Transrepression of the estrogen receptor promoter by calcitriol in human breast cancer cells via two negative vitamin D response elements

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Abstract

Calcitriol (1,25-dihydroxyvitamin D_3), the hormonally active metabolite of vitamin D, exerts its anti-proliferative activity in breast cancer (BCa) cells by multiple mechanisms including the downregulation of the expression of estrogen receptor α (ER). We analyzed an ~3.5 kb *ER* promoter sequence and demonstrated the presence of two potential negative vitamin D response elements (nVDREs), a newly identified putative nVDRE upstream at −2488 to −2473 bp (distal nVDRE) and a previously published sequence (proximal nVDRE) at −94 to −70 bp proximal to the P1 start site. Transactivation analysis using *ER* promoter deletion constructs and heterologous promoter–reporter constructs revealed that both nVDREs functioned to mediate calcitriol transrepression. In the electrophoretic mobility shift assay, the vitamin D receptor (VDR) showed strong binding to both nVDREs in the presence of calcitriol, and the chromatin immunoprecipitation assay demonstrated the recruitment of the VDR to the distal nVDRE site. Mutations in the 5′ hexameric DNA sequence of the distal nVDRE resulted in the loss of calcitriol-mediated transrepression and the inhibition of protein–DNA complex formation, demonstrating the importance of these nucleotides in VDR DNA binding and transrepression. A putative nuclear factor-Y (NFY) binding site, identified within the distal nVDRE, led to the findings that NFY bound to the distal nVDRE site interfered with the binding of the VDR at the site and reduced calcitriol-mediated transrepression. In conclusion, the *ER* promoter region contains two negative VDREs that act in concert to bind to the VDR and both nVDREs are required for the maximal inhibition of *ER* expression by calcitriol. The suppression of *ER* expression and estrogen-mediated signaling by calcitriol in BCa cells suggests that vitamin D may be useful in the treatment of ER+ BCa.

Key Words
- vitamin D
- calcitriol
- transrepression
- estrogen receptor
- nVDREs
- estrogen signaling
- breast cancer cells

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Introduction

Breast cancer (BCa) is the most common cancer in women in the USA (Siegel *et al.* 2012). Estrogens drive the proliferation of mammary epithelial cells and therefore promote the growth of estrogen receptor-positive (ER+) BCa cells. Estrogens act by binding to the estrogen receptor α (ER) to regulate gene expression. Therapy for ER+ BCa, therefore, includes hormonal treatments such as those with aromatase inhibitors that inhibit estrogen synthesis and selective *ER* modulators that block estrogen action in the breast by binding to the *ER* and acting as antagonists (Burstein *et al.* 2010, Tomao *et al.* 2011). Another approach to inhibit the growth of ER+ BCa is to decrease the levels of ER, the nuclear receptor that mediates the growth-promoting effects of estrogens in breast epithelial cells using compounds that act as selective *ER* downregulators such as fulvestrant (McDonnell & Wardell 2010, Nilsson *et al.* 2011).

Calcitriol, the hormonally active form of vitamin D (1,25-dihydroxyvitamin D_3), plays an important role in calcium homeostasis through its actions (Feldman *et al.* 2013). In addition to these classical actions, calcitriol also exhibits anti-proliferative, pro-differentiating, and anti-inflammatory activities, indicating its potential use in the prevention and treatment of multiple cancers including BCa (Beer & Myrthue 2004, Deeb *et al.* 2007, Krishnan & Feldman 2011, Pereira *et al.* 2012, Welsh 2012). Interestingly, our studies reveal that in ER+ BCa cells calcitriol inhibits both estrogen synthesis and signaling, leading to a significant attenuation of estrogen stimulation of cell growth (Krishnan *et al.* 2010*a*). We have shown that calcitriol acts as a selective aromatase modulator (SAM) and decreases estrogen synthesis in BCa cells and the surrounding mammary adipose tissue by suppressing aromatase expression (Krishnan *et al.* 2010*b*, Swami *et al.* 2011). Furthermore, calcitriol and its analogs also downregulate *ER* expression in BCa cells (James *et al.* 1994, Simboli-Campbell *et al.* 1997, Swami *et al.* 2000), thereby causing a significant inhibition of estrogenic bioresponses in these cells, including the suppression of estrogen-stimulated cell growth (Demirpence *et al.* 1994, Swami *et al.* 2000).

Our earlier study (Swami *et al.* 2000) and that of Stoica *et al.* (1999) revealed that the negative regulation of *ER* expression by calcitriol occurred at the transcriptional level. This transcriptional repression is probably mediated through the binding of the vitamin D receptor (VDR) to one or more negative vitamin D response elements (nVDREs) present in the *ER* promoter. The current study explores the mechanism of the transcriptional repression of the *ER* gene by calcitriol. Recent advances in the genome-wide analysis of transcriptional regulation by calcitriol have revealed that the VDREs could be located both proximal and distal to the transcriptional start sites of target genes and many, in fact, are located in clusters hundreds of kilobases from their target genes (Pike & Meyer 2010, Pike 2011, Pike *et al.* 2011*a*). However, our current analysis demonstrates that the functional activities of two nVDRE sites within an ~3.5 kb promoter region of the *ER* gene recapitulate the magnitude of the downregulation of *ER* expression observed in BCa cells following calcitriol treatment. One of these sites, the proximal nVDRE identified previously by Stoica *et al.* (1999), is an imperfect palindromic sequence located at −94 to −70 bp of the *ER* gene with reference to the P1 start site, the major start site in ER+ BCa cells (deConinck *et al.* 1995). Herein, we report the identification and characterization of another putative nVDRE (distal nVDRE) further upstream of the published proximal site that also contributes to the calcitriol-mediated transrepression of *ER*.

Materials and methods

Materials

The human BCa cell lines used in the study were obtained from the American Type Culture Collection (Rockville, MD, USA). Tissue culture media, supplements, and FCS were obtained from Gibco BRL, Lonza (Walkersville, MD, USA), and Mediatech, Inc. (Herndon, VA, USA). All antibodies were obtained from Santa Cruz Biotechnology. All other chemicals and reagents were procured from Sigma Chemical Co., unless otherwise indicated.

Methods

Cell culture The human BCa cell lines MCF-7, ZR75-1, and T47D were cultured at 37 °C under 5% CO_2 atmosphere using RPMI-1640 medium supplemented with 10% calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Plasmids *ER* promoter–reporter plasmids containing a series of 5′ deletions with reference to the P1 start site were a kind gift from Dr Ronald Weigel (formerly at the Stanford University and currently at the University of Iowa, Iowa City, IA, USA). The construction

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of these plasmids has been described previously (deConinck *et al.* 1995). The NFYA expression plasmids were a kind gift from Dr Roberto Mantovani (Universita di Milano, Milano, Italy).

Heterologous constructs for VDRE analysis We refer to the previously described VDRE by Stoica *et al.* (1999) (−94 to −70) as the proximal nVDRE and the newly discovered upstream site as the distal nVDRE (−2488 to −2473). A 230 bp KpnI–HindIII fragment (+230/0) from the plasmid pGL2-*ER* promoter (−3500 to +230) was cloned into the KpnI/SmaI sites of the heterologous Simian virus 40 (SV40) promoter-driven luciferase reporter vector pGL3 (Promega) to generate the nVDRE(null) construct. Oligonucleotides enclosing the proximal nVDRE (31 nucleotides), the distal nVDRE (27 nucleotides), and an oligonucleotide containing both nVDRE sequences (58 nucleotides) containing a MluI overhang at the 5′ end were synthesized (Operon Technologies, Alameda, CA, USA). Single copies of each of these oligonucleotides were annealed and ligated to the MluI site of the nVDRE(null) plasmid to generate the heterologous constructs containing the proximal, distal, or both nVDRE sequences. The orientation of each insert in the heterologous constructs was verified by sequencing.

Mutagenesis of the distal nVDRE sequence in the natural and heterologous *ER* promoter constructs Mutations in the 5′ half-site (potential RXR binding site) and the 3′ half-site (potential VDR binding site) in the distal nVDRE sequence were introduced into the −3500/+230/pGL2-Basic reporter construct using the GeneEditor site-directed mutagenesis system (Promega). To generate the heterologous mutant constructs, two pairs of oligonucleotides containing the same mutations in the distal nVDRE sequence with the MluI site overhangs were synthesized and cloned into the nVDRE(null) plasmid. Positive clones identified by sequencing were used in the transactivation assays.

Transient transfections and luciferase assays The various *ER* promoter-luciferase reporter constructs were transiently transfected into the ZR75-1 or MCF-7 cells using LipofectAmine (Life Technologies/Invitrogen). A renilla luciferase plasmid (pRLnull, Promega) was co-transfected to control for transfection efficiency. Following transfections, the cells were treated with a vehicle (0.1% ethanol) or 100 nM calcitriol for 24 h. Reporter and renilla luciferase activities were measured using the Dual Luciferase Assay Kit (Promega) (Peng *et al.* 2004).

Electrophoretic mobility shift assay Electrophoretic mobility shift assay (EMSA), as described previously (Peng *et al.* 2004), was used to analyze the binding of the VDR to the putative nVDREs in the *ER* promoter using complementary oligonucleotides for the distal and proximal nVDREs. ZR75-1 cell extracts were incubated for 15–20 min with 100 nM calcitriol and [^{32}P]-labeled oligonucleotide probes. For competition assays, a 500-fold molar excess of the corresponding unlabeled oligonucleotides was added to the reaction mixture before the addition of the labeled probes. In some reactions, 2 μg of an anti-VDR polyclonal antibody (H-81) or an anti-RXRα polyclonal antibody (D20) were added to the reaction mixture 40 min prior to the addition of the labeled probe. After additional incubation for 20 min, the DNA–protein complexes were separated by electrophoresis on 5% nondenaturing polyacrylamide gels in a Tris–borate–EDTA buffer and visualized by autoradiography (Peng *et al.* 2004).

Chromatin immunoprecipitation assay ZR75-1 and MCF-7 cells treated with a vehicle or 100 nM calcitriol for 2 h (for VDR) or 24 h (for RXRα and NFYA) were subjected to a chromatin immunoprecipitation (ChIP) analysis using the EZ-ChIP kit (Upstate/EMD Millipore Corp., Billerica, MA, USA) as described previously (Peng *et al.* 2004). Immunoprecipitation of the chromatin fragments was carried out with IgG or a mixture of polyclonal antibodies against the VDR (N-20, C-20, and H-81), a polyclonal antibody against RXRα (D-20), or a polyclonal antibody against NFYA (C-18). After washing and reverse cross-linking, qPCR was performed on the eluted DNA using primers designed to amplify a 178 bp *ERα* promoter fragment from −2581 to −2403 bp encompassing the distal nVDRE: forward, 5′-GTGGGTTTGGTTAACGAAGTG -3′; reverse, 5′-CCTTCTGGGATACATGTGGATCA-3′. QPCR data are expressed as the ratio of the amount of total immunoprecipitated DNA (bound) to the amount of input DNA. The PCR products were also visualized by agarose gel electrophoresis and ethidium bromide staining.

Western blot analysis Nuclear extracts of the BCa cells were subjected to 10% SDS–PAGE, transferred onto nitrocellulose membranes, and immunoblotted with an anti-mouse MAB to human *ER* (F-10; 1:500 dilution in 1% Carnation nonfat milk) as described previously (Swami *et al.* 2000). The blots were then probed with a HRP-conjugated anti-mouse secondary antibody, and the immunoreactive bands were detected using an ECL Kit (Amersham).

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Statistical analysis

All values are presented as means±S.E.M. Data were evaluated by ANOVA with Scheffe's test as the *post hoc* analysis or Student's *t*-test where appropriate using the GraphPad Prism 5 Software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Suppression of *ER* promoter activity by calcitriol in BCa cell lines

The plasmid containing the full-length *ER* promoter sequence (−3500 to +230) cloned into the pGL2-Basic vector was transiently transfected into various BCa cell lines along with a VDR expression plasmid (pSG5-VDR) and a renilla luciferase plasmid. Treatment of the cells with 100 nM calcitriol for 24 h resulted in significant decreases (~65–80%) in relative luciferase activity (Fig. 1A), demonstrating a substantial suppression of *ER* promoter activity by calcitriol in the various cell lines. In the ZR75-1 cells, calcitriol treatment decreased reporter activity in a dose-dependent manner (Fig. 1B). These data support our hypothesis that calcitriol transrepresses *ERα* transcription, probably acting through one or more nVDREs present in the *ER* promoter. Stoica *et al.* (1999) identified an imperfect palindromic sequence at −94 to −70 bp of the *ER* promoter with reference to the P1 start site and demonstrated that it acted as a nVDRE in reporter assays. Upon further *in silico* analysis, we have found another putative nVDRE upstream at −2488 to −2473 bp with reference to the P1 start site, hereinafter referred to as the distal nVDRE (Fig. 2A).

Deletion analysis of the *ER* promoter

To further define the nVDREs in the 3500 bp *ER* promoter fragment, deletion constructs of the promoter cloned into the pGL2-Basic vector were transiently transfected into the ZR75-1 cells and calcitriol suppression of reporter activity was determined. As shown in Fig. 2A, calcitriol treatment significantly inhibited the reporter activity of the full-length *ER* promoter-luc (−3500/+230 LUC), causing an ~72% decrease ($P<0.001$). The removal of the 5′ sequence from −3500 to −701 containing the distal nVDRE (−700/+230 LUC) significantly reduced the magnitude of calcitriol-mediated suppression when compared with the full-length promoter (~54 vs ~72% decrease, $P<0.05$), suggesting that the distal nVDRE sequence mediates a substantial portion of the negative regulation of the *ER* promoter by calcitriol. Interestingly, the removal of the −3500 to −701 fragment increased the basal reporter activity compared with the full-length promoter (~72% increase, $P<0.05$), suggesting that this region may contain binding sites for other negative regulators

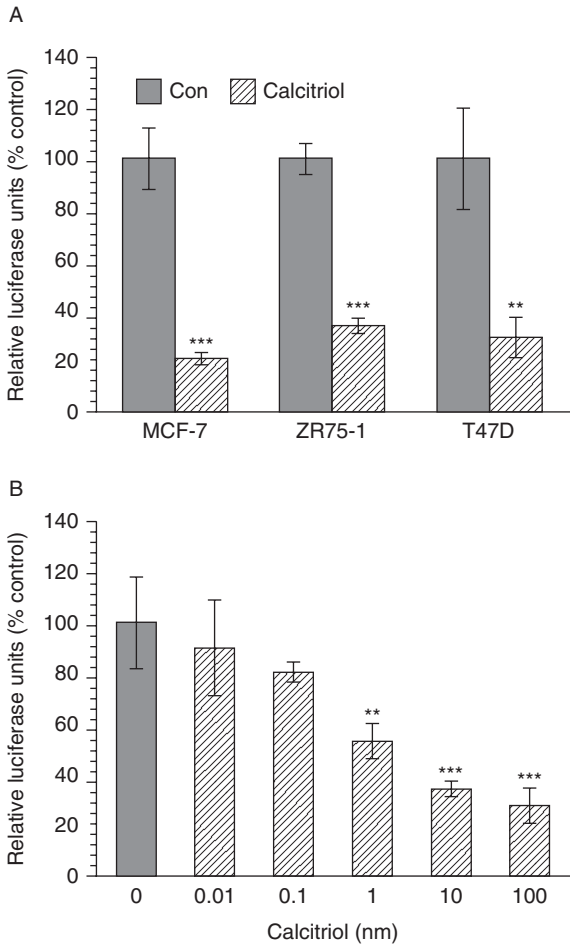

Figure 1
Transcriptional repression of the human *ER* promoter by calcitriol in breast cancer cell lines. (A) The human *ER* promoter-luciferase construct (~3.5 kb) was transiently transfected along with a VDR expression vector (pSG5-VDR) into the MCF-7, ZR75-1, and T47D human breast cancer cell lines. A renilla luciferase expression vector was co-transfected to control for transfection efficiency. The cells were treated with either a vehicle (Con) or 100 nM calcitriol for 24 h and the luciferase activity was assayed. Relative luciferase activity in the calcitriol-treated cells was compared with that of the vehicle-treated controls in each transfection that was set at 100%. Values represent means±S.E.M. of at least four independent transfections performed in triplicate. $^{**}P<0.01$ and $^{***}P<0.001$ when compared with the control. (B) Dose response of the *ER* promoter to calcitriol in the ZR75-1 human breast cancer cells. ZR75-1 cells transiently transfected with the human *ER* promoter, as described above, were treated with either a vehicle (Con) or various concentrations of calcitriol (B) for 24 h. Relative luciferase activity in the calcitriol-treated cells was compared with that of the vehicle-treated controls in each transfection that was set at 100%. Values represent means±S.E.M. of at least four independent transfections performed in triplicate. $^{**}P<0.01$ and $^{***}P<0.001$ when compared with the control.

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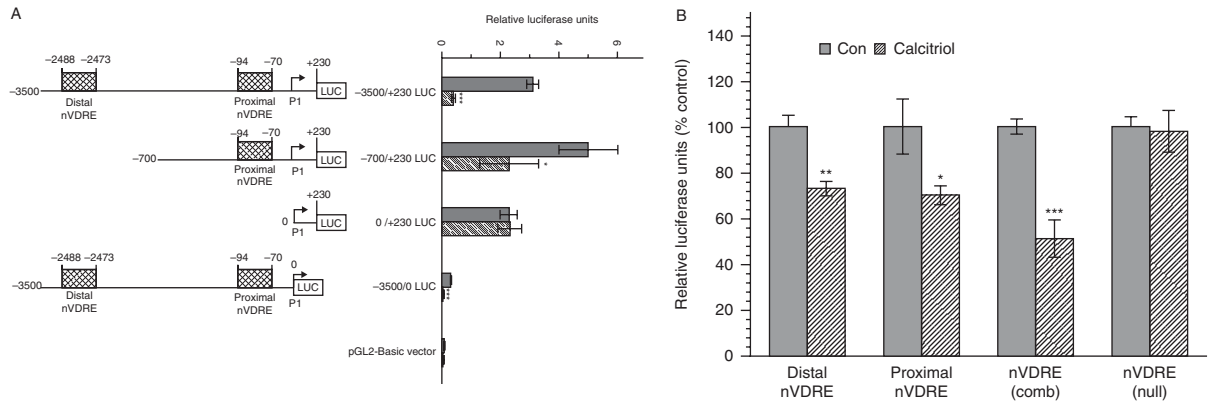

Figure 2

Transcriptional activity of *ER* promoter constructs. (A) Diagrammatic representation of the *ER* promoter (~3.5 kb) and the various deletions constructs cloned into the pGL2-Basic reporter vector (left panel) with reference to the location of the distal and proximal nVDRE sites. The numbers are relative to the P1 transcription start site that is set at 0. The constructs were transiently transfected into the ZR75-1 cells as described in the Subjects and methods section and treated with a vehicle (Con) or 100 nM calcitriol for 24 h (right panel). The values are expressed as a ratio of the *ER*-luc normalized to renilla-luc activity in order to correct for transfection efficiency. Values represent means±S.E.M. of at least four independent transfections performed in triplicate. $*P<0.05$ and $***P<0.01$ when compared with the control. (B) Reporter activity of the heterologous constructs of the distal and proximal nVDREs. Single copies of the wild-type distal or proximal or both *ER* nVDREs in the sense direction were cloned into the pGL3-promoter–reporter vector and transfected into the ZR75-1 cells as described in the Subjects and methods section. The transfected cells were treated with either a vehicle or 100 nM calcitriol for 24 h. A null reporter containing a nonsense sequence was used as a negative control to assess the effects of calcitriol on the heterologous constructs. Relative luciferase activity in the calcitriol-treated cells was compared with that of the vehicle-treated controls in each transfection that was set at 100%. Values represent means±S.E.M. of at least four independent transfections performed in triplicate. $*P<0.05$, $**P<0.01$, and $***P<0.001$ when compared with the control.

of *ER* promoter activity. Deletion of the entire 5′ promoter region (0/+230 LUC) eliminating both nVDREs completely abolished the calcitriol-mediated suppression of reporter activity, demonstrating that both distal and proximal nVDREs are necessary for the full suppressive effect of calcitriol. Deletion of a 230 bp fragment from the P1 start site containing the 5′ untranslated leader of the *ER* gene (−3500/0 LUC) greatly reduced (~90% decrease, $P<0.01$) the basal promoter activity when compared with the basal activity of the full-length 3500 bp promoter including the 230 bp fragment, indicating that this 230 bp region contained a powerful simulator of *ER* expression. However, even with the reduction in the basal promoter activity of this deletion construct, significant calcitriol-mediated further suppression was still evident (~85% decrease in calcitriol-treated cells vs basal activity of the −3500/0 construct, $P<0.001$).

Conferment of calcitriol responsiveness to a heterologous promoter by *ER* nVDREs

To further demonstrate that the two nVDRE sequences specifically respond to calcitriol, heterologous constructs containing a single copy each of the proximal and distal nVDRE sequences or both VDRE sequences were transiently transfected into the ZR75-1 cells along with pSG5-VDR and the renilla luciferase plasmid and reporter activity was assessed. Treatment with 100 nM calcitriol caused an ~25% decrease in reporter activity in cells transfected with a construct containing either the distal nVDRE or the proximal nVDRE alone (Fig. 2B). In cells transfected with a construct containing both the nVDREs (nVDRE(comb)), calcitriol-induced suppression of reporter activity was higher compared with the constructs with just either the proximal or distal nVDRE (~49% decrease, $P<0.05$). In ZR75-1 cells transfected with the pGL3 promoter vector lacking any VDRE sequence (nVDRE(null)), calcitriol did not suppress reporter activity (Fig. 2B).

Mutational analysis of the nVDRE sequences

A mutational analysis was carried out to confirm that the suppression of the *ER* promoter by calcitriol was mediated through the distal nVDRE sequence present in the *ER* promoter (Fig. 3A and B). Stoica *et al.* (1999) demonstrated that when the proximal nVDRE sequence was mutated to give rise to a nonsense sequence inserted 5′ of a SV40 promoter-chloramphenicol acetyl transferase reporter plasmid, calcitriol suppression of reporter activity was lost following transfection of this heterologous promoter construct into MCF-7 cells. In the current study, we used site-directed mutagenesis to create point mutations in the

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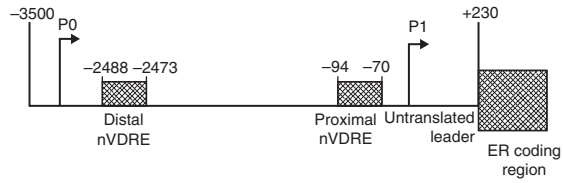
distal nVDRE. We generated mutants with base changes in both the 3′ half-site (3′MUT) and 5′ half-site (5′MUT) of the distal nVDRE (Fig. 3C). The mutations were created both in the native promoter context (−3500 to +230 *ER* promoter-luc) and in the heterologous construct in the pGL3 vector. Various wild-type (WT) and mutant constructs were transfected into the ZR75-1 cells and calcitriol responsiveness was assessed by measuring reporter activity. As shown in Fig. 3D, calcitriol treatment caused a significant suppression of activity in the native WT promoter construct as well as in the 3′MUT construct. Mutations in the 5′ half-site of the distal nVDRE (5′MUT) in the full promoter construct resulted in an approximately fivefold decrease in the basal promoter activity. Furthermore, a loss of calcitriol-mediated suppression of promoter activity was also observed with the 5′MUT construct (Fig. 3D). Similar results were observed when the same mutations were created in the distal nVDRE in the heterologous construct (Fig. 3E). Mutations in the 5′ half-site but not in the 3′ half-site abolished the suppressive effect of calcitriol, suggesting that the 5′ hexameric sequence is required for calcitriol suppressor activity via the distal nVDRE (Fig. 3E).

A *ER* promoter



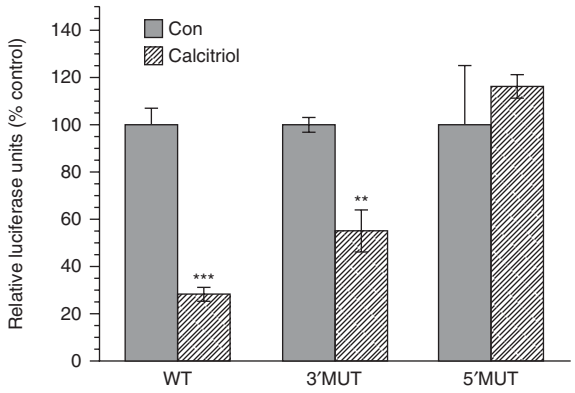
B nVDRE Sequences

Distal nVDRE	GTGCAA TCG GAGGCA
Proximal nVDRE	CAGGCAaggcaacagtcccTGGCCG

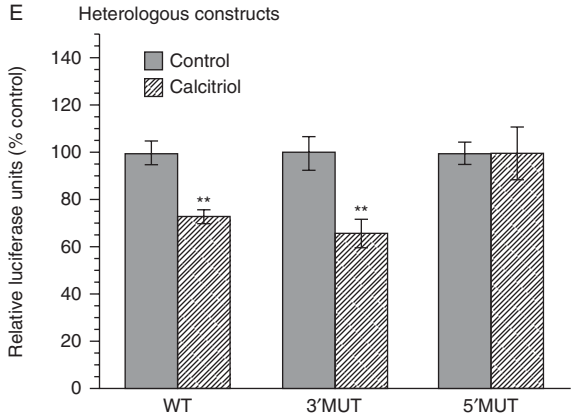
C Mutations in distal nVDRE

Wild-type (WT)	GTGCAA TCG GAGGCA
3′ mutation (3′MUT)	GTGCAA TCG t aG eCA
5′ mutation (5′MUT)	t T tCgA TCG GAGGCA

D Full promoter constructs



E Heterologous constructs



Analysis of the binding of the VDR to the nVDREs by EMSA

To demonstrate the binding of the VDR to the nVDREs in the *ER* promoter, we carried out EMSA (Fig. 4). In this assay, we used extracts of ZR75-1 cells that contained endogenous RXR and VDR as well as the VDR expressed by the vector pSG5-VDR that was transfected into the cells. Lane 1 in Fig. 4A and B represents the free probe. As shown in Fig. 4A, when incubated with the radiolabeled distal nVDRE probe, the formation of a protein–DNA complex was detected (Fig. 4A, lane 2). The addition of calcitriol substantially increased the intensity of this complex (Fig. 4A, lane 3). Competition with an unlabeled distal nVDRE oligonucleotide showed a decrease in the band intensity of the complex (Fig. 4A, lane 4). The addition of the anti-VDR antibodies clearly inhibited complex formation (Fig. 4A, lane 5). Similarly, the addition of the anti-RXRα antibodies also inhibited complex formation (Fig. 4A, lane 6). However, with both antibodies, the presence of lower-molecular-

Figure 3
Distal nVDRE sequences and mutations. A schematic representation of the *ER* promoter with the location of the two putative nVDREs (A) and their sequences (B). Individual bases were changed in the distal nVDRE sequence to create 5′ (5′MUT) and 3′ (3′MUT) mutations, shown in lower case (C). Transcriptional activities of the mutant constructs in the full-length promoter as well as heterologous constructs were assessed by transient transfections in the ZR75-1 cells. (D) Functional activity of the mutations in the native promoter and (E) activity of the heterologous constructs with the various mutations. All plasmids were transiently transfected into the ZR75-1 cells followed by treatment with either a vehicle (Con) or 100 nM calcitriol for 24 h. Relative luciferase activity in the calcitriol-treated cells was compared with that of the vehicle-treated controls in each transfection that was set at 100%. Values represent means±S.E.M. of at least four independent transfections performed in triplicate. $^{**}P<0.01$ and $^{***}P<0.001$ when compared with the control.

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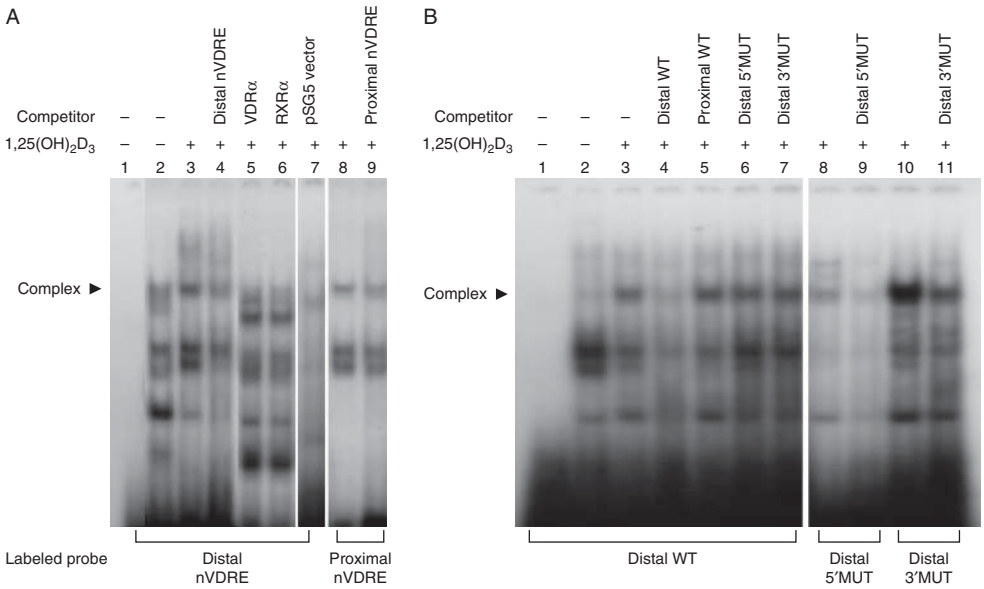

Figure 4

EMSA demonstrating the binding of the VDR to the *ER* nVDREs. (A) The nucleotides corresponding to the distal (lanes 1–7) and proximal (lanes 8 and 9) nVDREs were annealed and end-labeled with [g^{32}P] ATP using T_4 polynuleotide kinase. The labeled probes were then incubated with 5 μg of extracts of ZR75-1 cells containing the pSG5-VDR expression vector in the absence (lanes 1 and 2) or presence (lanes 3–9) of calcitriol. Competition with 500× unlabeled probe can be seen in lanes 4 (distal nVDRE) and 9 (proximal nVDRE). Blockades by an anti-VDR antibody and an anti-RXRα antibody of the distal nVDRE binding are shown in lanes 5 and 6. Lane 1 represents the free probe (no binding) and lane 7 represents the pSG5-empty vector (minimal binding). (B) Nucleotides corresponding to the WT distal nVDRE (lanes 1–7), the distal 5′MUT nVDRE (lanes 8 and 9), and the distal 3′MUT nVDRE (lanes 10 and 11) were annealed and end-labeled with [g^{32}P] ATP. Lane 1 represents the free WT distal nVDRE probe and lanes 2 and 3 represent those with and without calcitriol. Lanes 4–7 represent competition with various unlabeled probes as indicated. Specific binding of the VDR to the labeled 5′MUT and 3′MUT is shown in lanes 8–11. These mutations are the same as those introduced into the heterologous promoter constructs shown in Fig. 3.

weight complexes of unknown significance was detected (Fig. 4A, lanes 5 and 6). Lane 7 in Fig. 4A represents a control reaction using extracts from cells transfected with the pSG5 vector alone. The incubation of cell extracts with the radiolabeled proximal nVDRE probe led to the formation of a radiolabeled band, indicating protein–DNA complex formation (Fig. 4A, lane 8), and the band intensity was reduced upon competition with the unlabeled proximal nVDRE (Fig. 4A, lane 9).

Figure 4B depicts the competition exhibited by both WT and mutant nVDREs on complex formation. The intensity of the band representing the complex formed with a labeled distal nVDRE probe was reduced when competition was carried out with an unlabeled WT distal VDRE sequence (Fig. 4B, lane 4) but not with an unlabeled proximal VDRE sequence (Fig. 4B, lane 5) or unlabeled distal nVDRE 5′ or 3′ mutant sequences (Fig. 4B, lanes 6 and 7). When we used a radiolabeled distal nVDRE 5′ mutant sequence as the probe, the intensity of the complex formed (Fig. 4B, lane 8) was much weaker compared with that of the complex formed with the radiolabeled WT distal nVDRE (Fig. 4B, lane 3). In alternate experiments, when a radiolabeled distal nVDRE 3′ mutant sequence was used as the probe (Fig. 4B, lane 10), the complex formed had as much or more band intensity than that observed with the radiolabeled WT distal nVDRE probe (Fig. 4B, lane 3). These data suggest that the nucleotide bases in the 5′ hexameric half-site of the distal nVDRE are important for RXR–VDR heterodimer complexing with DNA. Competition exhibited by the unlabeled distal nVDRE 5′ and 3′ mutant oligonucleotides reduced the intensity of the complexes formed with their corresponding radiolabeled oligonucleotides (Fig. 4B, lanes 9 and 11).

ChIP analysis of the binding of the VDR and RXR to the distal nVDRE

To demonstrate that calcitriol recruits the VDR to the distal nVDRE in the native *ER* promoter in intact cells, we carried out a ChIP analysis. ZR75-1 and MCF-7 cells exposed to 100 nM calcitriol for 2 h were subjected to a ChIP assay. As shown in Fig. 5A, the *ER* promoter distal nVDRE region was co-immunoprecipitated with the anti-VDR (Fig. 5A, lanes 3 and 4) and anti-RXRα (Fig. 5A,

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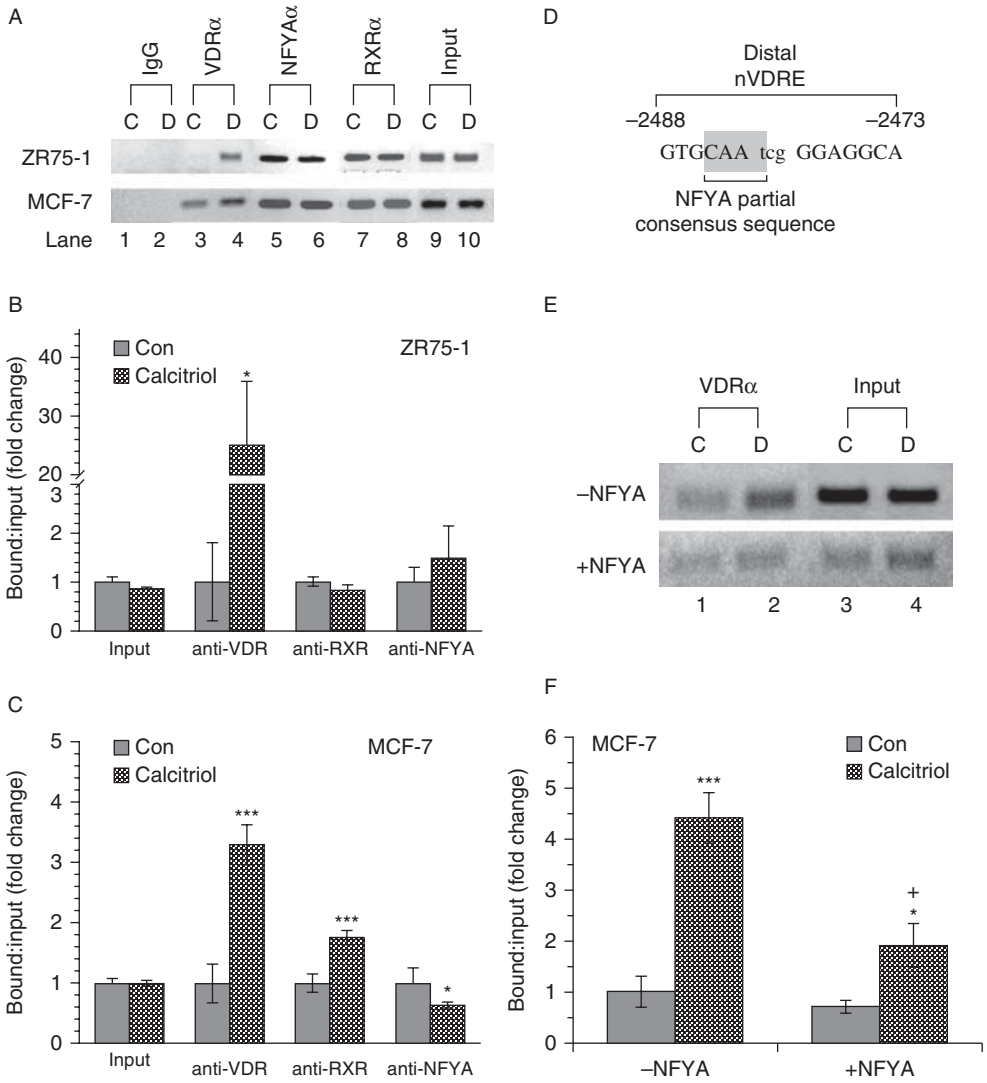

Figure 5

ChIP assay demonstrating the binding of the VDR, RXR, and NFYA to the distal nVDRE site in the *ER* promoter. (A) ZR75-1 and MCF-7 cells grown in 100 mm dishes were treated with a vehicle (0.1% ethanol) or calcitriol for 2 h and subjected to a ChIP assay as described in the Subjects and methods section. After immunoprecipitation, the samples were analyzed by qPCR using primers designed to amplify a 178 bp *ERα* promoter fragment from −2581 to −2403 bp encompassing the distal nVDRE. Representative agarose gels: C, control, vehicle-treated cells and D, calcitriol (100 nM)-treated cells. Lanes 1 and 2 represent immunoprecipitation with nonspecific IgG, lanes 3 and 4 with a mixture of anti-VDR antibodies, lanes 5 and 6 with an anti-NFYA antibody, and lanes 7 and 8 with an anti-RXRα antibody. Lanes 9 and 10 represent the input DNA. (B and C) DNA immunoprecipitated with the indicated antibody in the ZR75-1 (B) or MCF-7 (C) cells was analyzed by qPCR amplifying the distal *ER* nVDRE region. The qPCR data are normalized to the amount of chromatin in the immunoprecipitation reaction (input). Data are represented as a ratio of the immunoprecipitated (bound) to input DNA. The bound:input ratio was set at 1 for vehicle treatment in each immunoprecipitation and was 0.14±0.07, 4.4±0.6, and 16.6±1.3 respectively for VDR, NFYA, and RXR immunoprecipitations in the ZR75-1 cells and 0.04±0.005, 33.3±4.6, and 11.5±1.2 respectively for VDR, NFYA, and RXR in the MCF-7 cells. Values represent means±S.E.M. of three to six determinations. $^{*}P<0.05$ and $^{***}P<0.001$ when compared with the corresponding controls. (D) DNA sequence of the distal *ER* nVDRE and its flanking regions. The shaded area demonstrates the presence of a partial NFY binding site that overlaps the distal nVDRE site in the *ER* promoter. (E and F) ChIP analysis of the binding of VDR to the distal *ER* nVDRE site in the MCF-7 cells overexpressing NFYA. A NFYA expression plasmid (pSG5-NFYA) was transiently transfected into the MCF-7 cells. Untransfected (−NFYA) or NFYA-transfected (+NFYA) cells were treated with a vehicle (C) or 100 nM calcitriol (D) for 2 h, and a ChIP assay was carried out using a mixture of anti-VDR antibodies as described in the Subjects and methods section. (E) A representative agarose gel. (F) QPCR data of the ChIP analysis described in (E). The bound:input ratio was set at 1 for vehicle treatment in the VDR immunoprecipitation, which was 0.7±0.22 in the untransfected (−NFYA) cells and 0.6±0.09 in the NFYA-transfected cells. Values represent means±S.E.M. of three determinations. $^{*}P<0.05$ and $^{***}P<0.001$ when compared with the corresponding controls. $^{+}P<0.05$ compared with the calcitriol-treated +NFYA cells.

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lanes 7 and 8) antibodies, but not with IgG (Fig. 5A, lanes 1 and 2). In immunoprecipitation reactions with the anti-VDR antibodies, significantly more PCR product was generated in cells treated with calcitriol (D) when compared with the vehicle control (C) (Fig. 5A, lanes 4 vs 3), indicating that calcitriol induced the recruitment of the VDR to the distal nVDRE sequence in the *ER* promoter. Calcitriol induced an ~25-fold increase in the PCR product generated from DNA immunoprecipitated with the anti-VDR antibodies in the ZR75-1 cells (Fig. 5B) and an ~3.5-fold increase in the same in the MCF-7 cells (Fig. 5C). In contrast, calcitriol treatment did not affect the amount of PCR product generated from DNA immunoprecipitated with the anti-RXRα antibodies in the ZR75-1 cells (Fig. 5B), while a modest (approximately twofold) but statistically significant increase was observed in the MCF-7 cells (anti-RXR, Fig. 5C).

Assessment of the role of NFY in the repression of *ER* transcription by calcitriol

We identified a putative binding site for nuclear factor Y (NFY), a transcription factor known to bind to the CCAAT box in the promoter regions of target genes to activate gene transcription (Kabe *et al*. 2005). As shown in Fig. 5D, a partial consensus NFY binding sequence (−2486 to −2482 bp) is present within the sequence of the 15 mer distal nVDRE (−2488 to −2473 bp). We, therefore, considered the possibility that calcitriol-mediated repression of *ER* transcription is at least in part due to the competitive binding of the VDR and NFY to overlapping sites at the distal nVDRE locus. This would result in the displacement of NFY by the VDR, thereby attenuating the activation of *ER* transcription by NFY. Alternatively, occupancy of the site by NFY would diminish the ability of calcitriol to repress *ER* transcription by binding to the distal nVDRE site. To investigate this potential interaction, we carried our ChIP and transactivation assays.

Figure 5A (lanes 5 and 6) shows that the *ER* promoter distal nVDRE region was co-immunoprecipitated with an anti-NFYA antibody, suggesting the binding of endogenous levels of NFYA to this site. Calcitriol treatment did not affect the amount of PCR product generated from DNA immunoprecipitated with the anti-NFYA antibody in the ZR75-1 cells (Fig. 5B, anti-NFYA), while a small but statistically significant decrease was observed in the MCF-7 cells (Fig. 5C, anti-NFYA). In a separate experiment, a NFYA expression plasmid was transfected into the MCF-7 cells, following which a ChIP analysis was carried out using the anti-VDR antibodies in cells treated with a vehicle or calcitriol (Fig. 5E and F).

In the absence of NFYA overexpression with only endogenous NFYA levels (Fig. 5E, −NFYA), calcitriol induced an approximately fourfold increase in the amount of PCR product generated from the immunoprecipitated DNA (Fig. 5F, −NFYA). However, NFYA overexpression (Fig. 5E, +NFYA) significantly reduced the calcitriol-induced increase in the PCR product generated from DNA immunoprecipitated with the anti-VDR antibodies (Fig. 5F, +NFYA, $P<0.05$), suggesting an interference of the ligand-induced recruitment of the VDR to the distal nVDRE site by NFYA.

In transactivation assays, we transiently transfected the NFYA expression plasmid along with pSG5-VDR and the full-length *ER* promoter–reporter construct (−3500 to +230 *ER* promoter-luc) into the ZR75-1 and MCF-7 cells and measured reporter activity following calcitriol treatment. As expected, calcitriol treatment resulted in a repression of reporter activity (~55%) in the vector-transfected ZR75-1 cells (Fig. 6A). Co-transfection of the WT NFYA expression plasmid did not stimulate the basal promoter activity. However, NFYA co-expression greatly reduced the calcitriol-mediated suppression of *ER* promoter activity. However, a significant calcitriol-mediated suppression of reporter activity still remained when a mutant form of NFYA was co-expressed (Fig. 6A). Similar results were observed when the effect of NFYA overexpression on *ER* promoter activity was examined in the MCF-7 cells (Fig. 6B). We also carried out western blot analysis to determine the changes in *ER* protein levels due to calcitriol treatment in the ZR75-1 cells (Fig. 6C) and MCF-7 cells (Fig. 6D) transfected with vector, WT NFYA, and mutant NFYA expression plasmids. The data were similar to the changes observed in *ER* promoter activity. Co-expression of the WT NFYA but not of the mutant NFYA or vector abolished the calcitriol-induced suppression of *ER* protein levels (Fig. 6C and 6D). These data indicate that although NFYA by itself did not act as a positive regulator of *ER* transcription in this experimental setting, its co-expression resulted in the loss of the suppression of *ER* gene expression by calcitriol, suggesting that it probably competes for binding to the distal nVDRE.

Discussion

The factors and mechanisms that control *ER* levels are important in determining the magnitude of estrogenic actions in BCa cells. Studies conducted in our laboratory (Swami *et al*. 2000) and other laboratories (James *et al*. 1994, Simboli-Campbell *et al*. 1997, Stoica *et al*. 1999)

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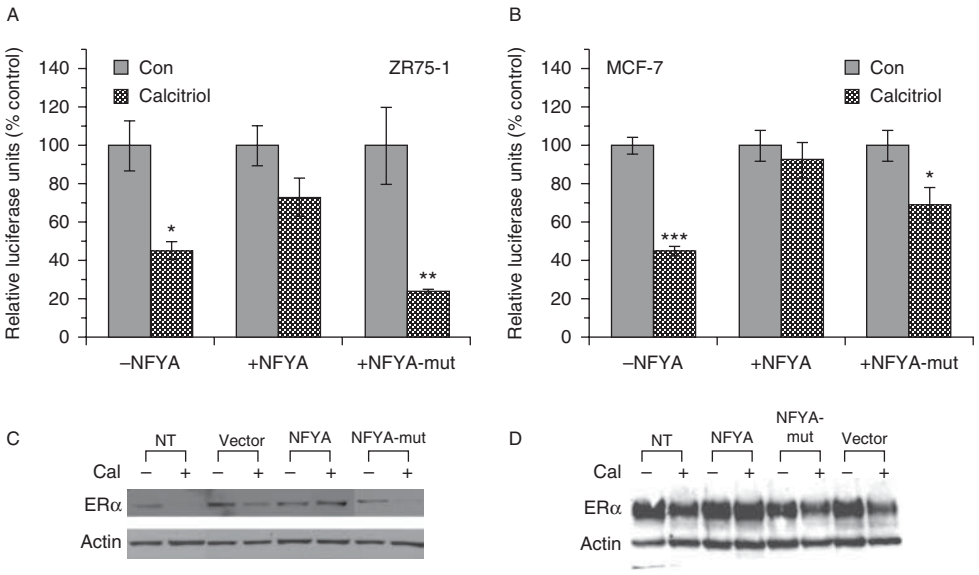

Figure 6

Interference of NFY with calcitriol suppression of *ER* promoter activity and *ER* expression. ZR75-1 (A) and MCF-7 (B) cells were transiently transfected with a WT *ER* promoter construct (~3.5 bp) in the absence or presence of pSG5-NFYA or pSG5-NFYA-mut plasmids as described in the Subjects and methods section. The cells were treated with a vehicle (Con) or calcitriol (100 nM) for 24 h and luciferase activity was measured. Relative luciferase activity in the calcitriol-treated cells was compared with that of the vehicle-treated controls in each transfection that was set at 100%. Data represent means ± S.E.M. of at least four independent transfections, performed in triplicate. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ when compared with their respective controls. (C) ZR75-1 cells and (D) MCF-7 cells are representative western blots showing *ER* expression in the presence or absence of NFYA overexpression. Briefly, cells transiently transfected with the pSG5-NFYA or pSG5-NFYA-mut expression vectors were treated with a vehicle or calcitriol (100 nM) for a period of 48 h after transfection. Empty vector-transfected (Vector) cells and non-transfected (NT) cells were similarly treated and used as negative controls in the experiment. At the end of treatment, the cells were processed for western blot analysis as described in the Subjects and methods section.

have demonstrated that calcitriol downregulates *ER* expression in human BCa cells. We have shown that by decreasing *ER* levels, calcitriol causes a significant attenuation of estrogen-mediated bioresponses in MCF-7 cells, including reduction of estrogen stimulation of cell growth (Swami *et al.* 2000). The mechanism of calcitriol regulation of *ER* expression appears to be a direct repression of *ER* transcription by calcitriol (Stoica *et al.* 1999, Swami *et al.* 2000). In this study, we have further analyzed the mechanism of the transrepression of the *ER* gene by calcitriol.

The transcriptional regulation of target genes is accomplished by the binding of calcitriol binding to the VDR, which after heterodimerization with RXR binds to specific VDREs located in the promoter regions of target genes as well as to distant enhancers. VDREs typically consist of a direct repeat (DR) of two hexameric core binding motifs separated by a spacer most often consisting of three (DR3) and sometimes four (DR4) nucleotides (Carlberg 2003, Haussler *et al.* 2011, Pike *et al.* 2011*b*). The VDR has also been shown to bind to some unconventional VDREs such as the DR-type VDRE with a larger spacer or an inverted palindromic arrangement (Carlberg 2003). In the case of transcriptional repression, however, direct VDR DNA binding or formation of a heterodimer with RXR may not always be required and the mechanism of repression may be target gene specific. For example, the repression of 25-hydroxyvitamin D-1α-hydroxylase (*CYP27B1*) gene transcription in the kidney by calcitriol involves the inhibition by the VDR of the actions of the transcription factor vitamin D interacting repressor, which is essential for regulating the basal CYP27B1 expression (Murayama *et al.* 2004). CYP27B1 repression has also been shown to involve calcitriol-induced epigenetic changes such as DNA methylation (Kim *et al.* 2007).

The current study and that of Stoica *et al.* (1999) have demonstrated the presence and functionality of two nVDREs in an ~3.5 kb promoter region of the *ER* gene. Recent genome-wide approaches have revealed that calcitriol regulation of target genes can be orchestrated not only by multiple regulatory regions located proximal to the promoters but often by many distal enhancers located several hundreds of kilobases away in either

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intergenic regions surrounding the transcription units or within the introns themselves (Pike & Meyer 2010, Pike 2011, Pike *et al*. 2011*a*). However, the current study shows that together the functional activities of the two nVDRE sites within the ~3.5 kb *ER* promoter region recapitulate the magnitude of the downregulation of *ER* expression observed in BCa cells following calcitriol treatment. These VDRE sites are referred to as the proximal nVDRE located at −94 to −70 bp with reference to the P1 start site and the distal nVDRE further upstream at −2488 to −2473 bp in the *ER* promoter. The proximal nVDRE, identified and characterized by Stoica *et al*. (1999), is an imperfect palindrome separated by 13 nucleotides, which maps in the region of a CTF/NF1 site in the *ER* promoter. The current study reports the presence and analysis of another upstream nVDRE (distal). The functionality of these nVDREs is strongly supported by the fact that these sequences confer calcitriol responsiveness both in the natural *ER* promoter and in a heterologous promoter setting. The individual proximal and distal nVDRE sites appear to contribute equally to the negative regulation of the *ER* gene by calcitriol as demonstrated by the fact that a heterologous construct containing both the nVDRE sites exhibited an ~50% decrease in reporter activity, recapitulating the magnitude of suppression reported by the measurement of *ER* mRNA and protein levels in BCa cells (Swami *et al*. 2000). The specific binding of the VDR to these nVDRE sites was demonstrated by EMSA. The addition of the anti-VDR and anti-RXR antibodies revealed the inhibition of protein–DNA complex formation in EMSA at the distal nVDRE site. Furthermore, the results of the ChIP assay demonstrated that calcitriol recruits the VDR to the distal nVDRE site in the context of the native *ER* promoter in BCa cells and that RXRα is also bound to this site most probably as a heterodimer with the VDR. Mutations in the 5′ half-site but not in the 3′ half-site of the distal nVDRE abolished the suppressive effect of calcitriol in transactivation assays and inhibited complex formation in the EMSA, suggesting that the 5′ hexameric sequence is required for calcitriol suppressor activity via the distal nVDRE.

Competition for binding with factors that stimulate transcription has been implicated in the repressive effects of calcitriol on the promoters of several target genes such as rat bone sialoprotein (Kim *et al*. 1996), runt-related transcription factor 2 (*RUNX2*/*Cbfa1*) (Drissi *et al*. 2002), interleukin 2 (Alroy *et al*. 1995), and human parathyroid hormone (h*PTH*; Koszewski *et al*. 2004, Jaaskelainen *et al*. 2005). The CCAAT box is one of the most typical elements for transcriptional activation. NFY is one among the different proteins that bind to CCAAT sequences on many promoter regions and recruit RNA polymerase II to these promoters (Kabe *et al*. 2005). Recently, NFY has been implicated in the positive regulation of $p27^{Kip1}$ promoter by calcitriol (Inoue *et al*. 1999, Huang *et al*. 2004) as well as in the suppression of the h*PTH* promoter by calcitriol (Koszewski *et al*. 2004, Jaaskelainen *et al*. 2005). Two consensus NFY binding sites have been identified in the h*PTH* promoter, and the repressive effect of calcitriol on h*PTH* gene transcription may involve the displacement of NFY binding to its proximal site by the VDR–RXR heterodimer (Koszewski *et al*. 2004). Cooperation between NFY bound to both the distal and proximal consensus sites causes synergistic transactivation of h*PTH* and calcitriol appears to attenuate this effect by disrupting NFY binding to its proximal consensus site (Koszewski *et al*. 2004). We have found a partial NFY binding site (CAAT) at −2486 to −2482 bp present within the sequences of the distal nVDRE on the *ER* promoter (−2488 to −2473 bp). This finding prompted us to investigate the possibility that the calcitriol-mediated repression of *ER* transcription is at least in part due to the competitive binding of the VDR to the NFY site and thereby attenuates the transcriptional activity of NFY.

Our analysis of the role of NFY in calcitriol-mediated transrepression of *ER* revealed several interesting findings. Our ChIP data demonstrate that endogenous levels of NFY bind to the distal nVDRE site in both ZR75-1 and MCF-7 cells. Although the data obtained from the MCF-7 cells suggest that calcitriol treatment, probably through the recruitment of the VDR to the distal nVDRE site, reduces endogenous NFY binding to this site, we did not detect this in the ZR75-1 cells. NFY overexpression in the MCF-7 cells clearly reduced calcitriol-induced VDR recruitment to this site. In transactivation assays, unlike the case of the h*PTH* promoter, the activity of which was robustly stimulated by NFY expression, we did not find a stimulation of *ER* promoter activity even when NFYA was overexpressed in either the ZR75-1 or MCF-7 cells. However, the overexpression of the WT NFYA but not the mutant NFYA resulted in the loss of calcitriol-mediated suppression of *ER* promoter activity and of *ER* protein expression in these cells. NFY has a stringent requirement for the CCAAT sequence and its flanking nucleotides for efficient DNA binding. A majority of the NFY binding sequences have the intact CCAAT core. However, in the case of a few NFY binding sites with a one-nucleotide difference in the core, a much lower DNA binding affinity of NFY has been reported

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(Mantovani 1999). It is possible that the lack of the intact CCAAT core sequence within the distal nVDRE might have contributed to less-than-optimal binding of NFY to the distal nVDRE site and the absence of a positive stimulation of *ER* transcription by NFY. It is also possible that other NFY binding sites that might be present further upstream were lacking in the *ER* promoter constructs that we tested, contributing to the absence of stimulation of the basal promoter activity by NFY. Our data suggest that even though we did not observe positive regulator activity, NFYA did bind to its putative binding site present within the distal nVDRE and inhibited the recruitment of the VDR complex to the distal nVDRE site, thereby reducing the repressive effect of calcitriol on *ER* transcription. It is interesting to note that as reported by Stoica *et al*. (1999) the proximal nVDRE also overlaps a potential binding site for the CCAAT box binding transcriptional stimulator CTF/NF1. However, it should be emphasized that when either the distal or proximal nVDRE was placed upstream of the SV40 promoter in a heterologous construct, it still acted independently as a negative response element, indicating that *ER* repression by calcitriol may not be solely due to interference with the stimulatory activity of positive factors such as NFY and CTF/NF1.

Calcitriol inhibits the proliferation of BCa cells through a variety of mechanisms and suppresses tumor growth in animal models of BCa (Deeb *et al*. 2007, Krishnan & Feldman 2011, Welsh 2011). We have shown that calcitriol acts as a SAM, decreasing aromatase expression in BCa cells and the breast adipose tissue surrounding a breast tumor, thereby reducing the local production of estrogens within the breast (Krishnan *et al*. 2010*b*, Swami *et al*. 2011, 2012). In addition to suppressing estrogen synthesis, the current study demonstrates that calcitriol also transrepresses *ER* through the nVDRE elements present in the *ER* promoter, leading to a decrease in the magnitude of estrogenic bioresponses (Swami *et al*. 2000). The combined actions of calcitriol to inhibit both estrogen synthesis and estrogen signaling through the *ER* lead to the diminution of the proliferative stimulus for BCa growth provided by estrogens. These observations raise the possibility that calcitriol may be useful in the prevention and/or treatment of ER+ BCa.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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