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Inhibition of BET proteins impair estrogen mediated growth and transcription in breast cancers by pausing RNA polymerase advancement

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Abstract

Purpose—Estrogen (E₂)-induced transcription requires coordinated recruitment of estrogen-receptor α (ER) and multiple factors at the promoter of activated genes. However, the precise mechanism by which this complex stimulates the RNA polymerase-II activity required to execute transcription is largely unresolved. We investigated the role of bromodomain (BRD) containing bromodomain and extra-terminal (BET) proteins, in E₂-induced growth and gene activation.

Methods—JQ1, a specific BET protein inhibitor, was used to block BET protein function in two different ER-positive breast cancer cell lines (MCF7 and T47D). Real-time PCR and ChIP assays were used to measure RNA expression and to detect recruitment of various factors on the genes, respectively. Protein levels were measured by western blotting.

Results—JQ1 suppressed E₂-induced growth and transcription in both MCF7 and T47D cells. The combination of E₂ and JQ1 down-regulated the levels of ER protein in MCF7 cells but the loss of ER was not responsible for JQ1 mediated inhibition of E₂ signaling. JQ1 did not disrupt E₂-induced recruitment of ER and co-activator (SRC3) at the E₂-responsive DNA elements. The E₂-induced increase in histone acetylation was also not altered by JQ1. However, JQ1 blocked the E₂-induced transition of RNA polymerase-II from initiation to elongation by stalling it at the promoter region of the responsive genes upstream of the transcription start site.

Conclusions—This study establishes BET proteins as the key mediators of E₂-induced transcriptional activation. This adds another layer of complexity to the regulation of estrogen-induced gene activation that can potentially be targeted for therapeutic intervention.

Keywords

Estrogen; Transcription; BET proteins; JQ1; Estrogen receptor; RNA polymerase II

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Introduction

Transcriptional activation of genes by estrogen (E_2 ; 17 β -estradiol) is a highly regulated process that requires the coordinated interaction of estrogen receptor α (ER) with estrogen responsive elements (EREs), followed by the binding of co-regulators and other chromatin modifying factors [1,2]. Co-regulators, which can be co-activators or co-repressors, can recruit other cofactors with diverse enzymatic activities required for modulating the transcriptional process [3]. E_2 -liganded ER and the p160 family of co-activators (SRC1, SRC2, SRC3) form a dynamic transcription activation complex [1,4] that interacts with CBP and p300 co-activators [5] possessing intrinsic histone acetyl-transferase (HAT) activity [6-8]. Addition of acetyl groups specifically occurs at the lysine residues of the N-terminal histone proteins by HAT activity, resulting in enhanced accessibility of the chromatin to the transcriptional machinery [9]. These chromatin alterations eventually lead to the synthesis of the RNA of E_2 responsive genes by RNA polymerase II (RNAP) [4,10]. However, the components and the events responsible for “reading” the hyper-acetylated histone chromatin that are necessary to integrate RNAP activation in the context of E_2 -regulated genes, are poorly understood.

Studies have established that dual bromodomain containing bromodomain and extra-terminal (BET) proteins, which include BRD2, BRD3 and BRD4, play important roles in stimulation of transcription by RNAP [11-13]. The bromodomains of the BET proteins bind to acetylated lysines of the histones, whereas the extra-terminal (ET) domain of the protein can interact independently with multiple other proteins [14]. BRD4 interacts with the positive transcription elongation factor b (PTEFb) [11], which is responsible for the transcriptional elongation by RNAP [15]. BRD2 and BRD3 also bind to hyper-acetylated histone 3 (H3) and histone 4 (H4) and permit transcriptional elongation by RNAP [13]. Interest in the BET proteins has increased recently due to the discovery of the potential therapeutic selective inhibitor of BET bromodomains, JQ1, which can block the interface of the bromodomain responsible for recognizing the acetylated lysines in histones [16]. JQ1 has been used extensively as a chemical tool to delineate the effects of BET proteins in various disease specific studies [17,18,16,19-22].

However, the roles of BET proteins in E_2 -induced transcription in the ER positive breast cancers have not been examined before. We studied the effect of BET protein inhibition using JQ1 on the growth and the transcriptional regulation of estrogen (E_2)-stimulated genes in two distinct ER positive breast cancer cell lines (MCF7 and T47D). These cells regulate the ER differently [23]. By studying three established and well characterized E_2 -regulated genes namely, pS2 (TFF1) [1,2], GREB1 [24], and XBP1 [25,26], this study demonstrates the key role of the BET proteins in E_2 -induced transcription by ER.

Materials and Methods

Cell Culture and Reagents

Cell culture media were purchased from Invitrogen Inc. (Grand Island, NY) and fetal calf serum (FCS) was obtained from HyClone Laboratories (Logan, UT). The ER α + breast cancer cells MCF-7:WS8 (mentioned as MCF7 hereafter) were derived from MCF7 cells

[27] obtained from Dr. Dean Edwards, San Antonio, Texas and T47D A:18 cells (mentioned as T47D hereafter) were derived from T47D cells as reported previously [28]. The cells were maintained in phenol red-containing RPMI media supplemented with 10% FCS. Three to four days prior to treatment the cells were cultivated in phenol red-free media containing 10% charcoal dextran treated FCS. BET protein inhibitor, JQ1 (cat # 4499) was purchased from Tocris Bioscience, (Bristol, UK) and the inactive JQ1 stereo-isomer (cat # A8181) was purchased from Apex Bio Technologies (Boston, MA). All the experiments were performed at least three times, in triplicate to confirm the results.

Cell growth assay

Cell growth assays were performed by measuring the total DNA per well in 24 well plates. Eight thousand cells were plated per well and treatment with indicated concentrations of compounds was started after 24 hours, in triplicates. Media with specific treatments were changed every 48 hours. Cells were harvested after six days of treatment in hypotonic buffer solution followed by sonication. Total DNA was measured using a fluorescent dye (Hoechst 33258) in the DNA quantitation kit (Cat # 170-2480; Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

RNA isolation and real time PCR

TRIzol reagent (Invitrogen, Carlsbad, CA) and RNeasy kit (Qiagen, Valencia, CA, USA) were used to isolate total RNA according to the manufacturer's instructions. Real-time PCR was performed as previously described [29]. Briefly, cDNA was generated from 1 µg of total RNA in a total volume of 20 µl using High Capacity cDNA Reverse Transcription kits (Applied Biosystems, Foster City, CA). Subsequently, the cDNA was diluted to 500 µl and RT-PCR was performed using ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). Each well contained 10 µl SYBR green PCR master mix (Applied Biosystems, Foster City, CA), 125 nM each of forward and reverse primers and 5 µl of diluted cDNA in 20 µl final volume. The change in expression of transcripts was determined as described previously and used the ribosomal protein 36B4 mRNA as the internal control [29]. Details of the primers used for TFF1, GREB1, XBP1, ER alpha and 36B4 genes can be found in supplementary table 1.

Western blotting

RIPA buffer containing protease inhibitors (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors I and II (EMD Chemicals Inc. San Diego, CA) was used to extract whole cell protein lysates. 15-20 µg of total protein was run on the gels and transferred onto nitrocellulose membranes. Membranes were subsequently blocked with 5% non-fat dry milk in tris-buffered saline and probed with primary and secondary antibodies. Specific bands were visualized using west-pico chemi-luminescence (Thermo-Fisher, Rockford, IL, USA). The antibodies used were: ER alpha (# sc-544; Santa Cruz Biotechnology, Dallas, TX), and beta actin (#A5441; Sigma, St. Louis, MO).

Chromatin immuno-precipitation (ChIP) assay

ChIP assay was performed as described previously [29]. Briefly, cells were treated with the indicated treatments for 45 minutes and cross-linked using 1.25% paraformaldehyde for 15 minutes and subsequently cross-linking was stopped with 2 M glycine. Nuclei were isolated from cells that were re-suspended in SDS-lysis buffer followed by sonication and centrifugation at $14,000 \times g$ for 20 minutes at 4°C. The supernatants were diluted 1:10 with ChIP dilution buffer. Pre-clearing of the samples were performed by normal rabbit IgG and protein A magnetic beads (Upstate cell signaling solutions, Temecula, CA) followed by over-night incubation using specific antibodies against ER alpha(1:1 ratio of sc-7207 and sc-543; Santa Cruz Biotechnology, Dallas, TX), SRC3 (sc-13066; Santa Cruz Biotechnology, Dallas, TX), acetylated histone 4 (06-866; Upstate cell signaling solutions, Temecula, CA) and RNA polymerase II (sc-56767; Santa Cruz Biotechnology, Dallas, TX). The immuno-complexes were pulled down using protein A magnetic beads (Upstate cell signaling solutions, Temecula, CA). Beads bound to immuno-complexes were thereafter washed and precipitates were extracted twice using freshly made 1% SDS and 0.1M NaHCO₃ followed by de-crosslinking. DNA fragments were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA). RT-PCR was performed using 2 µL isolated DNA, using loci specific primers. Details of the primer sequences for the promoters and gene coding region can be found in the supplementary table 2. The data are represented as fold enrichment over normal IgG pull down for each treatment sample after calculating the percent input. The “traveling ratio” of the RNAP, which is a measurement of its elongation function [30], was calculated as the ratio of fold enrichment of RNAP at the promoter and the coding region for each gene.

Statistics

Statistical significance of our data was calculated using the paired Student's “t”-test wherever relevant. A p-value of < 0.05 was considered as statistically significant.

Results

JQ1 blocks E₂-induced cell proliferation and ER mediated gene activation in MCF7 and T47D breast cancer cells

Most estrogen receptor positive breast cancers depend upon estrogen for cell proliferation and growth. We examined the dose dependent effect of the BET protein inhibitor JQ1, on E₂-induced growth of MCF7 and T47D breast cancer cells. JQ1 inhibited E₂-induced growth of both the breast cancer cell lines in a concentration dependent manner (figure 1A and 1B). In both cell lines, 500 nM JQ1 was sufficient to block the E₂-induced growth completely over a six day period. This growth inhibition was due to a reduction in proportion of the cells in “S” phase cells by JQ1 as evidenced by the cell cycle analysis (supplementary figure S1A and S1B). Basal growth of the cells in the absence of E₂ was also moderately inhibited over this time period. Next, we examined the inhibitory effect of JQ1 on ER mediated transcriptional activation of representative E₂-responsive genes, TFF1, GREB1 and XBP1. Expression of all these genes was up-regulated by 1 nM E₂ after 6 hrs of treatment in both MCF7 and T47D cells, an effect that was almost completely inhibited by JQ1 (figures 1C, 1D, 1E, 1F, 1G, 1H). A well-defined dose-dependent inhibitory effect of

JQ1 was not evident; even the lowest concentration of JQ1 (125nM) blocked E₂-induced gene activation and the higher concentrations of JQ1 did not enhance this effect significantly.

Inhibition of E₂-induced gene activation by JQ1 is enantiomer specific and is rapidly reversible

To investigate specificity of JQ1 mediated inhibition we used the inactive enantiomer of JQ1 ((-)-JQ1; figure 2A) that is unable to bind the acetyl-lysine binding pocket of the bromodomain [16]. (-)-JQ1 (250 nM) failed to inhibit the E₂-induced gene activation of TFF1, GREB1 and XBP1 in both MCF7 and T47D cells (figure 2C, through 2H), when compared with the 250 nM JQ1 treatment in combination with E₂. E₂-induced growth over a period of six days, of both MCF7 and T47D cells also was not inhibited by (-)-JQ1 (figure 2B) unlike JQ1. To further examine if there was an extended inhibitory effect of JQ1 on E₂-regulated genes, even after the withdrawal of JQ1, we treated the MCF7 cells with E₂ (1 nM) +JQ1 (250 nM) for 2 hrs and then removed JQ1 from the media and maintained the cells under E₂ (1 nM) treatment for 30, 60 and 120 minutes. As expected, two hours of JQ1 treatment (250 nM) in combination with E₂ almost completely blocked the E₂ activation of the TFF1, GREB1 and XBP1 genes (figures 3A, 3B and 3C). However, withdrawal of JQ1 from the treatment rapidly (within 120 minutes) restored the hormone mediated activation of all the genes to a level comparable to E₂ treatment alone after 2 hours (figures 3A, 3B and 3C). Interestingly, the kinetics of the activation of these genes after JQ1 withdrawal (figures 3A, 3B and 3C) and with E₂ treatment alone (figures 4E, 4F and 4G) were the same. Thus, JQ1 mediated inhibition is not only reversible but its constant presence is necessary for its effectiveness.

JQ1 and E₂ combination drastically reduces ER levels in MCF7 cells but is not responsible for the inhibitory effect of JQ1

To examine the effect of JQ1 plus E₂ combination treatment on the expression of ER, we determined the levels of ER protein and mRNA in E₂+JQ1 treated MCF7 and T47D cells. Different regulation of ER protein expression was observed for the two cell lines as T47D cells did not show any remarkable change in ER protein levels after E₂+JQ1 treatment for 6 hrs (figure 4B). In contrast, MCF7 cells exhibited drastic reduction of ER protein after 6 hrs of E₂+JQ1 treatment (figure 4A), whereas the reduction was less evident in cells treated with E₂ or JQ1 alone. Opposing regulation of ER mRNA in MCF7 and T47D cells was observed; JQ1 and E₂ combination treatment suppressed the levels of ER mRNA in MCF7 (figure 4C) but not in T47D cells (figure 4D). However, treatment with JQ1 alone reduced the ER mRNA levels by 40% to 50% in both the cell lines (figures 4C and 4D). Conversely, E₂ alone increased ER mRNA expression in T47D cells but no change was observed in MCF7 cells. Since ER protein levels were significantly reduced in MCF7 cells treated with JQ1 plus E₂; we investigated if these changes in ER were responsible for the impairment of the E₂ gene regulation. We first focused on the early time points after hormone treatment and found that E₂ up-regulated TFF1 and XBP1 within 60 minutes and GREB1 within 120 minutes of treatment (figures 4E, 4F and 4G). JQ1 completely blocked this up-regulation at each time point, whereas expression of ER protein did not alter significantly during this time period (figure 4H) except for the 120 min treatment with JQ1 + E₂. To determine further if

exogenous ER expression can reverse the JQ1 mediated inhibition of E₂-regulated genes, we over-expressed ER in MCF7 cells using a tet-off adenovirus system [31] and treated the cells with vehicle, E₂, JQ1 and E₂+JQ1 for 18 hrs. Exogenous ER expression (supplementary figure S2A) was unable to rescue the JQ1-mediated inhibition of E₂ regulated genes (supplementary figure S2B, S2C and S2D), further confirming that the inhibitory effect of JQ1 was not dependent on the reduced ER expression in MCF7 cells.

JQ1 treatment does not disrupt E₂-induced recruitment of ER, SRC3 or histone acetylation status at the promoters of genes

To understand the mechanism by which JQ1 inhibited the E₂-induced transcription of responsive genes, we examined if JQ1 disrupted the recruitment of ER and/or the co-activator SRC3 at the promoters of the responsive genes after E₂ treatment. Recruitment of these factors at the promoter regions of TFF1, GREB1 or XBP1 genes harboring EREs (Figure 5A, 5E, and 5I) was investigated using ChIP assays in MCF7 cells. These ERE sites have been well characterized and recruitment of ER and other factors at these sites have been associated with E₂-induced transcription of these genes [25,26,32,24,33,1]. Recruitment of ER and SRC3 at these sites was evaluated after 45 minutes of treatment with vehicle, E₂, JQ1 and E₂+JQ1. As expected, E₂ treatment induced recruitment of both ER and SRC3 at the promoter EREs of each of the TFF1, GREB1 and XBP1 genes (Figure 5B, 5C, 5F, 5G, 5J, and 5K). This recruitment was associated with E₂-induced hyper-acetylation of histone H4 (Figure 5D, 5H and 5L). Treatment with JQ1 alone had no effect on these parameters. Interestingly, JQ1+E₂ also did not affect the E₂-induced recruitment of ER and SRC3 at the TFF1 and GREB1 promoters. In the case of XBP1, a slight suppression in recruitment of ER and SRC3 was observed in the presence of JQ1. Modest reduction in E₂-induced hyper-acetylation of H4 was observed by JQ1 in the TFF1 and GREB1 genes but not with XBP1 (Figure 5D, 5H, and 5L).

JQ1 blocks E₂-induced RNA polymerase II (RNAP) advancement from the promoters to the coding region of the gene

Since E₂-induced recruitment of RNAP and its elongation activity at the promoters of the responsive genes is necessary to stimulate the transcription process, we measured enrichment of the RNAP by ChIP assay followed by loci-specific qRT-PCR. To evaluate its density at the proximal promoter we designed primers for the loci that were upstream of the transcription start site. For TFF1 and GREB1, these were the same regions that contained the ERE. For XBP1, we used primers for 300bp upstream region, which recruits higher level of RNAP than the enhancer region (–13kb) containing the ERE [26]. To assess the elongation activity of the RNAP enzyme, we evaluated its enrichment (density) at the coding region of the gene body of all the three genes. The density of RNAP on the gene body positively correlates with its transcriptional elongation activity in MCF7 cells [34]. Therefore, separate primers were used to assess the RNAP recruitment in the coding region of each gene as outlined in the Figure 6A, 6E and 6I. The results revealed that JQ1 marginally (19%-35%) suppressed E₂-induced RNAP recruitment at the proximal promoter of all the genes tested (Figure 6B, 6F and 6J). In contrast, RNAP recruitment was notably lower (~80%) in the coding region of the gene body for TFF1 and GREB1 in presence of JQ1 and E₂. For XBP1, we observed a 45% reduction in RNAP recruitment in the gene body (Figure 6C, 6G and

6K) compared with E₂ only. A measure of transitional activity from initiation to elongation of RNAP can be represented by the “traveling ratio”, which depicts the ratio of RNAP association with the promoter to the coding region [30]. Evaluation of the traveling ratio showed that E₂ treatment was associated with a substantial reduction in the traveling ratio for TFF1, GREB1 and XBP1 (Figure 6D, 6H and 6L), indicating proficient transcription elongation activity. The reduction in traveling ratio by E₂ was fully reversed by JQ1 treatment for TFF1 and GREB1, but only slightly for XBP1 gene (Figure 6D, 6H and 6L). Interestingly, XBP1 also showed high recruitment of RNAP at the promoter and in the coding region even in the absence of E₂, which was not reversed by JQ1 alone (Figure 6J and 6K). In fact at the promoter region of XBP1 gene, JQ1 alone enhanced the recruitment of the RNAP as compared with vehicle (Figure 6J).

Discussion

Histone acetylation at the promoters of E₂ induced genes is one of the key chromatin modifications required for the activation of transcription [8,10]. Based on our data we postulate (Figure 7) that the BET proteins, which can recognize the acetylated histones in the chromatin [35,36] and allow transcriptional elongation by RNAP [12,37], are essential for the E₂- induced gene activation in ER positive breast cancer cells. While this manuscript was being prepared a similar study was published [38] that reported BRD4 dependent elongation activity of RNAP in estrogen-induced gene activation. Our data is consistent with the findings of the study [38], and in addition, we show that BRD2 is also recruited at the promoters of estrogen regulated genes (supplementary figure 5). These observations underscore the critical role of multiple BET proteins in estrogen regulation of genes in breast cancers and other estrogen target tissues.

JQ1, a small molecule that mimics acetylated lysine, can selectively block the bromodomains of the BET proteins, (BRD2, BRD3 and BRD4) [16]. Expression of each BET protein was confirmed in MCF7 and T47D cells (supplementary figure 4). Using JQ1 as a tool to block BET protein function we demonstrate complete inhibition of E₂- induced transcription and growth (figure 1). The stereo-isomer of JQ1, ((-)- JQ1), which is unable to bind to the bromodomains [16], failed to inhibit E₂ induced gene activation as well as growth (figure 2). Thus, recognition of the histone acetylated lysines by the dual bromodomains of BET proteins is a key event in the E₂- regulation of the genes. Furthermore, our results indicated (supplementary figure 3) a direct inhibitory role of JQ1 since *de novo* protein synthesis was not a pre-requisite for the inhibitory effect of JQ1 on the estrogen-regulated genes.

Our results (figure 4) on the effect of JQ1 on ER protein expression revealed opposing regulation in MCF7 and T47D cells. Differential E₂ regulation of ER in these cells has been previously reported from our laboratory [23], where ER mRNA is down-regulated by E₂ in MCF7 cells but up-regulated in T47D cells [23]. In addition, ER protein is known to undergo ubiquitin mediated degradation by E₂ in MCF7 and other cells [39-41]. In this study we provide evidence that BET proteins are involved in maintaining the basal ER mRNA levels in both MCF7 and T47D cells as JQ1 treatment down-regulated the ER mRNA in both the cell lines (figure 4C and D). However, short term (6 hours) combination treatment of E₂

and JQ1 reduced the ER protein levels in MCF7 cells only (figure 4A and B). This outcome was most likely due to the additive effect of reduced mRNA by JQ1 and E₂- mediated ER protein degradation. While the loss of ER in MCF7 cells was not responsible for the inhibitory effects of JQ1 (figure 4E-G and supplementary figure 2), the contrasting regulation of ER protein in MCF7 and T47D cells in presence of JQ1+E2 indicates alternative mechanisms for the maintenance of ER protein levels in diverse breast cancer cells.

Since JQ1 did not disrupt either the E₂ -induced recruitment of ER and SRC3 at the promoters or the histone hyper-acetylation in the region (Figure 5), BET protein function is not clearly essential for either the binding of ER at the ERE or formation of the initial transcription complex at the promoters, including the recruitment of RNAP (Figure 6B, F and J). In contrast, BET protein function was indispensable for the activation and advancement of the RNAP onto the coding region of the E₂-regulated genes as JQ1 treatment, which blocked recruitment of BET proteins (supplementary figure 5), and stalled RNAP at the promoters upstream of the transcription start sites (Figure 6C, G and K). This establishes BET proteins as an essential component for integrating the E₂-ER/co-activator complex with RNAP activation.

Phosphorylation of serine-2 at the carboxy terminal domain (CTD) of RNAP by positive transcription elongation factor b (PTEF_b), a heterodimer of CDK9 and cyclin T1, precedes transcription elongation function [42,43]. BET proteins, particularly BRD4, are known to interact with PTEF_b [37,11], and positively influence transcription by RNAP. Besides BRD4 other BET protein family members, BRD2 and BRD3, have also been shown to couple histone acetylation with transcription [13]. Interestingly, BRD4 protein has also been suggested to activate RNAP independent of PTEF_b [44,45]. Since JQ1 can bind to all the BET protein family members [16], more than one member may play an important role in the E₂-mediated transcriptional elongation by RNAP. The transcription elongation is characterized by CDK9 mediated serine-2 phosphorylation of CTD of RNAP [46,47,15] and increased density of RNAP into the coding region of the gene body [34]. Recruitment of CDK9 and subsequent activation of RNAP of the estrogen regulated genes (including TFF1 and GREB1) [48] in MCF7 cells have been previously reported [48]. The observations that BET proteins can interact with CDK9 [37,11], coupled with our data strongly suggest that BET proteins are responsible for engaging CDK9 at the promoters of the E₂ responsive genes. Recent studies have underscored the importance of BET proteins; their association with CDK9 have also been reported in the context of interferon stimulated genes [49]. These observations attain clinical significance because we recently showed that constitutive high cMYC transcription mediated by CDK9 is a critical determinant of endocrine-therapy resistance breast cancers [50]. Therefore, it is reasonable to speculate that inhibition of BET proteins may prove to be clinically beneficial for endocrine therapy refractory breast cancers. Indeed, JQ1 has been successfully used in pre-clinical studies *in vivo* [18,16] including for breast cancers driven by cMYC [51].

Consistent with the functional role of BET proteins, our results (Figure 6D, H and L) demonstrate that the traveling ratio of RNAP (a measure of the rate of transition from initiation to elongation) at the promoters of E₂-induced genes was reversed in the presence

of JQ1. The E₂-induced reduction in the traveling ratio (Figure 6D, H and L) indicated a high density of RNAP at the coding region, which was consistent with high transcription activity. This effect was completely reversed to the vehicle treated levels by JQ1 treatment for TFF1 and GREB1 (Figure 6D, and H), suggesting stalling of the RNAP at the promoter region. For XBP1 (Figure 6L), very little change was observed in the traveling ratio in presence of JQ1+E₂ versus E₂ alone, despite ~40% decrease in RNAP association in the coding region in the presence of JQ1+E₂. XBP1 gene expression may have an E₂ and BET protein independent component, as evident from the high RNAP recruitment at the promoter and coding region even in the vehicle treated cells. Moreover, treatment with JQ1 alone was unable to reverse this RNAP recruitment (figure 6J and K). This emphasizes the possibility that not all E₂-induced genes may be exclusively regulated in a BET protein dependent manner and other mechanisms may also play important roles. Nonetheless, JQ1 blocked the E₂-induced growth of breast cancer cells (figure 1A and B) by restricting the cells in the 'G1' phase of the cell cycle (supplementary figure 1A and B), suggesting BET proteins as a potential therapeutic target.

The E₂ liganded ER engages in a cyclic recruitment process of the receptor and other factors including the RNAP enzyme at the responsive promoters [1,4]. It is important to note that BET protein inhibition had a marginal but consistent reduction in the recruitment of RNAP at the promoter region (Figure 6A, F and J) that was also reported by another study evaluating interferon stimulated genes [49]. This effect may be due to disruption of the dynamics of the cyclic events or / and to a failure of re-initiation of transcription by RNAP. Further studies are needed to evaluate the role of BET proteins on the cyclical recruitment of the liganded ER and other factors. Nevertheless, the results indicate that the role of BET proteins is largely restricted to regulating advancement of the RNAP and the transcription elongation process without interfering with the initial recruitment of ER and other necessary factors.

In conclusion, our data revealed BET proteins as a factor integrating the ER transcriptional complex with the RNAP activation of E₂ regulated genes. This study, along with a recent report [38], enriches our understanding of hormone induced ER-mediated transcriptional regulation and defines BET proteins as a critical regulator of transcriptional activation of E₂-regulated genes in ER positive breast cancers. Our study further provides opportunities to explore new therapeutic targets for breast cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Cell Proliferation

Figure 1. JQ1 inhibits estrogen (E₂)-induced growth and transcription of E₂ responsive genes in MCF7 and T47D cells

Total DNA was measured to assess the growth of MCF7 and T47D cells after 6 days of treatment with E₂ alone or in combination with increasing concentrations of JQ1 in (A) MCF7 and (B) T47D cells. Levels of TFF1, GREB1 and XBP1 mRNA (C, D and E respectively) were assessed in MCF7 cells using real time PCR after 6 hours of treatment with Vehicle (Veh) or E₂ or indicated concentration of JQ1 in combination with E₂. Levels of TFF1, GREB1 and XBP1 mRNA (F, G and H respectively) were assessed in T47D cells

using real time PCR after 6 hours of treatment with Vehicle (Veh), E₂, JQ1 or indicated concentration of JQ1 in combination with E₂. (* p<.05 versus E₂ treatment alone)

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Figure 2. JQ1 mediated inhibition of estrogen (E₂)-induced growth and transcription in MCF7 and T47D cells is stereo-isomer specific

(A) Chemical structures of active (+)-JQ1 and inactive (-)-JQ1 compound. (B) Total DNA was measured to assess the growth of MCF7 and T47D cells after 6 days of treatment with vehicle or E₂ alone or in combination with 250 nM of active (+)-JQ1 or inactive (-)-JQ1 compound in MCF7 and T47D cells. Levels of TFF1, GREB1 and XBP1 mRNA (C, D and E respectively) were assessed in MCF7 cells using real time PCR after 6 hours of treatment with Vehicle (Veh), E₂ or in combination with 250nM of active (+)-JQ1 or inactive (-)-JQ1

compound. Levels of TFF1, GREB1 and XBP1 mRNA (**F**, **G** and **H** respectively) were assessed in T47D cells using real time PCR after 6 hours of treatment with Vehicle (Veh), E₂, or in combination with 250 nM of active (+)-JQ1 or inactive (–)-JQ1 compound.

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Figure 3. Rapid reversal of JQ1 mediated inhibition of estrogen (E₂)-induced transcription in MCF7 cells after JQ1 withdrawal

Levels of TFF1, GREB1 and XBP1 mRNA (A, B and C respectively) were assessed in MCF7 cells using real time PCR. Cells were treated for 2 hours with Vehicle (Veh), E₂, JQ1 or in combination with 250 nM of active (+)-JQ1. To assess the effect of JQ1 withdrawal JQ1+E₂ containing media was replaced after two hours with E₂ only media and harvested at 30, 60 and 120 minutes after the media change. (* p<.05 versus E₂ + JQ1 treatment)

Figure 4. Differential regulation of ER alpha by JQ1+E₂ in MCF7 and T47D cells

Levels of ER alpha protein was measured by western blot after 6 hours treatment with vehicle (Veh), E₂ or a combination of indicated concentration of JQ1 plus E₂ (10⁻⁹M) in (A) MCF7 and (B) T47D cells. The mRNA levels of ER alpha were assessed by quantitative real time PCR in (C) MCF7 and (D) T47 D cells in identically treated cells as for western blot. (* p<.05 versus vehicle treatment) Levels of TFF1, GREB1 and XBP1 mRNA (E, F and G respectively) were assessed in MCF7 cells using real time PCR. Cells were treated for indicated time period (15, 30, 60 and 120 minutes) with E₂ alone or in combination with (+)-

JQ1 (250 nM) . The cells treated with vehicle or (+)-JQ1 (250 nM) alone were harvested after 120 minutes. **(H)** Level of ER alpha protein was measured by western blot in MCF7 cells treated for indicated time period (15, 30, 60 and 120 minutes) with E₂ alone or in combination with (+)-JQ1 (250 nM) . The cells treated with vehicle or (+)-JQ1 (250 nM) alone were harvested after 120 minutes.

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Figure 5. Effect of JQ1 on E₂-induced recruitment of ER, SRC3 and hyper-acetylation of histone H4 protein at the promoters of E₂-responsive genes in MCF7 cells

ChIP assay followed by real-time PCR was used to assess the recruitment of ER alpha, SRC3 and histone acetylation status for the ERE promoter region of (A) TFF1 (E) GREB1 and ERE enhancer region of (I) XBP1 gene. The black bars below the ERE regions of the schematically represented genes depicts region amplified by real-time PCR. Fold enrichment versus IgG (normal rabbit) was calculated for ER alpha, SRC3 and acetylated H4 for TFF1 (B, C, and D respectively), GREB1 (F, G, and H respectively) and XBP1 (J, K, and L

respectively) after 45 minutes of treatment with vehicle (0.1% ethanol), E₂ (10⁻⁹M), (+)-JQ1 (250 nM) or JQ1+E₂. (* p<.05 versus E₂ treatment alone)

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Figure 6. Effect of JQ1 on E₂-induced recruitment of RNA polymerase-II enzyme (RNAP) at the proximal promoters and gene coding region of E₂-responsive genes in MCF7 cells

ChIP assay followed by real-time PCR was performed to assess the recruitment of RNAP at the promoter and gene coding region of (A) TFF1 (E) GREB1 and (I) XBP1 gene. The black bars below the schematically represented genes depicts region amplified by real-time PCR. Fold enrichment versus IgG (normal rabbit) was calculated for RNAP at the promoter and gene coding region TFF1 (B and C respectively), GREB1 (F and G respectively) and XBP1 (J and K respectively) after 45 minutes of treatment with vehicle (0.1% ethanol), E₂

(10^{-9} M), (+)-JQ1 (250nm) or JQ1+E2. The travel ratio of RNAP for **(D)** TFF1, **(H)** GREB1 and **(L)** XBP1 was calculated as mentioned in the materials and method section after 45 minutes of treatment with vehicle (0.1% ethanol), E₂ (10^{-9} M), (+)-JQ1 (250 nM) or JQ1+E2. The % numbers in B, C, F, G, J and K graphs correspond to percent decrease in RNAP enrichment in E₂+JQ1treated cells compared to E₂ treatment alone. (* p<.05 versus E₂ treatment alone)

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Figure 7.
Schematic presentation of the proposed model for the role of BET proteins in estrogen induced transcription