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Estradiol downregulation of the tumor suppressor gene *BTG2* requires estrogen receptor- α and the REA corepressor

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Abstract

B-cell Translocation Gene 2 (*BTG2/TIS21/PC3*) is an anti-proliferative tumor suppressor gene whose expression is significantly reduced in breast carcinomas, and in MCF-7 and T-47D breast cancer cell lines treated with estradiol (E2). In this study the mechanisms involved in E2 down regulation of *BTG2* gene expression were examined. Depletion of ER α by siRNA indicated that the receptor is required for E2 down regulation of *BTG2* mRNA levels, and cycloheximide experiments indicated that the effect of E2 on *BTG2* expression was independent of intermediary protein synthesis. Chromatin immunoprecipitation analyses revealed that ER α interacts with the *BTG2* promoter in a ligand-independent fashion whereas transfection experiments indicated that ER α 's DNA and ligand binding domains are required for E2 repression of *BTG* promoter activity. Surprisingly, histone deacetylase (HDACs) activity is essential for basal expression as evidenced by trichostatin A inhibition of *BTG2* mRNA levels. Estradiol treatment did not alter histone H3 acetylation although it did induce displacement of RNA polymerase II from the *BTG2* gene. Depletion of the ER specific corepressor REA (Repressor of Estrogen Receptor Activity) significantly abrogated E2-mediated *BTG2* repression. Taken together, our results reveal a requirement of HDAC activity for basal *BTG2* expression and the ER α -REA interaction for estrogen repression of the *BTG2* gene. The ability of E2-bound ER α and REA to suppress *BTG2* expression indicates a positive role for this corepressor in regulation of breast cancer cell proliferation.

Keywords

ER α ; *BTG2*; estrogen-mediated repression; breast cancer cell proliferation

B-cell translocation gene 2 (*BTG2*) is an antiproliferative (ARPO) tumor suppressor protein, because its overexpression leads to blockade of the cells at the G₁ phase of the cell cycle.^{1,2} It was originally identified in the rat pheochromocytoma PC-12 cell line undergoing neuronal differentiation induced by nerve growth factor, where it was classified as an immediate early gene whose expression could be controlled by mitogenic, differentiative and antiproliferative factors.^{3,4} Later, it was demonstrated that *BTG2* expression is induced by cytotoxic and genotoxic stress through a p53-dependent mechanism.^{2,5} Binding sites for p53 and NF- κ B are located within the *BTG2* promoter, and the latter correlates well to the regulation of *BTG2* expression by NF- κ B activators such as tumor necrosis factor- α (TNF α) and some members of the transforming growth factor- β (TGF β) family.^{6,7} Induction of *BTG2* expression inhibits S-phase entry via either retinoblastoma (Rb)-dependent⁸ or independent pathways,^{9,10} and this is attributed, at least in part, to transcriptional suppression of the cyclin D1 gene whose product is required for the G₁ to S phase transition.⁸ A loss of nuclear *BTG2* expression is observed in ER α positive human breast tumor samples,¹¹ and a correlation between breast

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tumor size and BTG2 expression has also been demonstrated.¹² In normal mammary gland, BTG2 expression is highly regulated by estrogen and progesterone.⁷ Levels of both RNA and protein are suppressed during lactation, but return to prepregnancy levels rapidly once lactation is terminated. Consistent with *in vivo* studies, a decrease in BTG2 mRNA has been observed for E2 and progesterone treated MCF-7 and T-47D breast cancer cell lines, respectively.⁷ Moreover, microarray analyses reveal that BTG2 mRNA levels are reduced in E2-treated MCF-7 cells.¹³ Taken together, these studies suggest that E2 dependent inhibition of BTG2 expression may help to promote cell cycle progression.

The 2 estrogen receptor genes (ER α and ER β) belong to the steroid receptor superfamily and encode transcription factors that regulate diverse physiological processes in mammary gland, bone, brain and cardiovascular tissues.¹⁴ In the presence of its cognate ligand 17 β -estradiol (E2), ER β undergoes a series of sequential events that include changes in conformation followed by dimerization and recruitment to the regulatory regions of genes by either interacting directly with an estrogen response element (ERE) or indirectly via tethering to other DNA binding proteins such as AP-1, Sp1 or NF- κ B. In the course of this process, ER α recruits coregulatory proteins that remodel chromatin structure and ultimately determine the fate of gene transcription. In general, ER α exerts its function via 2 distinct regions; the N-terminally located activation function 1 (AF1) domain is constitutive and the C-terminal activation function domain (AF2) is ligand dependent. Moreover, the contribution of AF1 and AF2 to ER α activity is both cell type and promoter specific.^{15,16} Depending on the nature of the ligand, particularly whether it is an agonist, pure antagonist or selective estrogen receptor modulator (SERM), binding to ER α induces its ligand binding domain to undergo a structural reorganization that dictates the ability of the receptor to interact with different coregulators (coactivators or corepressors) and therefore regulate gene expression.¹⁷

Although considerable progress in our understanding of the molecular mechanism of estrogen action has been achieved in recent years, the specific roles of estrogen receptors in transcriptional events related to abnormal mammary cell proliferation and carcinogenesis are poorly understood. The advent of microarray technology made possible the large scale identification of mRNAs sensitive to estradiol treatment.^{13,18-21} Although some of the up-regulated genes identified in these studies are important for cell cycle progression (*e.g.*, c-Myc, cyclin D1), a number of them (*e.g.*, GATA1, pS2, Cathepsin D, NRIP1) appear to have little relationship with regulation of cell proliferation or survival. Moreover, although most investigations of estrogen regulated gene expression in breast cancer have focused on defining mechanisms of transcriptional activation, recent microarray analyses of estradiol-treated MCF-7 cells revealed a significant number of down-regulated genes.¹³ Interestingly, a number of these encode proapoptotic proteins (*e.g.*, IEX-1, caspase 9) consistent with the ability of estrogens to promote cell survival.²² Likewise, others are negative cell cycle regulatory genes (*e.g.*, cyclin G2, p21), whose inhibition enables cells to progress through the cell cycle.²³ More recently chromatin immunoprecipitation (ChIP) based methods (*i.e.*, ChIP cloning or ChIP-chip) applied to MCF-7 cells have identified ER α genomic binding sites and further defined possible ER α negative regulatory regions.²⁴⁻²⁸ In one ChIP-chip study examining ER α and RNA polymerase II (Pol II) interactions with gene promoter regions, it was found that E2 treatment caused a reduction in Pol II binding to 16 of the 663 ER α target genes examined,²⁶ and this suggested that E2-ER α can play a direct role in suppressing gene expression. On the basis of the proportion of genes downregulated *versus* upregulated by E2 in MCF-7 cells (36-70% to 64-30%, respectively, reviewed in ref. ²⁹), it would appear that E2 repression of transcription may be as necessary as gene activation for appropriate regulation of cell proliferation and survival. This highlights the importance of understanding the mechanism(s) employed by agonist-bound ER α to repress gene expression.

The list of well-characterized genes for which mechanisms of negative regulation by agonist-bound ER have been determined is relatively small and our understanding of the mechanism(s) involved in such repressive processes is undoubtedly incomplete. In some instances, E2-bound ER α repression of gene expression is associated with interactions between ER α and transcription factors such as p53 and Nrf2.^{30,31} It also has been shown that E2-dependent recruitment of the NCoR corepressor is associated with many of these negatively regulated genes.³²⁻³⁵ Somewhat surprisingly, the SRC-2/GRIP1 coactivator has been found to be a key factor for repression of some genes³⁶ and a repression domain has been mapped within this coregulator indicating that distinct surfaces of the SRC-2/GRIP1 coregulator are utilized in activation and repression contexts.³⁷ In addition, the involvement of the CBP/p300 coactivators in negative regulation of *c-Myc* in quiescent cells recently has been reported,^{38,39} and this further suggests that a complex array of mechanisms employing coactivators and corepressors can be utilized to repress gene expression.

On the basis of the microarray evidence that E2 treatment reduces steady state levels of *BTG2* mRNA and the relative paucity of information on mechanisms of E2-ER α suppression of gene expression, we investigated the molecular mechanisms employed by agonist occupied ER to regulate the expression of the *BTG2* gene. Our studies demonstrate an unexpected requirement of histone deacetylase activity for basal *BTG2* mRNA expression and reveal a novel mechanism in which agonist-bound ER α in conjunction with the REA (Repressor of Estrogen receptor Activity) corepressor actively represses expression of the *BTG2* gene.

Material and methods

Chemicals

17 β -Estradiol and the partial antiestrogen 4-hydroxytamoxifen (4HT) were purchased from Sigma (St. Louis, MO), whereas the pure antiestrogen ICI 182,780 (ICI) was obtained from Tocris (Ellisville, MO). Trichostatin A (TSA) was obtained from Millipore (Millipore Corp, Bedford, MA) and cycloheximide (CHX) was from Sigma.

Plasmids and siRNAs

The plasmids that express human full length ER α (pCR3.1-hER α), ER β (pCXN₂-hER β) and also the expression plasmids for ER α lacking the A/B domain (pCR3.1-hER α -179C, amino acids 1-178 deleted), ER α with 3 mutations (K362D/V376D/L539A) in the ligand binding domain (pCR3.1-hER α -KVL), ER α with a double mutation (E203A/G204A) within the DBD (pCR3.1-hER α -DBD) and ER α containing the A/B and DNA binding domains (pCR3.1-hER α -N282G, amino acids 1-282) have been described previously.^{40,41} Luciferase constructs p2658 and p266 (nucleotides -1 to -2658 and -1 to -266 upstream of translational start site of the *BTG2* gene cloned in pGL3) were kindly provided by Dr. Alain Puisieux.⁶ All siRNAs were chemically synthesized by Ambion (Austin, TX) as oligonucleotide duplexes. Target siRNA sequences for ER α ,⁴¹ SMRT,⁴² NCoR,⁴² SRC-1,⁴³ SRC-2⁴⁴ and SRC-3⁴³ were described previously. Predesigned siRNA for CBP (ID no. 146409) was obtained from Ambion. The siRNA against REA was obtained as a SMART pool from Dharmacon Research (Lafayette, CO). Ambion's Negative control siRNA no. 2 was used as nonspecific siRNA control.

MCF-7 cell culture and treatments

The MCF-7 human breast cancer cell line was maintained in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). For treatments or siRNA transfections, 3×10^5 cells MCF-7 cells were plated in each well of a 6-well plate and grown overnight in phenol-red-free DMEM containing 10% FBS. For treatments with TSA, CHX or ER α ligands, 24 hr after plating cells were switched to phenol red-free DMEM containing 10%

stripped serum and treated for the indicated time periods. For transfection experiments, one day after plating, cells were transfected with 20-40 pmol/well siRNA using Oligofectamine (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Four hours after transfection, medium was switched to phenol-red-free DMEM containing 10% sFBS and 24-48 hr later cells were treated with vehicle or hormone for 4 hr. Concentrations of siRNA, hormone and other treatments and their duration are indicated in the figure legends.

ER α trans-activation assays

The HeLa human cervical cancer cell line was maintained in DMEM supplemented with 10% FBS. For ER α *trans*-activation assays, 3×10^5 HeLa cells were plated in each well of a 6-well plate and grown overnight in phenol-red free DMEM containing 5% sFBS, followed by transfection with the indicated plasmids using Lipofectamine (Invitrogen). Thereafter cells were treated with vehicle or 10 nM E2, and 16 hr later were harvested for luciferase activity assays using a luciferase assay kit (Promega) and a Luminoskan Ascent Thermo Labsystems apparatus (Thermo Electron, Milford, MA). Relative luciferase units were normalized to total protein content of the cell lysate measured by Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Quantitation of endogenous gene expression

For mRNA measurements, total RNA was isolated by TRIzol® reagent (Invitrogen) following the manufacturer's instructions and analyzed by RT-qPCR using an ABI Prism 7500 sequence analyzer (PE Applied Biosystems, Foster City CA). Predeveloped 20× primer and probe mix was purchased from Applied Biosystems (BTG2: Cat no. Hs00198887_m1 and 18s: Cat no. 4310893E). Primers for cyclin G2 mRNA³⁴ were obtained from Invitrogen. BTG2 and 18S was measured by employing TaqMan chemistry, whereas the SYBR method was used to measure cyclin G2.

Western blot analyses

Western blot analyses were performed essentially as described previously.⁴² Briefly, equal amounts of protein from cell lysates were resolved by SDS-PAGE, electro-transferred to nitrocellulose membrane and probed with primary antibodies (see below) and the appropriate HRP-conjugated secondary antibody. Signals were detected by chemiluminescence using ECL Plus (GE healthcare) with XO-1 blue film (Kodak; Branchburg, NJ). The primary antibodies are as follows: anti-ER α (HC-20, Santa Cruz Biotechnology), anti-SRC-1 (612378, BD Transduction Laboratories), anti-SRC-2 (610985, BD Transduction Laboratories), anti-SRC-3 (611095, BD Transduction Laboratories), anti-CBP (557021, BD Transduction Laboratories), anti-NCoR (06-892, Upstate), anti-SMRT (611386, BD Transduction Laboratories), anti-REA (A300-658A, Bethyl Laboratories, Montgomery, TX) and anti-actin (MAB1501R, Chemicon).

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as described earlier.⁴² Briefly, MCF-7 cells were grown in phenol red-free DMEM containing 5% sFBS for 48 hr and treated with 2.5 μ M α -amanitin for 90 min. After removal of α -amanitin, cells were washed twice with PBS and then treated with vehicle or 10 nM E2 for 45 min before cell fixation with formaldehyde. Chromatin was precipitated with antibodies against ER α (HC-20 & H-184), SRC-3 (C-20), NCoR (N-19 & C-20), RNA polymerase II (N-20 & H-224) or normal rabbit IgG; all antibodies were obtained from Santa Cruz Biotechnology. Antibodies to REA (A300-656A & A300-658A) were from Bethyl Laboratories and acetyl-histone H3 antibody (06-599) was from Upstate (Upstate Biotechnology, Lake Placid, NY). Purified, precipitated chromatin was quantified by qPCR using SYBR green chemistry and normalized against the input chromatin. Primer sequences used in the qPCR are provided in Table I.

Results

Regulation of *BTG2* gene expression by estradiol and estrogen receptors

A time course microarray analysis of estradiol (E2)-treated MCF-7 cells identified *BTG2* as an early estrogen down-regulated gene.¹³ Levels of *BTG2* mRNA were reduced to 40% of their starting levels within 4 hr of E2 exposure and remained suppressed for up to 48 hr after hormone treatment. Our first objective was to verify that E2 treatment suppresses *BTG2* mRNA expression in MCF-7 breast cancer cells and to determine the ability of partial and pure ER antagonists to affect *BTG2* gene expression. As shown in Figure 1, 4 hr treatment of MCF-7 cells with 10 nM E2 resulted in a $(65 \pm 6)\%$ reduction in *BTG2* mRNA levels, consistent with previous microarray results.¹³ In contrast, RT-qPCR analyses demonstrated that *BTG2* mRNA expression was unaffected by 4 hr exposure to either 100 nM 4HT or ICI under similar culture conditions, and this suggests that unliganded ER α does not significantly affect *BTG2* gene expression. To test whether estrogen's suppressive effect on *BTG2* mRNA expression is mediated by an estrogen-regulated intermediate, MCF-7 cells were exposed to the translation inhibitor cycloheximide for 4 hr before treatment with vehicle or E2 for 4 hr. Quantitation of *BTG2* mRNA levels revealed that cycloheximide was unable to block E2 downregulation (Fig. 1b), indicating that E2 suppression of *BTG2* mRNA levels does not require the synthesis of an intermediary protein.

To assess the involvement of ER α in E2-directed *BTG2* mRNA repression, cellular ER α levels in MCF-7 cells were depleted by specific siRNA and then cells were treated with vehicle or estrogen. Depletion of ER α completely abrogated the E2-mediated repression of *BTG2* mRNA expression (Fig. 2a), indicating that this receptor is required for E2 to inhibit *BTG2* gene expression. The efficacy of the siRNA for inhibition of ER α expression was demonstrated by Western blot analysis (Fig. 2b). Next, experiments were initiated to determine if the proximal *BTG2* promoter was responsive to regulation by ER α and E2. With the exception of the identification of functional p53 and retinoic acid receptor (RAR) response elements located upstreams of the translational start site,^{6,45} little is known about transcription factor interactions with the *BTG2* promoter. Therefore, 2 luciferase reporter constructs consisting of nucleotides -2658 $\rightarrow$ -1 (p2658) or -266 $\rightarrow$ -1 (p266) of the *BTG2* promoter cloned into the pGL3 luciferase expression vector were tested to determine their respective responsiveness to signaling by E2 and ER α in ER-negative HeLa cells. These constructs revealed that an active region of the *BTG2* promoter lies between 266 and 2658 nucleotides upstream of the translational start site, as an ~ 5 fold increase in luciferase activity was obtained with the p2658 compared to the p266 reporter plasmid (Fig. 2c). Transient transfection of ER α along with the p2658 reporter and subsequent treatment with E2 significantly decreased luciferase gene expression by $(47 \pm 4)\%$ compared to the vehicle-treated control (Fig. 2d). The extent to which estrogen suppresses the expression of the p2658 *BTG2* reporter is consistent with the results for ER α -positive MCF-7 cells obtained above in which E2 treatment significantly decreased *BTG2* mRNA levels by 51–65% in comparison to vehicle controls (Figs. 1a and 2a). Furthermore, treatment with the ER α antagonists 4HT and ICI prevented the E2-mediated downregulation of the p2658 reporter indicating a clear role for E2-ER α in the repression of *BTG2* promoter activity (data not shown). It was also noted that ectopic expression of ER β in HeLa cells supported E2 repression of the p2658 reporter gene (Fig. 2e), and this indicates that either isoform of ER is sufficient to downregulate *BTG2* gene expression in these E2 treated cells.

Interaction of ER α with the *BTG2* promoter

Estrogen receptors can interact with target genes directly or indirectly via tethering to certain other transcription factors [e.g., AP-1,^{46,47} Sp1,^{48,49} p53^{31,50} and NF- κ B⁵¹], and to determine whether potential interaction sites for ERs were present within 3000 nucleotide

upstream of the *BTG2* translation start site, MaT-Inspector software (<http://www.genomatix.de>) was used to inspect for estrogen response elements (EREs) and binding sites for other transcription factors known to interact with ERs. This computational analysis revealed six half ERE sites and potential binding sites for AP-1, Sp1, NF- κ B and p53 transcription factors (Fig. 3a). In addition, a recent genome-wide ER α ChIP-on-chip analysis suggested the presence of an ER α binding site ~2000 bp upstream of the *BTG2* translational start site²⁴; thus, a number of potential ER α interaction sites are located within the *BTG2* promoter region. Therefore, a ChIP-PCR scanning approach was employed to identify ER α binding regions within the endogenous *BTG2* gene in cells that permitted its E2 downregulation; this avoids the potentially confounding influence of transiently transfected reporter templates and overexpressed exogenous proteins. Sheared chromatin with an average size of 500 nucleotides was prepared from MCF-7 cells treated with vehicle or E2 for 45 min and immunoprecipitated with either ER α specific antibodies or normal rabbit IgG to assess nonspecific interactions. Purified DNA was quantitated by qPCR using sets of PCR primers designed to amplify regions of the promoter ~500-1000 bp apart, thereby covering an area from -3000 to +2000 nt with respect to the *BTG2* translational start site (Fig. 3a). One set of primers was intentionally designed to reside within the region implicated as an ER binding site in prior ChIP-on-chip analyses.²⁴ A control primer set far upstream of the *BTG2* transcriptional start site (located 7958 bp upstream of the translational start site), was used for PCR amplification as a negative control. Quantitative PCR of the immunoprecipitated chromatin samples with the P1-P6 primer sets (Table I and Fig. 3a) demonstrated E2-independent binding of ER α across ~3000 nt of the *BTG2* promoter and to a lesser extent downstream of the translational start site (Fig. 3b). The signal obtained for ER α is ~6-fold higher than for the IgG negative controls upstream of the translational start site, and declines downstream of this position. In contrast, the signal obtained for ER α with the P_{up} primers at the -7958 location was indistinguishable from the signal obtained for normal IgG indicating no specific receptor interaction at that site. As a positive control, the same chromatin samples were used to determine the occupancy of ER α at the known estrogen target gene, *pS2*, using primers adjacent to the ERE within the *pS2* promoter.⁵² In contrast to the *BTG2* results, E2 treatment induced an approximately 10-fold induction of ER α recruitment to the *pS2* promoter (data not shown), and this clearly distinguishes the hormone-independent nature of ER α 's interaction with the *BTG2* promoter from its E2-stimulated interaction with the *pS2* gene. Moreover, as estrogen repression of *BTG2* mRNA expression is not reflected in hormone-dependent recruitment of ER α to the gene, these results suggest that other E2-dependent events are required for *BTG2* down regulation.

Involvement of histone-deacetylases on *BTG2* gene expression

Deacetylation of histones is an important regulatory event frequently associated with gene repression. To assess the participation of HDACs in E2-dependent repression of *BTG2* expression, cells were pretreated with the general HDAC inhibitor trichostatin A (TSA) for 16 hr followed by 4 hr hormone treatment, and *BTG2* mRNA expression was measured thereafter by reverse transcription and qPCR. Surprisingly, TSA alone decreased *BTG2* mRNA expression to an extent similar to that obtained for E2 treatment (Fig. 4a), indicating that HDAC activity is required for basal *BTG2* expression. In TSA treated cells, E2 had no further effect on *BTG2* mRNA levels and this suggests that either TSA blocks the activity of HDACs required for E2 repression of *BTG2* expression or possibly TSA blocks the activity and/or expression of other factors required for basal *BTG2* mRNA expression and the low levels of the *BTG2* mRNA remaining after the TSA treatment cannot be further reduced by E2-bound ER α . These results contrast with those obtained for a previously characterized, estrogen down-regulated gene, cyclin G2.³⁴ Pretreatment with TSA blocks downregulation of cyclin G2 mRNA levels by E2 but does not affect basal expression of this gene (Fig. 4b).

To take a more global approach to determining whether HDAC activity is associated with E2 repression of *BTG2* mRNA expression, ChIP assays using an antibody against pan-acetylated histone H3 were used to investigate whether E2 treatment produced a deacetylation of histones at the *BTG2* promoter. Chromatin was prepared for MCF-7 cells treated with either vehicle or E2 for 45 min and immunoprecipitated DNA was quantitated using the P3-P6 primers upstream and downstream of the translational start site (Fig. 5a). As expected, high levels of acetylated histone H3 were observed for primer locations near the transcriptional start site in comparison to the IgG controls, with the highest levels obtained for the primer sets located immediately upstream and downstream of this region (Fig. 5b). However, the extent of total histone H3 acetylation was unaffected by E2 treatment suggesting that deacetylation of this histone in this region does not contribute to repression of *BTG2* gene expression. In contrast, ChIP assays employing antibody to RNA polymerase II (Pol II) revealed that Pol II binding, which is highest near the *BTG2* translational start site, is reduced in E2 *versus* vehicle-treated cells (Fig. 5c). The levels of acetylated histone H3 and Pol II were similar to the IgG negative control at the P1 and P2 regions defined as in Figure 3. Thus, the collective results indicate that E2 repression of the *BTG2* gene is associated with ER α interaction with a broad region of the *BTG2* promoter and displacement of Pol II from the *BTG2* gene.

Functional mapping of ER α domains required for estrogen repression of *BTG2*

To define the structural features of ER α that are required for repression of *BTG2* gene expression a series of ER α mutants encompassing either deletions or point mutations that affect known receptor functions (*e.g.*, DNA binding, ligand binding, coactivator interaction or transcriptional activation) were tested for their ability to inhibit the activity of the p2658 *BTG2* promoter-luciferase reporter gene. A schematic representation of the mutants used in these experiments is shown in Figure 6a. Hela cells were transfected with expression plasmids for wild type or mutant forms of ER α , along with the p2658 reporter construct followed by hormone treatment. In the case of the AF1 deletion mutant (ER α -179C), repression of the activity of the *BTG2* promoter was similar to that obtained for wild type ER α , indicating that *BTG2* repression by E2 does not require the ligand-independent AF1 domain of ER α (Fig. 6b). However, disruption of the DBD by a mutation within the P-box that blocks DNA binding ability effectively prevents this ER α mutant from repressing *BTG2* promoter activity. Next, the N282G ER α mutant which lacks the ligand binding domain (LBD) and consequently AF-2 was tested and found unable to repress *BTG2* gene expression suggesting that the LBD and/or AF2 is required for E2-dependent down regulation of *BTG2* gene expression by ER α . To further ascertain if an intact AF-2 domain within the LBD is necessary for repression of *BTG2*, a specific LBD mutant ER α -KVL which possesses 3 mutations that disrupt the integrity of the AF-2 coactivator binding groove was tested in the *BTG2 trans*-repression assay. The inability of ER α -KVL to repress the *BTG2*-luciferase reporter in E2-treated cells indicates that coregulators that interact with receptor via the coactivator binding groove are required for ER α to repress *BTG2* gene expression. Moreover, it also was noted that the activity of the p2658 reporter was greater in cells co-transfected with ER α -KVL than wild type ER α suggesting that this ER α mutant, perhaps through a basal interaction at the *BTG2* promoter, blocks repressive events that negatively affect the gene under basal conditions. Taken together, these experiments indicate that E2-induced *BTG2* gene repression requires the intact DBD and LBD of ER α , and suggest that coregulators that interact with ER α via its coactivator binding groove play an important role in the downregulation of this gene.

Coregulator requirement for E2 mediated *BTG2* gene regulation

Previous studies examining the inhibition of ER α target gene expression by unliganded or antagonist-bound receptors have implicated the corepressors NCoR and SMRT with transcriptional repression,⁵³⁻⁵⁵ and NCoR is recruited to the promoters of several E2-downregulated genes including *cyclin G2*, *VEGFR2*, *BMP7*, *ABCG2* and *BCL-3*.³³⁻³⁵

However, the p160 family coactivator SRC-2 (also known as TIF2 or GRIP1) has also been shown to be involved in E2-mediated repression of the *TNF α* gene,³⁶ and both coactivators and corepressors were therefore tested for their ability to mediate E2-ER α repression of the *BTG2* gene, focusing on the p160 family of coactivators (SRC-1, SRC-2 and SRC-3), the classical nuclear receptor corepressors NCoR and SMRT and the ER-specific corepressor REA. In addition, CBP/p300 which has been associated with repression of cMyc was tested.^{38,39} MCF-7 cells were transfected with specific siRNAs for each coregulator and cells subsequently were treated with hormone for 4 hr, followed by RT-qPCR for *BTG2* mRNA expression. Depletion of endogenous proteins by siRNA was verified by Western blotting (Fig. 7c). The effect of coregulator depletion was first examined with respect to effects on *BTG2* mRNA expression under basal conditions. Out of all the cofactors tested, only SRC-3 depletion induced a significant increase in basal *BTG2* mRNA expression, whereas depletion of NCoR, SRC-1 and SRC-2 produced modest but insignificant decreases in basal *BTG2* mRNA levels (Fig. 7a). The expected E2-dependent repression of *BTG2* mRNA expression in control siRNA treated cells, and the loss of this repression in cells depleted of ER α is demonstrated in Figure 7b. Estradiol-dependent repression of *BTG2* mRNA levels was not affected by depletion of any of the tested nuclear receptor coactivators (*i.e.*, SRC-1, SRC-2, SRC-3 or CBP). However, depletion of REA effectively abrogated E2-mediated *BTG2* downregulation, whereas inhibition of SMRT or NCoR expression modestly attenuated the ability of E2 to repress *BTG2* mRNA levels. Taken together, these data indicate that REA exerts a major effect in E2 repression of *BTG2* gene expression, with minor contributions by SMRT and NCoR.

To extend these results, ChIP assays were performed to examine the interaction of REA, NCoR and SRC-3 with the *BTG2* gene at positions corresponding to the P1 to P4 amplicons. Although low levels of NCoR and SRC-3 were detected at each of these positions, consistent with their respective abilities to exert effects on *BTG2* gene expression, there was no change in their interaction with the *BTG2* promoter following E2 treatment (data not shown). To verify that the ChIP assays were working properly, the same immunoprecipitated chromatin samples were examined with primers for the pS2 gene which revealed the expected E2-dependent recruitment of SRC-3 and release of NCoR.^{43,56} Surprisingly, REA was undetectable at any of the tested locations (P1-P4) of the *BTG2* gene. Although this result suggests that REA does not interact with the *BTG2* promoter, it is also possible that the available REA antibody is not suitable for ChIP assay or that the epitopes recognized by the REA antibody are masked by other factors located at the *BTG2* gene.

Discussion

It is now appreciated that control of cell proliferation by estrogen receptors is achieved not only by activating the expression of cell cycle regulatory and anti-apoptotic genes but also by down regulating genes that encode proteins with proapoptotic properties, or those which directly or indirectly inhibit cell cycle progression. Although considerable attention has been focused on delineating the mechanisms of agonist-dependent gene activation and antagonist-mediated gene repression by ER α , relatively little is known about how E2 represses gene expression. Our results demonstrate that E2 acts via estrogen receptors to directly repress the *BTG2* gene. This negative regulation is dependent on the ER specific corepressor REA, with lesser contributions from the general corepressors, NCoR and SMRT; it also is accompanied by displacement of Pol II from the *BTG2* promoter. Our data also demonstrate that HDAC activity, typically associated with gene repression, is required for maximal *BTG2* gene expression, and this indicates that HDACs can exert positive effects on gene expression in addition to their well established repressive effects.

Our ChIP experiments indicate that ER α interacts strongly with a broad region of the *BTG2* promoter region, and can be found at lower levels downstream of the presumed translational

start site. However, there was not any change in the level of ER α bound to the *BTG2* promoter associated with E2 treatment. Although this was unexpected, recent work by the Kraus laboratory recently analyzed estrogen-regulated promoters based on ER α and Pol II binding. Our work on *BTG2* indicates that it falls within the Class IVB promoter group that is characterized by reduced Pol II binding with no change in recruitment of ER α .²⁶ The E2-ER α repressed gene, E-cadherin, also shows no E2-dependent change in ER α occupancy at its promoter⁵⁷ suggesting that it may fall into the same category of E2 repressed genes as *BTG2*. An *in silico* search of the *BTG2* promoter region from -3000 to +1 by the Estrogen Responsive Genes Database (ERGDB) revealed no EREs; however, several ERE half-sites were detected as were binding sites for transcription factors (*e.g.*, AP-1, Sp1, p53) known to mediate indirect binding of ER α to genes. It has been demonstrated that the nature of ER α 's interaction with target genes can influence whether it activates or represses gene expression. Specifically, ER α interaction with the initiator and flanking sequences of the folate receptor (FR)- α core promoter is sufficient for E2 repression of this gene by E2.³² In contrast, when stronger ER binding sites are inserted into the context of the core promoter, E2 stimulates gene expression. Thus our data adds to the cumulative evidence demonstrating that nuclear receptors can employ diverse modes of interaction with target genes to inhibit their expression.

Repression of the *BTG2* gene by E2 does not require the ligand-independent AF1 domain of ER α , and this is similar to the finding for several other E2-ER α down regulated genes, such as *FR α* and vascular endothelial growth factor receptor (*VEGFR*)-2.^{32,33} However, in the case of *cyclin G2*, another E2-ER α inhibited gene, the N-terminal domain of ER α was found to be indispensable for repression, whereas the ligand-dependent AF2 domain was not required.³⁴ The ER α AF2 domain is essential for E2-dependent repression of *BTG2* gene expression, and together, these data suggest that ER α can employ different mechanisms to facilitate coregulator interactions with repressed genes. Interestingly, a requirement of the ER α DNA binding domain is common to a number of E2 repressed genes including *BTG2*.^{32,33,34} As noted above, there are several half-ERE sites within the *BTG2* promoter, and it is possible that the DBD enables ER α to bind directly to these elements. However, other investigators have shown that the DBD is essential for indirect interactions of ER α with DNA via tethering of the receptor to Sp1 and AP-1 transcription factors.^{58,59} Thus, we are unable to conclude that E2-ER α repression of *BTG2* requires direct interaction of the receptor with DNA, as it is possible that the DBD is required for maintaining the structural organization of the receptor and/or its ability to interact with other transcription factors and coregulators essential for *BTG2* gene repression.

The importance of ER α interactions with coregulators for *BTG2* repression was first suggested by the ability of the K362D/V376D/L539A mutations to abrogate E2-dependent repression of *BTG2*. Depletion of SMRT or NCoR by siRNA had a modest impact on E2 repression of *BTG2* mRNA expression, and this distinguishes *BTG2* from other E2 down regulated genes (*FR α* , *VEGFR2*, *cyclin G2*) that required NCoR for this purpose.³²⁻³⁴ In contrast, E2 repression of *BTG2* was significantly abrogated by depletion of the ER-specific corepressor, REA. Prior work on REA has focused on its ability to attenuate E2 induction of gene expression⁶⁰⁻⁶² and its ability to negatively regulate mammary gland proliferation and differentiation in mice.^{63, 64} Biochemical studies reveal that ER α interactions with REA are ligand-dependent and can be induced by either E2 or the antagonist, 4HT.⁶² In this regard, it is somewhat surprising that the tested antagonists, 4HT and ICI, did not repress *BTG2* gene expression, and this suggests that factors in addition to ER α may be required to facilitate REA repression of *BTG2* gene expression. Unlike other ER α corepressors such as SMRT and NCoR, REA does not employ NR (Nuclear Receptor) or CoNR (Corepressor-Nuclear Receptor) box motifs for its interaction with ER α or its repressive activity.⁶⁰ The repressive effects of REA on E2-stimulated ER α -dependent gene expression are achieved via competition between REA and the SRC-1 coactivator for binding to ER α , and consequently reductions in receptor-coactivator interaction.^{60,62} Knock-down of SRC-1 does not block E2 repression of the *BTG2* gene and

it therefore appears that REA repression in the context of E2-inhibited transcription differs mechanistically from its inhibitory effects on E2-induced genes.

In general, repression of gene expression is associated with recruitment of corepressors and their associated HDACs and consequently deacetylation of histones, compaction of chromatin structure and ultimately inhibition of transcription, and it has been shown that REA can interact with HDACs.⁶⁵ Interestingly, in the case of *BTG2*, treatment with the HDAC inhibitor TSA significantly reduced basal *BTG2* gene expression, and blocked further reduction by E2 treatment. Although it is possible that TSA treatment blocks E2-ER α suppression of *BTG2* gene expression either at the level of ER α -REA-HDAC or through an alternative repressive process, it also is possible that TSA inhibition of basal *BTG2* expression leaves little transcription to be inhibited by E2 treatment. The TSA inhibition of basal *BTG2* mRNA expression was unexpected, but other instances of a requirement of HDAC activity for gene transcription have been noted. For instance, transcription of selected interferon mediated immediate early genes that are regulated by signal transducer and activator of transcription-1 (STAT-1) and STAT-2 transcription factors requires HDAC activity,^{66,67} and HDAC1 is important for glucocorticoid receptor induction of reporter gene activation.⁶⁸

In addition to a requirement of HDAC activity for maximal expression of the *BTG2* gene, our depletion experiments also revealed that depletion of the SRC-3 coactivator increased basal *BTG2* expression. Interestingly, SRC-3 possesses intrinsic histone acetyltransferase activity,⁶⁹ and this further suggests that acetylation may reduce *BTG2* mRNA levels in MCF-7 cells, consistent with our observation of a requirement of deacetylation for basal *BTG2* expression. It is, however, also possible that the stimulatory effect of SRC-3 depletion represents an indirect effect via alterations in the expression of other cofactors that regulate *BTG2* mRNA production. Nonetheless, it is interesting to note that increased *BTG2* expression in SRC-3 depleted MCF-7 cells may be physiologically relevant as we have recently demonstrated that depletion of SRC-3 inhibits cell cycle progression and cellular proliferation (Karmakar and Smith, unpublished communication) which is anticipated for cells that express increased levels of the antiproliferative *BTG2* protein.

The ability of E2-bound ER α to stimulate cyclin D1 expression in breast cancer cells is well studied, and it is likely that estrogen stimulation of cellular proliferation represents the combined effects of direct ER α -mediated *BTG2* inhibition and cyclin D1 stimulation. The ability of ectopic *BTG2* expression to reduce the growth of MCF-7 breast cancer cells on soft agar and to increase the percentage of cells in the G1 phase of the cell cycle has demonstrated the importance of *BTG2* to control of breast cancer cell proliferation.⁷ Indeed, the importance of one transcription factor, ER α , being able to coordinately regulate the expression of these proteins is easily appreciated when one considers that exogenous expression of cyclin D1 can overcome cell cycle arrest induced by overexpression of *BTG2*.⁸ Conversely, in the absence of estrogens, low ER α activity results in low cyclin D1 expression and an increased level of *BTG2* which further inhibits cyclin D1 mRNA expression.⁸

In summary, this study provides evidence for E2-bound ER α and REA dependent down regulation of the *BTG2* gene. One of the questions that remain in this and other studies examining ER α repression of gene expression is what determines whether agonist-bound receptor binds to coactivator or corepressor. It appears logical that the identity of the target gene must play a major role and it is possible that target genes impart structural alterations in the receptor or alter the spatial relationship between receptors within an ER α homodimer and thereby influence receptor interactions with corepressors. This would be analogous to prior investigations in which it was demonstrated that the nature of the ER α binding site within a target gene influences receptor structure and interactions with coactivators.⁷⁰⁻⁷² Thus the specificity of ER α down regulation of gene expression may also provide an avenue to

specifically block this mode of gene regulation. Because the ability of E2-ER α to suppress BTG2 expression may be a major pathway for estrogen stimulation of ER α -positive breast cancer cell proliferation, we postulate that inhibition of ER α -REA interactions may have significant potential as a targeted therapeutic strategy for breast cancer.

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Abbreviations

AF, activation function
 BTG2, B-cell translocation gene 2
 CBP, cAMP response element-binding protein (CREB) binding protein
 ChIP, chromatin immunoprecipitation assays
 DBD, DNA binding domain
 E2, 17 β -estradiol
 ER α , estrogen receptor- α
 ERE, estrogen response element
 HDAC, histone deacetylase
 4HT, 4-hydroxytamoxifen
 LBD, ligand binding domain
 NCoR, nuclear receptor corepressor
 NP40, Nonidet P-40
 qRT-PCR, quantitative reverse transcription polymerase chain reaction
 REA, repressor of estrogen-receptor activity
 SDS, sodium dodecyl sulphate
 SERM, selective estrogen receptor modulator
 sFBS, charcoal-stripped fetal bovine serum
 SMRT, silencing mediator of retinoic and thyroid hormone receptor
 SRC, steroid receptor coactivator
 TSA, trichostatin A

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Figure 1.

Effect of 17β-estradiol and ERα antagonists on *BTG2* gene expression. (a) 3×10^5 MCF-7 cells in 10% sFBS were treated with vehicle (Veh, 0.1% ethanol), 10 nM E2, 100 nM 4-hydroxytamoxifen (4HT) or 100 nM ICI 182,780 (ICI) for 4 hr before RNA isolation for *BTG2* mRNA quantitation by RT-qPCR. (b) MCF-7 cells were pretreated with 10 μg/ml cycloheximide (+CHX) or its vehicle (-CHX) for 4 hr followed by an additional 4 hr treatment with vehicle or 10 nM E2. Cells were then harvested for RNA isolation and measurement of the *BTG2* mRNAs by RT-qPCR. In both experiments, data were normalized to corresponding 18S RNA values and are represented as the mean $\pm$ SEM of at least 3 independent experiments. * $p < 0.05$ versus vehicle.

Figure 2.

Involvement of estrogen receptors on E2 repression of *BTG2* gene expression. (a) MCF-7 cells were transfected with 10 nM *Silencer* negative control siRNA or siRNA for ERα, and 48 hr later were treated with vehicle (0.1% ethanol, Veh) or 10 nM E2 for 4 hr. Expression of *BTG2* mRNA was quantitated by RT-qPCR and normalized to 18S RNA. Values are the average ± SEM of 3 independent experiments done in duplicate. **p* < 0.05 versus vehicle. (b) Cell lysates from a parallel set of cells were analyzed by Western blot for expression of ERα and actin. (c) HeLa cells were transfected with 1 µg of p266 or p2658 plasmids, and 24 hr later, were treated with vehicle (0.1% ethanol, black bars) or 10 nM E2 (grey bars) for another day followed by measurement of luciferase activity. In similar experiments, HeLa cells were transfected with

100 ng of ER α (*d*) or 150 ng of ER β expression vectors (*e*) along with 1 μ g of p2658 luciferase plasmid, followed by 24 hr hormone treatment and measurement of luciferase activity. Values were normalized to total protein concentration. Experiments were done in duplicate and data represents the average $\pm$ SEM of 3 independent experiments. **p* < 0.05 *versus* vehicle-treated controls.

Figure 3.

Interaction of ER α with the *BTG2* promoter. (a) A schematic diagram indicates -3000 bp upstream and +3000 bp downstream of the *BTG2* start site, including the locations of predicted half-site EREs, and binding sites for p53, AP-1, Sp1 and NF- κ B and the positions of amplicons (P_{up} and P1-P6) used to monitor the ChIP assay. Numbered grey boxes represent exons. Primer sequences and locations of the amplicons are listed in Table I. The horizontal bar represents an estrogen receptor binding region identified in a genome wide ER α ChIP-on-chip study. (b) Chromatin was prepared from MCF-7 cells treated with either 0.1% ethanol (Veh) or 10 nM E2 for 45 min and then subjected to ChIP assay using antibodies for ER α or rabbit IgG. Immuprecipitated DNA was quantitated by qPCR using primers to amplify the indicated amplicons. Data represent an average $\pm$ SEM of 2 independent experiments.

Figure 4.

Effect of HDAC inhibition on estrogen regulation of *BTG2* gene expression. MCF-7 cells were grown in the absence or presence of 300 ng/ml TSA for 16 hr followed by 4 hr treatment with either vehicle (0.1% ethanol, black bars) or 10 nM E2 (grey bars). Thereafter, RNA isolated from treated cells was quantitated for *BTG2* (a) and cyclin G2 (b) mRNA levels. All experiments are done in duplicate and results represent the average $\pm$ SEM of 3 independent experiments. * $p < 0.05$ versus each group's vehicle-treated control (*t*-test).

Figure 5.

ChIP assay of acetylated histone H3 and Pol II at the 5' end of the *BTG2* gene in the presence or absence of E2. (a) Schematic diagram showing the position of primers used for amplification of immunoprecipitated chromatin (▲). MCF-7 cells were treated with vehicle or E2 for 45 min and then subjected to immunoprecipitation with normal rabbit IgG or antibodies to either anti-acetyl histone H3 (b) or anti-Pol II (c). The resulting chromatin was quantitated by qPCR using the indicated primers. Results obtained were normalized to input DNA and plotted on the x-axis in proportion to the relative distance between the qPCR amplicons. Experiments were repeated 2-3 times, and results are presented as the average $\pm$ SEM.

Figure 6.

Assessment of ERα functional domains required for repression of the *BTG2* promoter. (a) Schematic diagram showing wild type ERα and various mutants used in these experiments. (b) HeLa cells were transfected with 100 ng of wild type or mutated ERα along with 1 μg of p2658 reporter plasmid. Twenty-four hours after transfection, cells were treated with vehicle (0.1% ethanol) or E2 (10 nM) and 24 hr thereafter were harvested for luciferase activity measurements. Data are expressed as the percentage change in BTG2-luciferase activity in E2 *versus* vehicle-treated cells. Experiments were repeated 3 times in duplicate and data represent the average ± SEM.

Figure 7.

Effect of coregulator depletion on estrogen suppression of *BTG2* gene expression. MCF-7 cells were transfected with Ambion's *Silencer* no. 2 negative control siRNA or siRNA targeting ERα (10 pmol), SMRT (20 pmol), NCoR (20 pmol), REX (40 pmol), SRC-1 (20 pmol), SRC-2 (20 pmol), SRC-3 (20 pmol) or CBP (40 pmol) and 48 hr later were treated with vehicle (0.1% ethanol) or 10 nM E2 for an additional 4 hr in all cases except for SRC-2 siRNA, which was treated 24 hr after transfection. Cells were then harvested for RNA isolation and measurement of *BTG2* mRNA expression by RT-qPCR. (a) Basal levels of *BTG2* expression in vehicle-treated cells were calculated relative to the level of *BTG2* mRNA obtained for control siRNA-transfected cells which was arbitrarily set to 100%. (b) Percentage of *BTG2* mRNA levels remaining after E2 treatment was calculated compared to each group's vehicle-treated control. All experiments are done in duplicate and the results represent an average of 3 independent experiments $\pm$ SEM. The solid and dashed lines represent *BTG2* mRNA levels determined for control siRNA transfected cells treated with vehicle and E2, respectively. (c) Parallel cultures of cells were harvested and whole cell lysates were prepared for analyses of the respective protein levels by Western blot. Blots are representative of 3 separate experiments.

TABLE I
LOCATION OF AMPLICONS AND PRIMERS USED FOR qPCR ANALYSIS OF ChIP ASSAY

Amplicon	Amplicon location ¹	Forward	Reverse
P1	-2613 to -2492	5'-GTTTTTCACATTTAGGTGCTGAACAA-3'	5'-GCCATGGAGACCAGGTGTCA-3'
P2	-1912 to -1829	5'-GATCATGGGCAAGCTGTTGA-3'	5'-TGTGCTCCAGGGTCACTCTTC-3'
P3	-1105 to -1024	5'-CCACTGGGTTTTAGTCACCTTACA-3'	5'-AACTTCTACCCAAACCCGTGAAG-3'
P4	-595 to -512	5'-AAAAGCGGTAAAATGGTGACAACAC-3'	5'-CCCTTTCTGTGATTCCACACAGCAT-3'
P5	+539 to +660	5'-GGTGCAGTCGAGCCCTTTTCA-3'	5'-GGGCCCCAAATTCCAACTTG-3'
P6	+1486 to +1572	5'-TGCTCCACAGAGCACTACAAACAC-3'	5'-TGTGGTTGATGCGAATGCA-3'
P _{up}	-8029 to -7953	5'-TGTGTCTCTCCAAGTGGCCATCT-3'	5'-GGTATCAGCATTTCCAAGTGTCTATCTG-3'

¹The location of the amplicon is given relative to the *BTG2* translational start site defined by Rouault *et al.*²