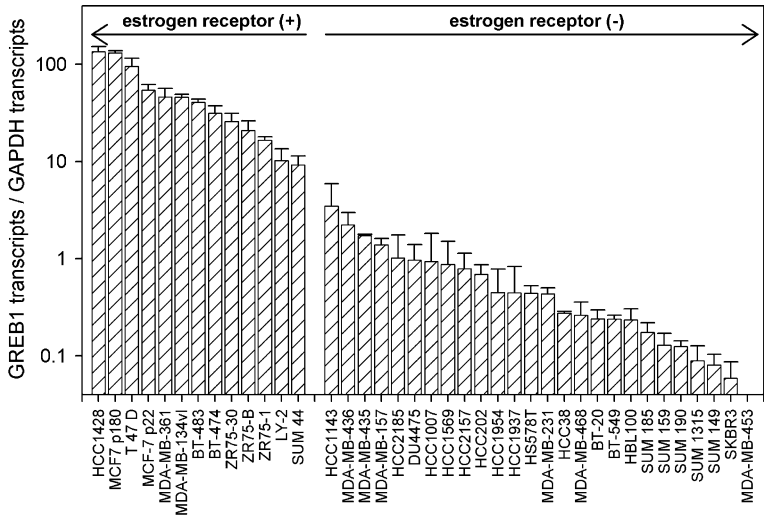


Figure 2. GREB1 mRNA expression levels correlate with ERα status in 39 breast cancer lines and sublines of know estrogen receptor status. Quantitative real-time PCR was performed as described in the Methods section and data are plotted as the ratio of the log of GREB1 transcripts/log GAPDH transcripts in decreasing order from left to right.

efficiency of these GREB1 siRNAs was verified by transfecting MCF-7 cells grown in 10% FBS with the siRNAs, both individual or as a pool of duplexes. GREB1 mRNA levels were determined 48 h later revealing similar efficiencies. GREB1 mRNA levels were suppressed approximately one tenth the level in cells transfected with a control siRNA duplex with no homology to any known sequence (Figure 6a). To further test the efficacy and specificity of our siRNA duplexes, MCF-7 cells were cultured for 4 days in steroid depleted medium, transfected with the siRNAs and then 24 h later treated with various doses of E2. After 4 days RNA was isolated, and assayed for GREB1, PS2 and 36B4 mRNA levels by real-time PCR. Transfection with GREB1 siRNA duplexes suppressed E2 induced expression of GREB1 (77% decrease at 10^{-9} M E2) (Figure 6b) but had no effect on PS2 or 36B4 levels (Figure 6c and d) suggesting the GREB1 siRNA duplexes are specific for that gene. Data are the results of quadruplicate samples (duplicate biologic samples from two separate experiments) assayed in triplicate.

To determine if the suppression of GREB1 mRNA levels by the siRNAs would have any effect on the growth of the cells, proliferation assays were conducted in parallel with the RNA studies described above. MCF-7 cells were cultured for four days in steroid

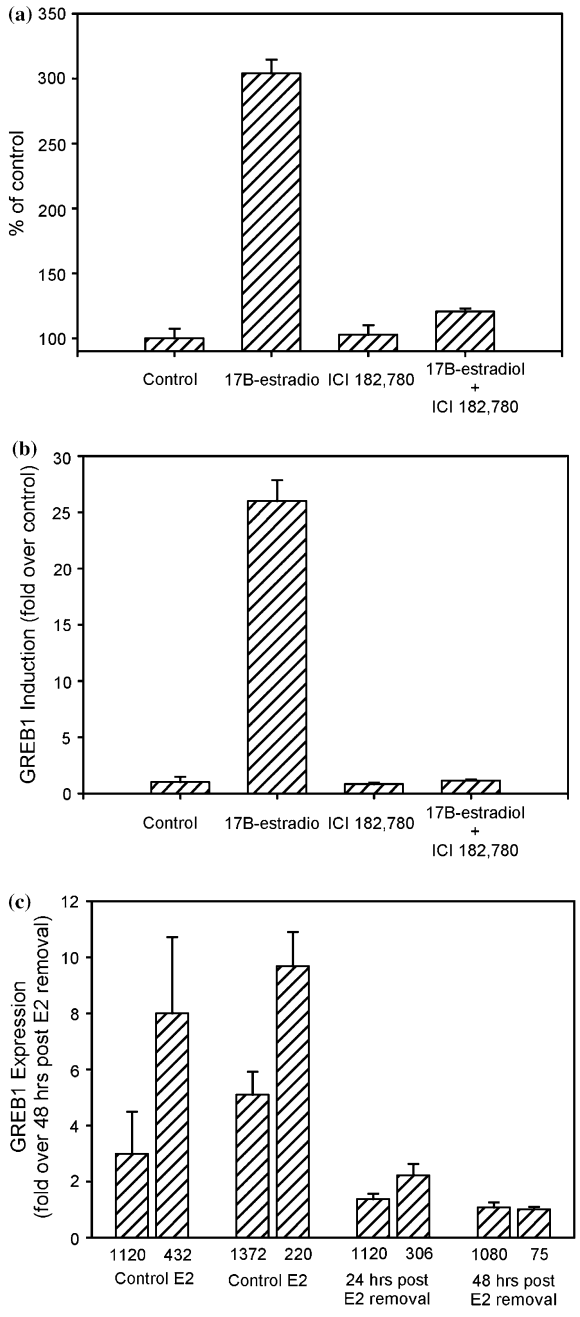


Figure 3. The anti-estrogen ICI 182,780 suppresses E2 induced cell growth and GREB1 induction in MCF-7 cells. MCF-7 cells were culture in steroid-depleted conditions and treated with: vehicle control, E2 (10^{-9}), ICI 182,780 (10^{-7} M), or the combination. After 4 days, cell number (a) was determined and compared GREB1 mRNA levels (b) at 24 h. Shown are the mean and standard deviation of duplicate samples assayed in triplicate. (c) GREB1 is regulated by E2 *in vivo*. MCF-7 xenografts were withdrawn from E2 supplementation, tumors assayed for GREB1 expression 24 or 48 h later and compared to control (continued E2 supplementation). Tumor volumes (expressed in mm^3) are shown under each bar.

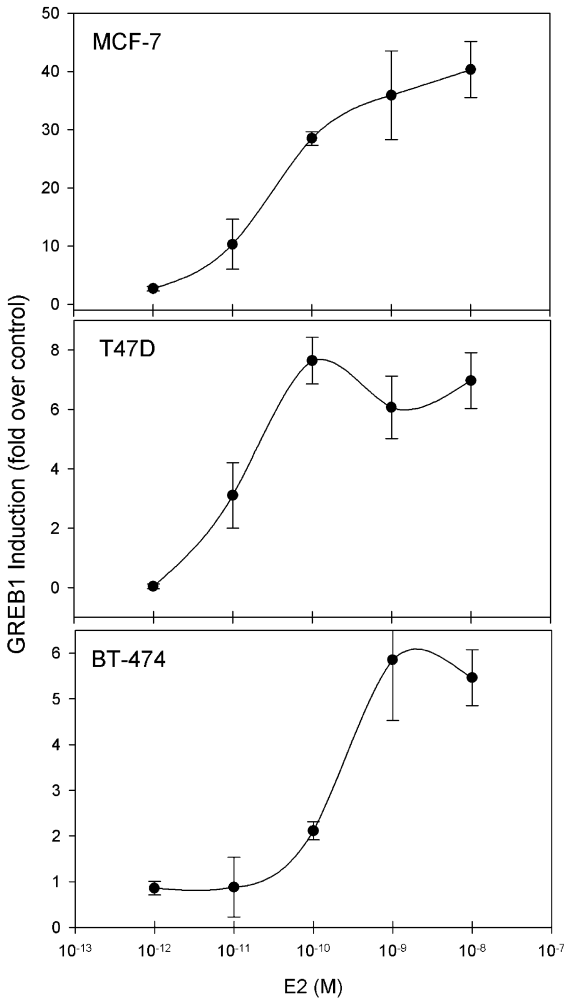


Figure 4. GREB1 induction is dose dependent. ERα-positive breast cancer cell lines were cultured in steroid depleted conditions and then treated with a dose range of E2 (10^{-12} to 10^{-9} M). After 24 h, RNA was extracted and assayed for GREB1 mRNA as described in the methods section. The mean and standard deviation of duplicate samples assayed in triplicate are shown.

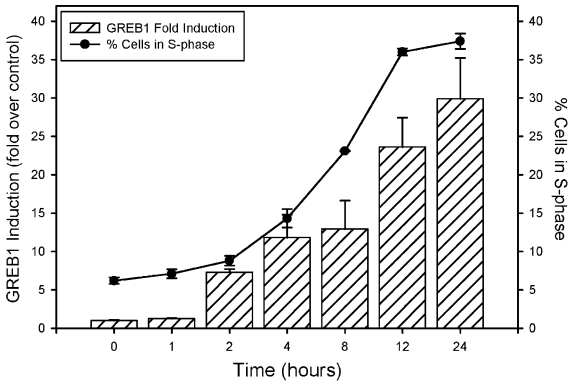


Figure 5. Time course of GREB1 induction and correlation with increased S-phase fraction. MCF-7 cells were cultured in steroid depleted conditions and then treated with E2 at 10^{-9} M. Samples were collected at 1, 2, 4, 8, 12 and 24 h and assayed for GREB1 mRNA levels by real-time PCR and S-phase fraction by flow cytometry. The mean and standard deviation of duplicate samples assayed in triplicate are shown.

hormone free medium, then transfected with the siRNA duplexs. After 24 h, the cells were treated with a dose range of E2 (10^{-12} to 10^{-9} M) and assayed for growth on days 2–5. As expected, E2 induced the growth of MCF-7 cells treated with control siRNA (Figure 7a). Cells grown continuously in the steroid depleted medium continued to grow slowly, indicative of a low level of residual estrogen, and transfection with the GREB1 siRNA had minimal effects. Transfection with the GREB1 siRNA had a dramatic effect on cells treated with E2. Not only was the normal stimulation of proliferation completely abolished, but growth was suppressed to below that seen in the cells grown continuously in the steroid depleted medium. There was a dose response relationship for this suppression that mirrored that for proliferation seen in the cells transfected with the control siRNA (Figure 7b).

Discussion

Previous studies to identify E2 regulated genes have focused on the gene expression patterns induced by estrogens and antiestrogens in single cell lines [17–22]. Our approach was to compare the gene expression patterns induced in three separate ERα-positive, estrogen dependent breast cancer cells lines grown under identical conditions, using multiple estrogen agonist and antagonist signals to narrow down the number of candidate genes for detailed characterization. We postulated that differentially regulated genes that are involved in estrogen induced growth could exhibit common patterns of regulation in parallel to effects on proliferation in all three cell lines, in contrast to those genes that are regulated by estrogen but not directly involved in cellular proliferation. Using this approach we identified three

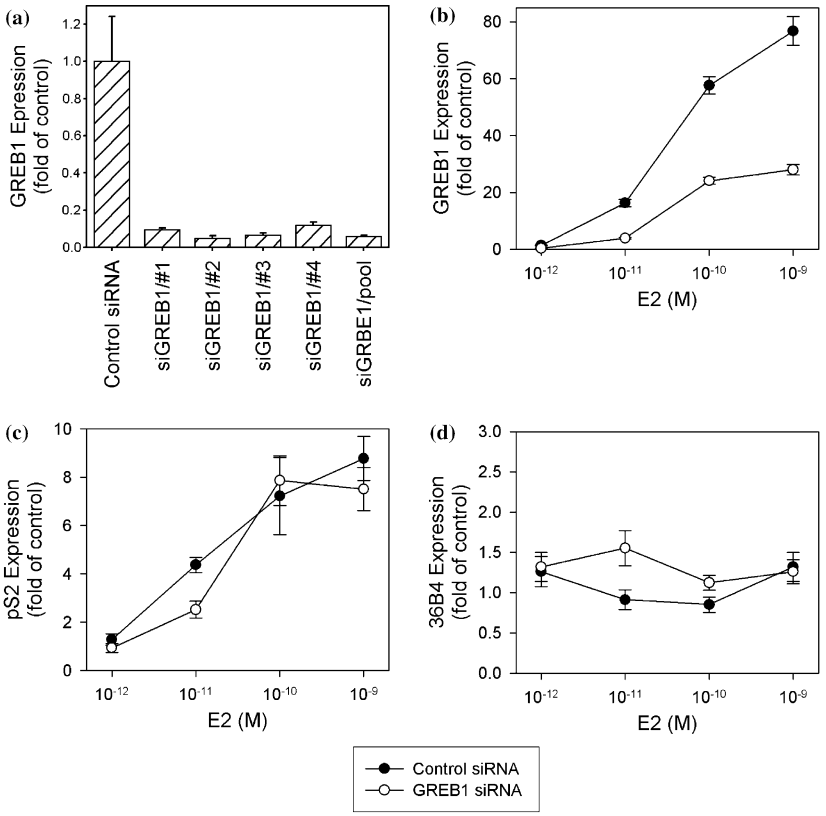


Figure 6. Efficacy and specificity of GREB1 siRNA duplexes. (a) MCF-7 cells grown in 10% FBS were transfected with individual or a pool of duplexes and after 24 h assayed for GREB1 mRNA. MCF-7 cells were grown in steroid depleted medium, transfected with the siRNAs, after 24 h treated with E2 and assayed for GREB1 (b), pS2 (c) and 36B4 (d) mRNA on day 4. Results represent the mean and standard deviation of quadruplicate samples (duplicate biologic samples from two separate experiments) assayed in triplicate.

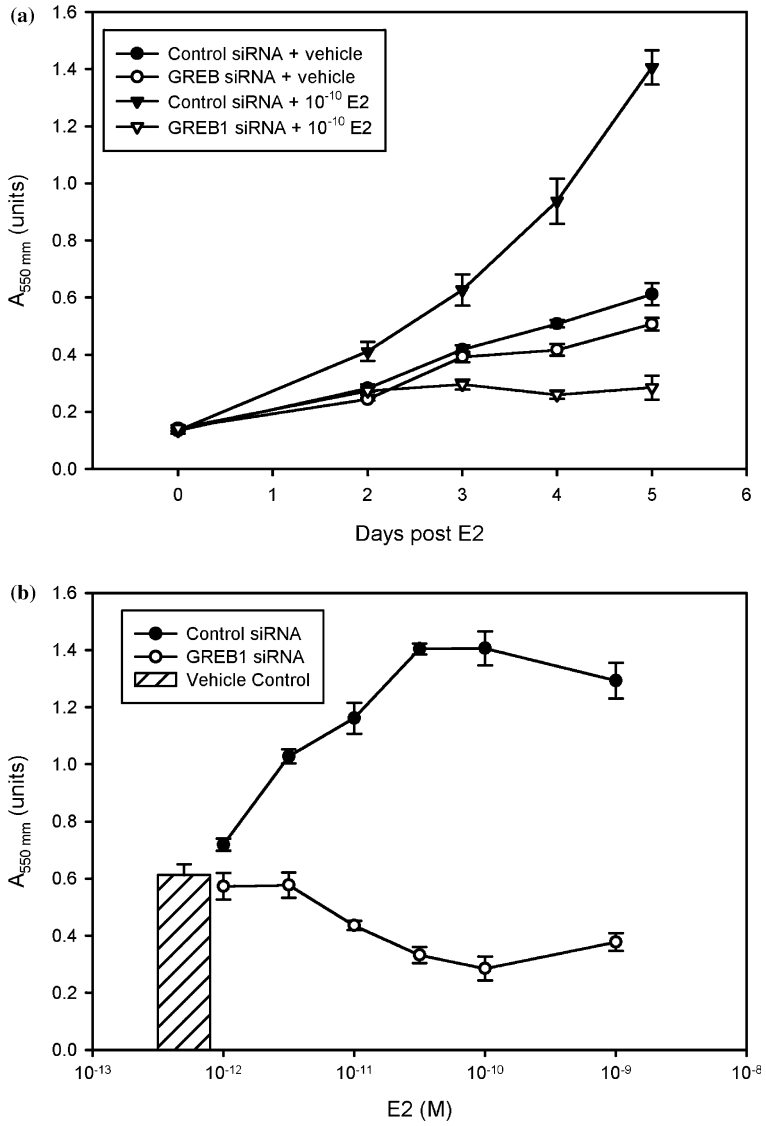


Figure 7. Suppression of GREB1 by siRNA reduces cell growth. MCF-7 cells were culture in steroid-depleted conditions, transfected with a pool of synthetic siRNA duplexes specific to GREB1 or a control siRNA, and after 24 h the cells were treated with a dose range of E2 (10^{-12} to 10^{-9} M). Changes in cell number were then assayed on days 2–5 by crystal violet staining. Growth curves for cells transfected with the control or anti GREB1 siRNAs treated with either E2 (10^{-10} M) or the vehicle control (0.1% ethanol) are shown in panel A. The dose response relationship for estrogen induced changes in cell number after 5 days of culture for cells transfected with either the control or GREB1 siRNA are shown in panel B. Each point represents the mean and standard deviation five replicate wells.

genes that were differentially expressed in MCF-7, BT-474 and T47D breast cancer cells treated for 24 h with E2 after rigorously removing exogenous estrogens: pS2, SDF-1 and GREB1.

Previously it has been shown that the pS2 gene, which is regulated by estrogen *in vitro* [23] and *in vivo* [24], is not required for estrogen induced cell proliferation and most likely plays a role protection of the gastrointestinal mucosal integrity [13]. This may be the underlying reason why pS2 expression in primary breast cancers does not provide a significantly better prediction of hormonal therapy than estrogen receptor status alone [25]. In contrast, studies suggest that SDF-1 may play a role in breast cancer pathogenesis. The cytokine SDF-1 is the principle ligand for the chemokine receptor CXCR4, a coreceptor with CD4 for the human immunodeficiency virus type 1 [26]. It has been shown that SDF-1 is a chemotactic factor and plays an important role in breast cancer metastasis by mediating actin polymerization and pseudopodia formation resulting in cell migration [27]. Recently, Hall et al. have shown that SDF-1 is induced by estrogen in MCF-7 cells and the BG-1 ovarian carcinoma cell line and induces cellular proliferation via autocrine/paracrine interactions with the CXCR4 receptor [14]. Our results confirm their conclusion that SDF-1 is an estrogen regulated gene in MCF-7 cells and show similar regulation in two additional ERα-positive/estrogen dependant cell lines.

In the current study we have focused on GREB1 because its cellular function and its role in breast cancer have not been established. It was originally discovered in the human brain [28] and the mouse homolog was

discovered soon after on chromosome 12 [29]. The human gene is located in the short arm of chromosome 12 (p25.1) where it spans roughly 108 kb. The longest transcript codes for a putative 1949-aa protein with at least four transmembrane domains and a N-myristoylation domain (http://www.ncbi.nlm. nih.gov/IEB/Research/Acembly). Gosh et al. were the first to show that GREB1 is an estrogen regulated gene [15]. They showed that it is an early response gene directly regulated by ER and suggested that it plays an important role in hormone-responsive tissues and cancer [15]. These results have been confirmed recently by Lin et al. who found GREB1 to be one of the few ER responsive genes shared by MCF-7 and T-47D cells and show using chromatin immunoprecipitation that ligand bound ER binds to the predicted estrogen response element in the putative GREB1 promoter region [30].

Having identified GREB1 as a gene consistently regulated in parallel with proliferation by estrogenic and anti-estrogenic treatments in three independent cell lines, and that its expression is significantly associated with ERα positivity in breast cancer cell lines, we set out to determine if GREB1 might be involved directly in estrogen stimulated growth. We used the powerful technique of siRNA mediated gene 'knockdown' and found that when GREB1 expression was suppressed, not only were the cells no longer stimulated to proliferate by E2, but in fact their growth was inhibited by E2 in a dose dependent fashion. This effect does not seem to be the result of a global alteration of the response of the cells to E2 since the induction of pS2 was not affected by GREB1 suppression. This result also suggests that the growth effects are not simply the result of global effects on mRNA levels or transcription, an idea further supported by the fact that 36B4 levels remain unaltered. While it remains possible that the GREB1 siRNA's may be affecting the levels of other genes that we have not examined, the fact that four independent GREB1 targeted siRNA's have very similar effects makes this quite unlikely. The use of the irrelevant siRNA duplex provides a control for the global, target-independent effects of transfection with siRNAs which have been reported [31].

It is possible that suppressing GREB may simply suppress proliferation, irrespective of whether that growth is stimulated by E2. Since GREB1 is itself an estrogen responsive protein, this is somewhat of a semantic issue and is quite difficult to test since none of the estrogen receptor negative breast cancer cells express GREB1 at significant levels, making them poor systems in which to test the effects of GREB1 suppression independent of the effects of estrogen. The function of GREB1 remains a mystery and few hints to its function are provided by analysis of the structure of the protein.

Much work remains to be done to establish the role of GREB1 in estrogen stimulated proliferation but the data presented here suggest the exciting possibility that GREB1 may be a valuable new target for the treatment of breast cancer.

Acknowledgements

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