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Original Article

Calcitriol induces estrogen receptor α expression through direct transcriptional regulation and epigenetic modifications in estrogen receptor-negative breast cancer cells

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Abstract: Patients with estrogen receptor (ER) α-negative breast tumors have a poor prognosis and are not suitable for hormone therapy. Previously, we demonstrated that calcitriol, the active metabolite of vitamin D, induces ERα expression and re-establishes the response to antiestrogens in ER-negative breast cancer cells. However, the mechanisms involved in this process have not been elucidated. Therefore, the present study was undertaken to investigate the mechanisms implicated in the calcitriol-induced ERα expression in ER-negative breast cancer cells. Using EMSA and ChIP assays, we found that the calcitriol/vitamin D receptor (VDR)/retinoic X receptor (RXR) complex binds to putative vitamin D response elements (VDREs) in the ERα gene promoter region. In addition, we established by a fluorometric assay that calcitriol decreased DNA-methyltransferase and histone deacetylase activities. Flow cytometry and qPCR analyses showed that co-treatment of calcitriol with inhibitors of the histone deacetylase and DNA methyltransferase, and genistein significantly increased ERα expression, compared to that observed with the compounds alone. In conclusion, the calcitriol-dependent ERα induction in ER-negative breast cancer cells results from binding of the VDR-RXR complex to VDREs in the ERα gene promoter region, including the downregulation of enzymes with chromatin-remodeling activities. These results may bring forth novel mechanistic knowledge into the actions of calcitriol in ERα-negative breast cancer.

Keywords: Calcitriol, ERα, VDR, epigenetic modulator, ER-negative breast cancer

Introduction

The estrogen receptor (ER) α plays a critical role in the pathogenesis, progression, and treatment of breast cancer [1]. Approximately two-thirds of breast cancer tumors overexpress ERα, and thus, appropriately respond to antiestrogen compounds. By contrast, the lack of ER expression in tumors has been associated with early recurrence, development of metastasis, high tumor grade, and poor prognosis [2-4]. The repression mechanisms of ERα in breast tumors have been linked to hyperactivation of the mitogen-activated protein kinase-signaling pathway or miRNAs gene expression deregulation [5-8]. Similarly, other studies in the field have indicated that epigenetic events play a crucial mechanistic role in ERα downregulation. Evidence supporting this fact is that treatment with inhibitors of histone deacetylase (HDAC) and DNA methyltransferase (DNMT), as well as with the soybean genistein, can re-establish ERα gene expression in ERα-negative breast cancer cells [9-15].

In addition, we have demonstrated that calcitriol, a natural compound, was able to induce the ERα protein expression. The functionality of this receptor was confirmed by upregulating the expression of an estrogen inducible gene. Interestingly, the calcitriol-induced ERα restored the inhibitory proliferative ability of the ER antagonists in ERα-negative breast cancer cells [16].

Epidemiological studies have demonstrated the association between the high incidence of ER-negative and triple-negative breast tumors with low calcitriol levels, the active form of vitamin D [17, 18]. Indeed, the vitamin D receptor (VDR) knock-out mice had a higher incidence of ER- and progesterone receptor-negative mammary tumors when compared with their wild-type counterparts after treatment with a carcinogen [19]; suggesting that vitamin D favors a more differentiated tumor phenotype. In this regard, besides calcium homeostasis and bone mineralization, calcitriol regulates many physiological processes, including important anticancer effects [20]. Most of these actions are mediated via activation of the nuclear VDR, which binds to vitamin D response elements (VDREs) and regulates the transcription of target genes [21]. VDREs consist of two hexameric sequences, commonly arranged as direct repeats with variable numbers of spacing nucleotides. These response elements are designated DR3 or DR4-type depending on the number of intermediate nucleotides [22, 23].

Recent studies have shown that the calcitriol anticancer properties may also involve epigenetic control mechanisms related to histone modifications and DNA methylation [24, 25]. Supporting this, it has been shown that the treatment of breast cancer cells with vitamin D increased phosphatase and tensin homolog (PTEN) expression, a tumor suppressor gene, through the reduction of methylation levels within the gene promoter region and downregulation of DNMT1 gene expression and enzymatic activity [26].

Therefore, this study was designed to get an insight into the mechanism by which calcitriol induces ERα expression in ERα-negative breast cancer cells. The results give evidence that calcitriol/VDR/RXR complex binds to VDREs in the ERα gene promoter region and decreases DNMT and HDAC enzymatic activities in these cancer cells. In addition, ERα gene and protein expression was significantly augmented to a greater extent in cells co-treated with calcitriol and the epigenetic modulators than in the cells treated in the presence of each compound alone. The changes in DNA methylation and histone modification, as well as, the direct regulation of gene transcription by calcitriol successfully induced ER expression.

Material and methods

Reagents

Calcitriol, trichostatin A (TSA), 5-aza-2'-deoxycytidine (AZA) were purchased from Sigma (St. Louis, MO, USA) and genistein (GE) from Calbiochem (Millipore MA, USA). Cell culture media was from Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Hyclone Laboratories Inc. (Logan, UT, USA). Trizol and the synthetic oligonucleotides for quantitative real-time polymerase chain reaction (qPCR) were acquired from Invitrogen (Thermo Fisher Scientific Inc, MA, USA). The TaqMan Master Reaction, probes, plates, and reverse transcription (RT) system were all from Roche (Roche Applied Science, IN, USA).

Cell culture

The SUM-229PE cells (RRID:CVCL_5594, Asterand, San Francisco, CA) were cultured in Ham's F-12 media supplemented with 5% heat-inactivated-FBS, 10 mM HEPES, 1 μg/ml hydrocortisone, 5 μg/ml insulin, 1% antimycotic, and cultured under standard cell culture conditions, 5% CO_2 at 37°C. The HeLa, SK-BR-3, HCC1806, and HCC38 (RRID: CVCL_0030, CVCL_0033, CVCL_1258, and CVCL_1267, respectively, ATCC, Manassas, VA, USA) established cell lines were cultured and maintained following indications from the supplier.

DMEM-F12 media supplemented with 5% charcoal-stripped-heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin was used for the experimental procedures. All experiments were carried out between the 3rd to 5th passages after thawing.

In silico screening for VDREs in ERα gene promoter region

Analysis for detection of putative regulatory sequences present in the human ERα gene (NCBI Reference Sequence: NG_008493.2)

Table 1. Oligonucleotides used in EMSAs

Double-stranded probe name	Complementary sequences
S1	5'-GGGAGGTTGAGGCGGGTGGATCACC-3'
	5'-GGTGATCCACCCGCCTCAACCTCCC-3'
S4	5'-CAGGAGAGGGTAGGGAGGGAAGCCA-3'
	5'-TGGCTTCCCTCCCTACCCTCTCCTG-3'
S5	5'-GGACAAGGCTCACCAAGATGAGTTT-3'
	5'-AAACTCATCTTGGTGAGCCTTGTCC-3'
S6	5'-AGAAAGTGGTCAAGAGGTGGATCCA-3'
	5'-TGGATCCACCTCTTGACCACTTTCT-3'
S7	5'-ATCCTAGCCCAAGTGAACCGAGAAG-3'
	5'-CTTCTCGGTTCACTTGGGCTAGGAT-3'
DR3	5'-AGCTTCAGGTCAAGGAGGTCAGAGAGCT-3'
	5'-AGCTCTCTGACCTCCTTGACCTGAAGCT-3'
DR4	5'-GAAGTTCAGCGAAGTTCA-3'
	5'-TGAACTTCGCTGAACTTC-3'

The putative VDREs are underlined.

within a region encompassing approximately 5000 bp upstream from the transcription start site [27] was performed using MatInspector software (Genomatix, www.genomatix.de) [28].

Electrophoretic mobility shift assay (EMSA)

Following the manufacturer's instructions, nuclear extracts were obtained from SUM-229PE and HeLa cells using the Cell Nuclear Extraction Kit (Sigma, St. Louis, Missouri, USA). Protein concentrations were determined using the Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA, USA). Nuclear extracts (5 μg) were incubated in the absence or presence of calcitriol (1×10^{-7} M) for 15 min. EMSAs were performed following Gel Shift Assay System (Promega) instructions. ^{32}P end-labeled probes containing the putative VDREs from the human ERα gene promoter (S1, S4, S5, S6, and S7) were synthesized, and canonical DR3- and DR4-type VDRE were used as positive controls [29] (**Table 1**). Per reaction, 17.5 fmol of each labeled probe were added to the nuclear extracts. For supershift assays, pre-incubation of 1 μg of specific antibodies against VDR (sc-13133, Santa Cruz Biotechnology, USA) or the retinoid X receptor (RXRα/β/γ) (sc-774 Santa Cruz Biotechnology) with samples were performed for 1 h, before incubation of the labeled probes with nuclear extracts. For competition studies, a 200-fold excess of the unlabeled double-stranded competitor oligonucleotide was added for each reaction. The DNA-protein complexes were loaded on 4% non-denaturing polyacrylamide gels. Subsequently, the gels were transferred onto filter paper, dried, and exposed to a film developed by autoradiography.

Chromatin immunoprecipitation (ChIP)-quantitative PCR assays

SUM-229PE cells were treated with calcitriol (1×10^{-7} M) or vehicle for 24 h. The ChIP assay was performed according to EZ Chromatin Immunoprecipitation Assay Kit (Millipore, MA, USA). Cross-linking of was carried out in 1% formaldehyde for 7 min at room temperature. To stop the reaction, 2.5 M glycine was added for 5 min. The cells were washed and harvested with cold PBS containing Protease Inhibitor Cocktail II. After the samples were centrifuged, the pellets were resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1) and sonicated for 5 cycles of 20 s each (Ultrasonic Processor model GEX500, Geneq Inc., Montreal, Canada). The cellular debris was eliminated by centrifugation, and salmon sperm DNA/protein G-agarose was added at 4°C for 1 h. The antibodies against VDR, RXR RXRα/β/γ (sc-13133 X and sc-774 X, respectively; Santa Cruz Biotechnology) or nonspecific IgG (sc-2025, Santa Cruz Biotechnology) were added to chromatin supernatants and incubated at 4°C overnight with shaking. For immunoprecipitation, salmon sperm DNA/protein G-agarose was added to samples at 4°C overnight. After, several washes were performed with 1 ml of the following buffers: low-salt wash buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, and 2 mM EDTA), high-salt wash buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 0.1% SDS, 1% Triton X-100, and 2 mM EDTA), LiCl wash buffer (250 mM LiCl, 20 mM Tris-HCl pH 8.1, 500 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 2 mM EDTA), and two washes with TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). To reverse cross-link, 5 M NaCl was added to DNA-protein complexes and the input samples were incubated at 65°C over-

night. Then, the samples were incubated with proteinase K for 1 h at 45°C. To purificate the DNA samples we used the High Pure PCR Product Purification Kit (Roche, Switzerland), and subsequent PCR reaction was carried according to the following protocol: Initial denaturation at 94°C for 2 min followed by 35 cycles each with denaturation at 94°C for 25 s. Annealing at 50-57°C (depending on sequences) for 45 s, and extension at 72°C for 2 min. Final extension at 72°C for 7 min. The primer sequences used in ERα ChIP analysis were: S1-F, CAGACTTAAGCCTCAAGATTG; S1-R, TAAGTAGCTGGGATTACAGGT; S4-F, CATGTTTGAGAATGGTGACTC; S4-R, GGAAAGTATACTGTGCTACGC; S5-F, GGCGTAGCACAGTATACTTTC; S5-R, CTGCAATTGTTCACTATTGAC; S6-F, CACTCTAGGAGCACCTCAGTA; S6-R ATAGCTCAGTTGACTGCTTTC; S7-F, GACGCATGATATACTTCACCT; S7-R, CATACCAAACATAACCTCAGG. DNA was analyzed by 2% agarose gel electrophoresis.

Real time RT-PCR

The cells were treated with calcitriol (1×10^{-7} M), AZA (6×10^{-7} M), TSA (1.4×10^{-6} M), GE (2.5×10^{-5} M), their combination, or the vehicle alone (0.1% ethanol or DMSO) during 24 h. Afterward, Total RNA was isolated from samples by using Trizol reagent and was copied to cDNA with the transcriptor RT system. Real-time PCR was performed using the LightCycler 480 from Roche (Roche Diagnostics, Mannheim, Germany) according to the following protocol: Initial DNA denaturation at 95°C for 10 min, proceeded by the primers' annealing and DNA synthesis using Taq DNA polymerase by 45 amplification cycles consisting of 10 s at 95°C, 30 s at 60°C, and 1 s at 72°C. The following oligonucleotides were used: ERα-F, CCTTCTTCAAGAGAAGTATTCAAGG; ERα-R, GTTTTTATCAATGGTGCACTGG; DNMT-3A-F, ACTACATCAGCAAGCGCAAG; DNMT3A-R, CACAGCATTCATTCCTGCAA; HDAC1-F, CGGTGCTGGACATATGAGAC; HDAC1-R, TGGTCCAAAGTATTCAAAGTAGTCA. The gene expression of RPL-32 (ribosomal protein L32) was used to normalize of data: RPL32-F, GAAGTTCCTGGTCCACAACG; RPL32-R, GAGCGATCTCGGCACAGTA.

Analysis of ERα protein levels

SUM-229PE cells were incubated in the absence or presence of calcitriol (1×10^{-7} M, during 48 or 72 h), TSA (1.4×10^{-6} M, 12 h before finishing the 48 h of treatment), or AZA (6×10^{-7} M, 24 h before finishing the 72 h of treatment) alone or combined (calcitriol with epigenetic modulators). ERα protein levels were analyzed by flow cytometry. After treatment, cells were harvested and fixed with ethanol 70% (v/v). Then, the samples were washed and incubated with 0.5% v/v Triton X-100 for 15 min. Next, cells were incubated with mouse anti-ERα monoclonal antibody (1:200, Santa Cruz) for 1 h. For detection of ERα, cells were washed and incubated with Alexa-488 rabbit anti-mouse mAb (Molecular Probes, Eugene OR) at room temperature for 30 min. After washing, cells were acquired on a FACSCanto II Flow cytometer (Beckton Dickinson, San Jose, CA, USA). To gate out cell aggregates, a forward scatter area (FSC-A) vs. forward scatter height (FSC-H) was done. Next, tumor cells were selected using a FSC-A vs. SSC-A dot plot graph, excluding cell debris. A total of 10,000 events were acquired from the gate of tumor cells, and the expression of ERα molecule was measured using the mean fluorescence intensity (MFI) from the corresponding histograms.

HDAC activity

HDAC enzymatic activity was evaluated using an HDAC assay kit (Abcam, Cambridge, UK). Nuclear extracts (10 μg) from SUM-229PE cells were prepared with the HDAC assay substrate and incubated in the absence or presence of calcitriol (1×10^{-7} M), TSA (1.4×10^{-6} M), or its combination at room temperature for 5 min. Fluorescence intensity was measured in a fluorescent microplate reader (BioTek, Winooski, VT, USA) at excitation of 360 nm and emission of 460 nm for 60 min at 1-2 min intervals.

DNMT activity

DNMT activity was evaluated using a DNMT assay kit (Abcam) according to the manufacturer's instructions. Nuclear extract (10 μg) was incubated with DNMT assay substrate and assay buffer. Later, the samples were added with calcitriol (1×10^{-7} M), AZA (6×10^{-7} M), or a combination of both. Then, capture antibody, detector antibody, and enhancer solution were incubated at room temperature for 60 min, 30 min, and 30 min, respectively. The samples were washed, and subsequently, it was added Fluorescence Development Solution and incubated at room temperature for 1-3 min. Fluorescence intensity was measured in a fluo-

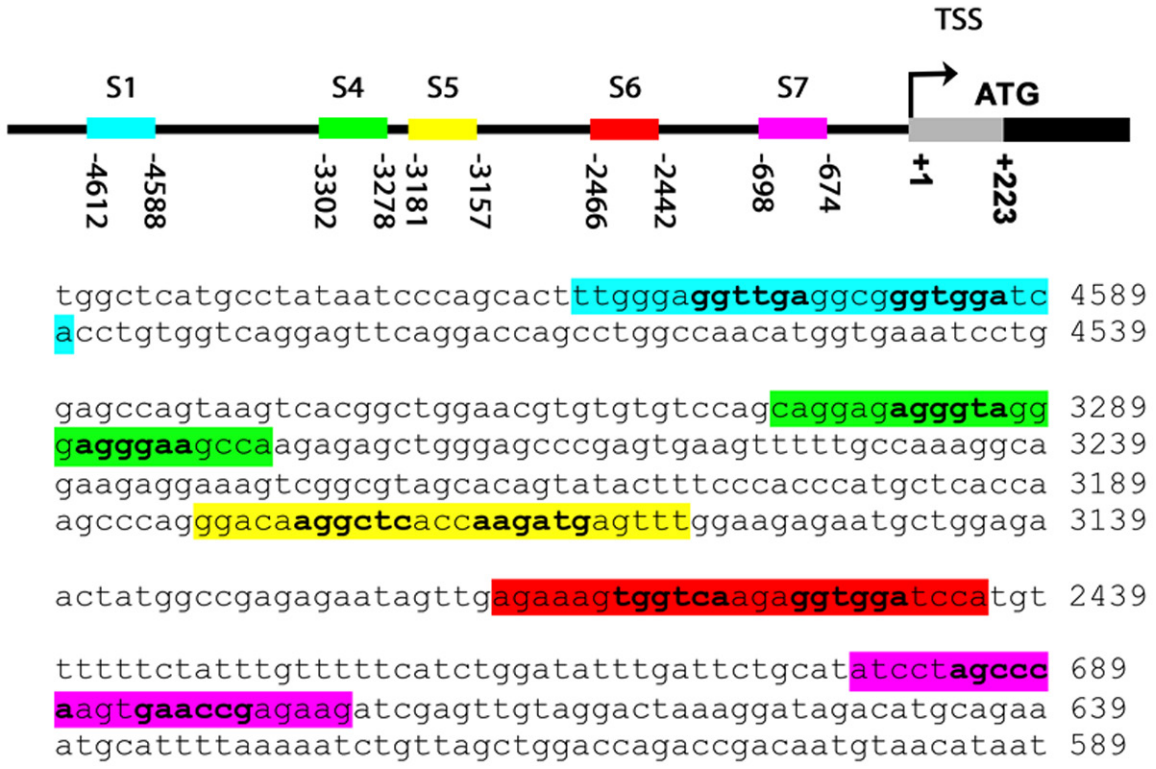

Figure 1. The human ERα gene promoter contains putative vitamin D responsive elements. Schematic representation of VDREs found on ERα gene promotor region. The putative VDREs are highlighted in different colors and the hexanucleotide repeats are in bold. TSS: transcriptional start site, S: sequence.

rescent microplate reader at an excitation of 530 nm and emission of 590 nm within 2-10 min.

Statistical analyses

One-way ANOVA followed by the Student-Newman-Keuls or Holm-Sidak methods was used to multiple comparisons test using a specialized software package (SigmaStat, Jandel Scientific). Data are expressed as the mean ± standard deviation (S.D.). The results were considered significant at $P<0.05$.

Results

The human ERα gene promoter region contains putative VDREs

In order to identify the presence of VDREs in the human ERα gene promoter region, an analysis *in silico* was carried out. Screening the 5000 bp upstream of ERα gene promoter region, using the MatInspector program, identified the presence of seven putative DR1-, DR3-, DR4- and DR5-type VDREs (named S1-S7) (**Figure 1**). The sequence S2, DR1-type, was not analyzed in this study since the matrix similarity was less than the search threshold (<8.0). It is known that the VDR-RXR complex recognizes with greater affinity DR3 and DR4-type elements. Therefore, the S3 sequence, which is a DR5-type, was also discarded. The sequences S4 to S7 corresponded to the DR3-type and the sequence S1 to the DR4-type, both located upstream from the reported transcriptional start site of exon 1B in the ERα gene promoter region [27, 30].

The VDREs in the ERα gene promoter region interact with VDR and RXR

To determine if the sequences identified with MatInspector interact with endogenous VDR, EMSAs were performed. The sequence-specific labeled probes S4 (**Figure 2A**, lane 6), S1, S5, S6, and S7 (**Figure 3A**, lane 2, 9, 13, and 17, respectively) and the nuclear extracts from SUM-229PE cells formed bands, indicating the interaction between the protein and DNA. These bands showed similar size to those

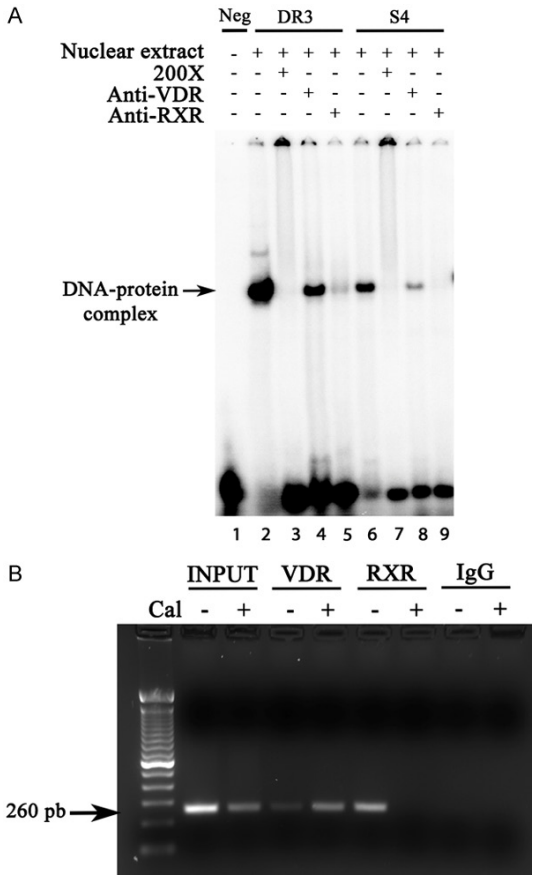

Figure 2. The VDR is recruited *in vitro* and *in vivo* to VDREs in ERα gene promoter region. A. Nuclear extracts of SUM-229PE cells treated with calcitriol (1×10^{-7} M) were incubated with the S4 (VDRE of DR3-type) ^{32}P-labeled sequence-specific. The probe was incubated with VDR or RXR antibodies or an excess of unlabeled specific sequences. Negative control (Neg): ^{32}P-labeled sequence in the absence of nuclear extract. Each blot is representative of at least three experiments. B. ChIP analysis was performed on SUM-229PE cells incubated in the presence of calcitriol (1×10^{-7} M) or ethanol as its vehicle (-) during 24 h. Soluble chromatin was immunoprecipitated with the antibodies against VDR or RXR and subjected to PCR analysis with the appropriate set of primers flanking the S4 in the ERα gene promoter region. A non-specific IgG was used as negative control. INPUT was a positive control (genomic DNA). Results are representative of at least two separate experiments.

formed with consensus DR3 (**Figure 2A**, lane 2; **Figure 3A**, lane 21) and DR4-type VDREs (**Figure 3A**, lane 6) used as positive controls [29]. As depicted, the DNA-protein complexes practically disappeared when nuclear extracts were incubated with a 200-fold excess of unlabeled DR3 (**Figure 2A**, lane 7; **Figure 3A**, lane 10, 14, and 18) or DR4 probes (**Figure 3A**, lane 3). This effect was also observed in positive controls (**Figure 2A**, lane 3; **Figure 3A**, lane 7), confirming the specificity of the DNA-protein interaction. In addition, nuclear extracts were incubated with the labeled sequence-specific probes and DR3 or DR4-type sequences in the presence of antibodies against VDR or RXR. A significant reduction in the formation of DNA-protein complex was observed in the presence of an anti-VDR antibody, except for the sequence-specific labeled probes S7 (**Figure 2A**, lane 8 and **Figure 3A**, lanes 4, 11, 15, and 19). Notably, when using the anti-RXR antibody, no band depicting DNA-protein complex formation was detected, suggesting the complete dissociation of the complex or the total hindrance between the DNA and the transcription factor (**Figure 2A**, lane 9 and **Figure 3A**, lanes 5, 12, 16 and 20). The above data demonstrated the specific binding of the labeled sequences with the endogenous VDR/RXR heterodimer.

Similar results were obtained with the sequence-specific labeled probe S4 and the nuclear extracts from HeLa cells, another ER-negative cell line (**Figure 3B**, lane 6). It should be noted that two bands are observed, presumably due to the production of alternative forms of the receptors, as has been previously observed [31]. The DNA-protein complexes were confirmed by the reduction and disappearance of the band in the presence of the VDR and RXR antibodies, respectively (**Figure 3B**, lane 8 and 9, respectively). These data demonstrated that DNA-protein interaction can be observed in another cell line with ER-negative phenotype.

In order to confirm if VDR was able to bind the VDREs in the ERα gene promoter *in vivo*, ChIP assays were performed. To induce ERα expression, SUM-229PE cells were incubated with or without calcitriol for 24 h. After, the VDRE-containing regions of ERα gene promoter were amplified and analyzed by qPCRs using the sequence-specific primers. **Figure 2B** shows an amplicon of 260-bp PCR obtained from the PCR-amplified region (-3302 to -3278) of the ERα gene promoter (**Figure 1**), using the S4 specific primers. An increase in the relative amount of the amplified PCR product was observed by calcitriol treatment in the presence of the anti-VDR antibody, which indicated

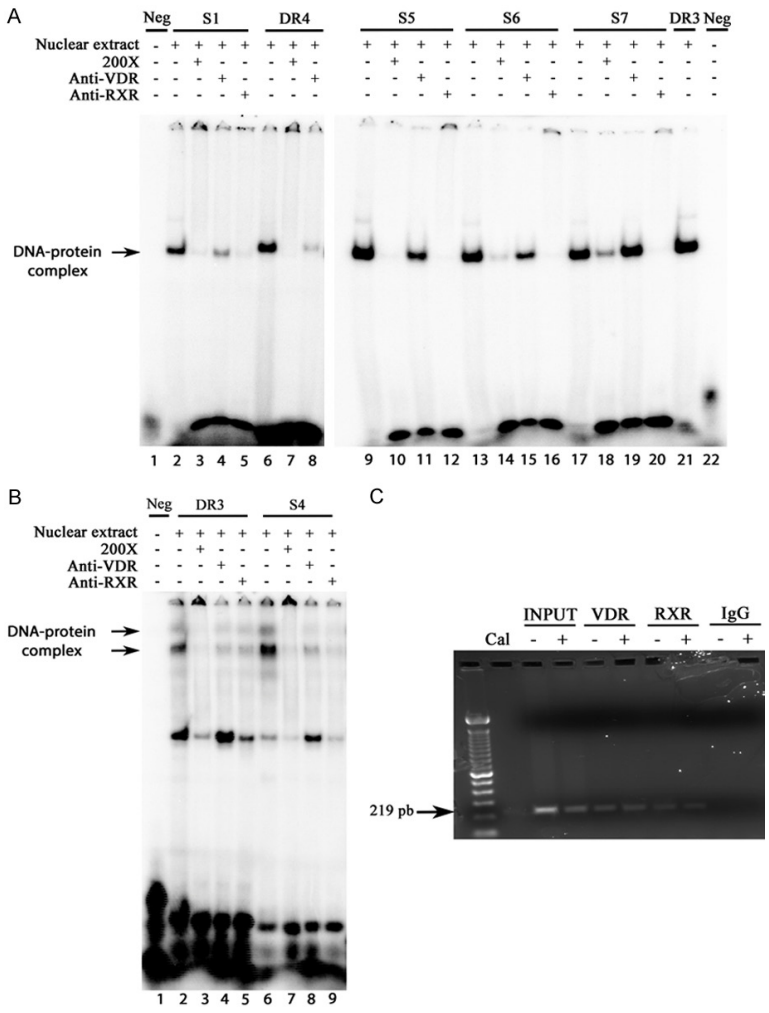

Figure 3. The VDR is recruited to VDREs in ERα gene promoter region. Nuclear extracts of (A) SUM-229PE or (B) HeLa cells treated with calcitriol (1×10^{-7} M) were incubated with the (A) S1 (VDRE of DR4-type), S5-S7 (VDRE of DR3-type) ^{32}P-labeled sequences-specific (B) S4 (VDRE of DR3-type). The probes were incubated with VDR or RXR antibodies or an excess of unlabeled specific sequences. Negative control (Neg): ^{32}P-labeled sequence in the absence of nuclear extract. Each blot is representative of at least three experiments. (C) ChIP analysis was performed on SUM-229PE cells incubated in the presence of calcitriol (1×10^{-7} M) or ethanol as its vehicle (-) during 24 h. Soluble chromatin was immunoprecipitated with the antibodies against VDR or RXR and subjected to PCR analysis with the appropriate set of primers flanking the S5 in the ERα gene promoter region. A non-specific IgG was used as negative control. INPUT was a positive control (genomic DNA). Results are representative of at least two separate experiments.

the association of activated VDR to DNA strands within intact chromatin. Interestingly, this PCR product was not amplified when the anti-RXR antibody was used in homogenates of calcitriol-treated cells. As a control for PCR specificity, we used amplification of input DNA. As expected, the PCR products were not detected in the presence of non-specific IgG. Using the S5 specific primers, we obtained a 219-bp PCR product (**Figure 3C**), comprising the region between -3181 to -3157 of the ERα gene promoter (**Figure 1**). In the 219-bp PCR product, the VDR-RXR complex interaction within the promoter region was confirmed with specific antibodies (**Figure 3C**). VDREs corresponding to S1, S6, and S7 did not form PCR products. These data clearly indicate that the VDR-RXR complex is directly bound to VDREs within the ERα gene promoter region.

Epigenetic mechanisms involved in calcitriol effects on ERα gene and protein expression

Epigenetic modulators such as TSA, AZA, and genistein (GE) contribute to ERα transcriptional regulation through epigenetic mechanisms linked to histone modifications and DNA methylation in ERα-negative breast cancer cells [9, 13, 32]. Considering this, we investigated whether the induction of ERα expression by calcitriol might involve epigenetic mechanisms. For this reason, the expression of ERα was assessed in breast cancer cells treated with calcitriol in the presence or absence of TSA, AZA, or GE. Consistent with previous studies, we demonstrated that the treatment with calcitriol or the epigenetic modulators alone significantly increased ERα gene expression in breast cancer cells with ER-negative phenotype [9, 13, 16] (**Figure 4**). Notedly, the induction of ERα gene expression by calcitriol was observed in different breast cancer cell lines (**Figure 4A**). Further, treatment with calcitriol in the presence of the epigenetic modulators was followed by a significantly higher increase in ERα mRNA than with compounds

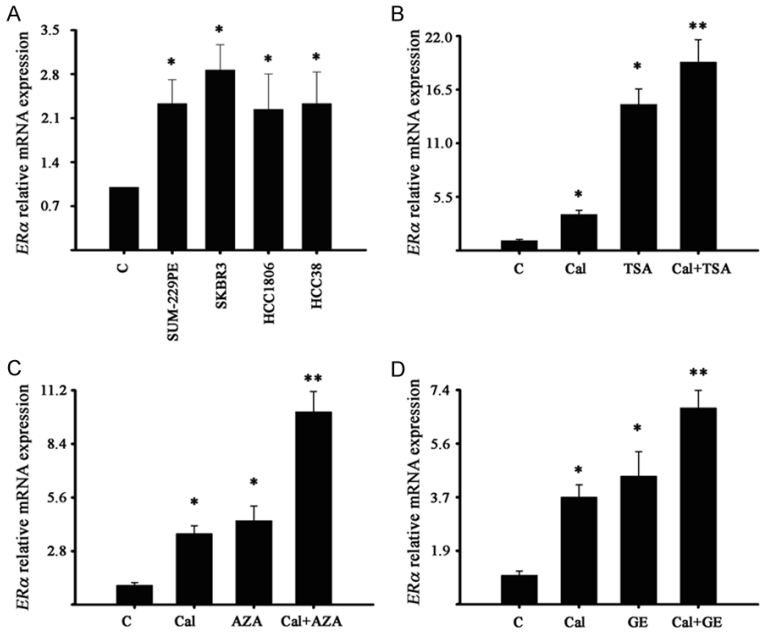

Figure 4. Calcitriol combined with epigenetic modulators induced ERα mRNA expression in a greater to extent than compounds alone in ERα-negative breast cancer cells. (A) Different ER-negative phenotype breast cancer cells were incubated in the absence (C) or presence of calcitriol (Cal, 1×10^{-7} M). (B) SUM-229PE cells were treated as previously described with trichostatin A (TSA, 1.4×10^{-6} M), (C) 5-aza-2'-deoxycytidine (AZA, 6×10^{-7} M), or (D) genistein (GE, 2.5×10^{-5} M) alone or combined for 24 and 48 h. Quantitative real time PCR was performed to measure relative transcription of ERα. Results are shown as the mean ± S.D. of ERα/RPL32 mRNA normalized ratio of three independent experiments per triplicate. Data were normalized to 1 for vehicle-treated cells. *P<0.05 vs. C. **P<0.05 vs. each compound alone.

alone in the SUM-229PE cells (**Figure 4B-D**). To corroborate that the increase of ERα gene expression is also reflected at the protein level, we evaluated ERα protein expression by flow cytometry. For this, we analyzed only the combination of calcitriol with AZA or TSA. In a similar manner as in qPCR studies, we observed a significant induction in ERα protein expression by calcitriol, TSA, or AZA (**Figure 5**). However, the presence of the epigenetic modulator significantly increased calcitriol effects on the expression of ERα at protein level compared to that in the absence of epigenetic compounds (**Figure 5**). These results suggest an important role of calcitriol in ERα reactivation via epigenetic modulation in ERα-negative breast cancer cells.

Calcitriol inhibits DNMT and HDAC activity

Calcitriol-mediated epigenetic effects are linked to histone modifications and DNA methylation [24]. To further study the epigenetic mechanisms involved in calcitriol-induced ERα re-expression in ERα-negative breast cancer cells, we assessed the effect of this hormone on the activity and expression of DNMT and HDAC, two of the main epigenetic regulatory enzymes. **Figure 6** shows that calcitriol treatment did not affect DNMT3A or HDAC1 gene expression. In contrast, the AZA and TSA alone downregulated DNMT3A and HDAC1 mRNA expression levels, respectively, as previously reported [13, 33]. The combined treatment did not modify AZA or TSA effects (**Figure 6A** and **6B**). Afterward, we assessed the effect of calcitriol alone or combined with the epigenetic regulators on total DNMT and HDAC activity. We found that calcitriol and the combined treatments significantly reduced total DNMT and HDAC activity. Both AZA and TSA were used as controls [9, 13] (**Figure 6C** and **6D**). These results suggest that calcitriol stimulates ERα expression through the decrease of HDACs and DNMTs enzymatic activity.

Discussion

Calcitriol actions are mediated by its nuclear VDR, which preferentially acts as a heterodimer with RXR subtypes on specific promoter sequence regions in target genes [34, 35]. Calcitriol exerts its antiproliferative activity in breast cancer cells by multiple mechanisms, including the regulation of gene expression [36, 37]. In fact, several studies have shown that the antiproliferative effects of calcitriol and its analogues on ER-positive human breast cancer cells are mediated through the downregulation of ER expression and disruption of estrogen dependent signaling pathways [38-41]. This transcriptional repression is regulated by the binding of the VDR to two negative VDREs in the ER promoter region of the ER-positive breast cancer cells [42]. In contrast, we have previ-

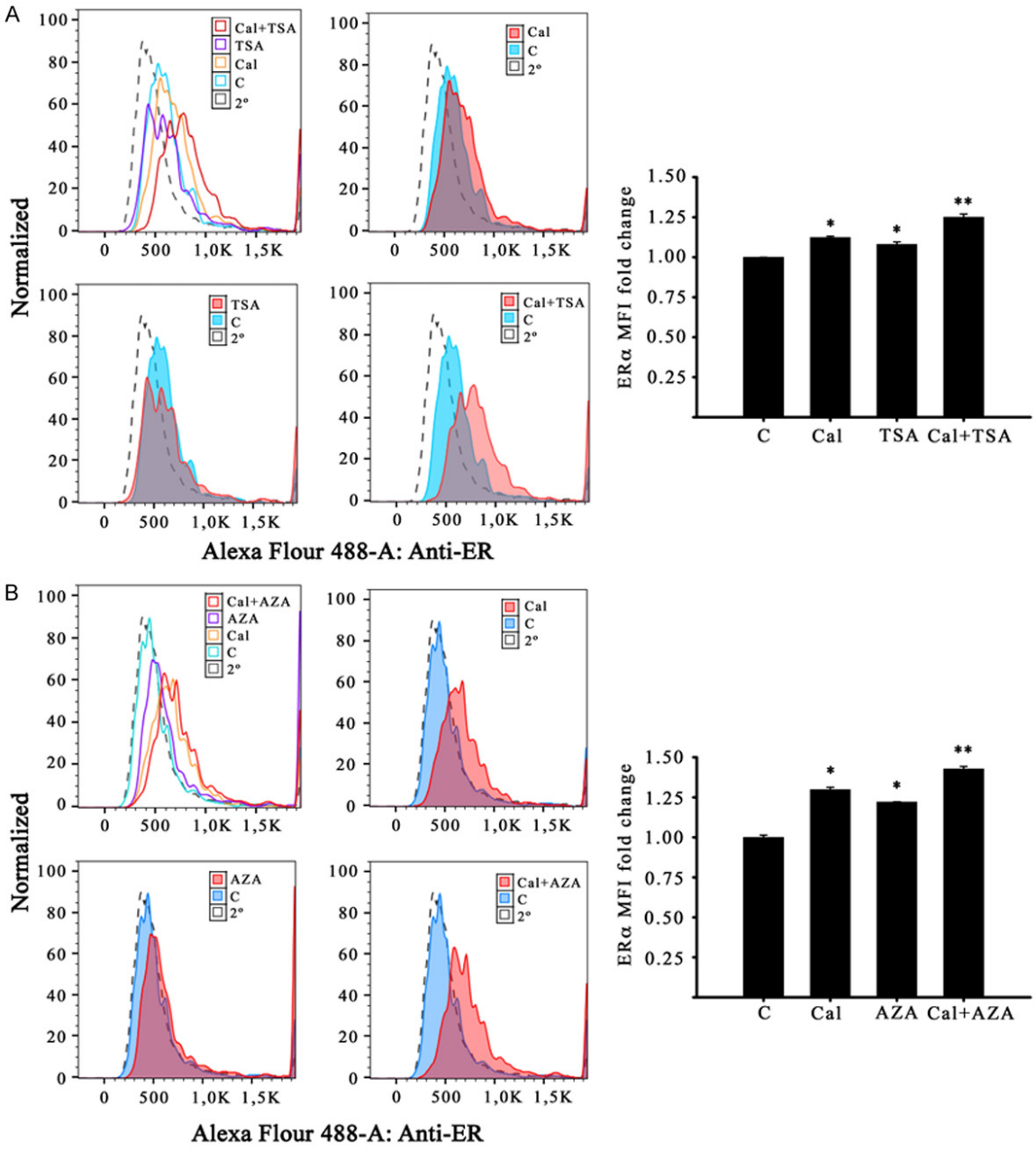

Figure 5. The combined treatment with calcitriol plus the epigenetic modulators increased ERα protein expression levels in ERα-negative breast cancer cells. Flow cytometric analysis of ERα protein was performed after treatment of cells with calcitriol (Cal, 1×10^{-7} M), and (A) trichostatin A (TSA, 1.4×10^{-6} M, during 48 h) or (B) 5-aza-2'-deoxycytidine (AZA, 6×10^{-7} M, during 72 h) alone or combined. Mean fluorescence intensity (MFI) values for the expression of ERα were obtained from the corresponding histograms. Dashed lines indicate the secondary antibody (2°), used as an internal control of MFI. Vehicle-treated cells (C) were arbitrarily given a value of 1 (bar chart). Results are the mean ± S.D. of three different experiments. *P<0.05 vs. C, **P<0.05 vs. each compound alone.

ously demonstrated that calcitriol induced the expression of functionally active ERα in both primary and established ERα-negative breast cancer cell lines [16], which results in the reestablishment of the sensitivity to an antiestrogen treatment. However, the mechanisms by which calcitriol induces the ER expression in ER-negative breast cancer cells still remain unexplored. In the present study, we demonstrated by EMSA that activated VDR was able to bind the putative VDREs identified by an *in silico* analysis. Furthermore, the results of the *in*

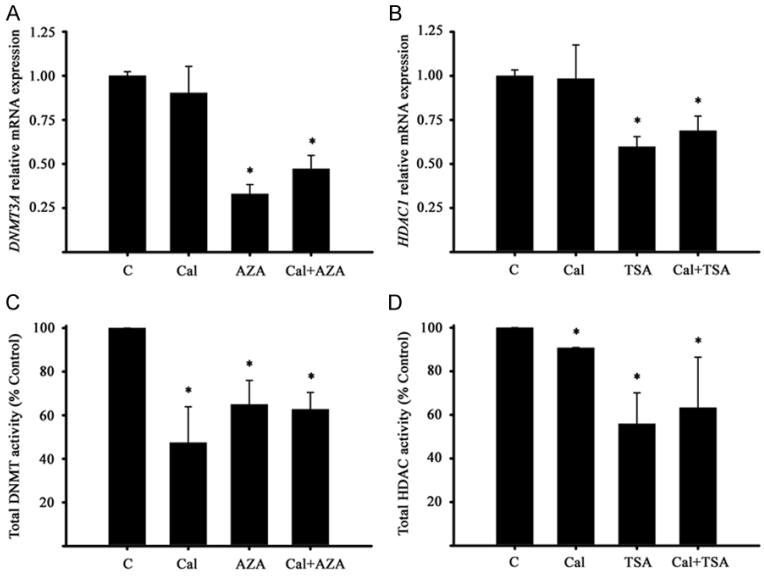

Figure 6. Calcitriol regulated DNMT and HDAC enzymatic activity in ER-negative SUM-229PE breast cancer cells. The cells were treated in the absence (C) or presence of calcitriol (Cal, 1×10^{-7} M), trichostatin A (TSA, 1.4×10^{-6} M), and 5-aza-2'-deoxycytidine (AZA, 6×10^{-7} M) alone or the combination of calcitriol with each epigenetic modulator for 24 and 48 h. (A) DNMT3A and (B) HDAC1 gene expression were determined by quantitative real time PCR. Total (C) DNMT and (D) HDAC activity was evaluated by using a Fluorometric Assay. Each bar represents the mean ± S.D. of three independent experiments. *P<0.05 vs. C.

vivo ChIP experiments confirmed the recruitment of VDR and RXR to the putative S4 and S5 sequences. This could be explained by the sum of the following factors: the preferential binding of VDR/RXR complex to DR3 type sequences and the fact that S4 and S5 DNA fragments share high homology with the consensus sequence. Interestingly, it was not possible to amplify PCR products using the S4 specific primers when incubating the lysates of calcitriol-treated cells with the RXR antibody in the ChIP assay, indicating the no co-immunoprecipitation of this transcription factor with the S4 sequence in the presence of calcitriol. This may be due to a cyclic dynamic process of activated VDR/RXR-DNA binding, which results in intermittent specific localization of the calcitriol-transcription factors complex to this promoter in space and time. We found supporting previous evidence to these results in the 5-Lipoxygenase gene transcription induced by calcitriol, where the PCR product obtained from the ChIP with RXR changed at different times and regions within the promoter [43]. Another study also showed the absence of PCR product amplification when ChIP analysis in the presence of calcitriol and RXR antibody was performed in the ether à go-go gene promoter [44]. Alternatively, this observation could also mean that without the ligand, RXR is located at this VDRE sequence in the ER promoter, and when the ligand is present, a conformational change occurs within the VDR-RXR heterodimer that impede this region to co-immunoprecipitate with the RXR. Probably, since the binding of the VDR to its ligand results in a conformational change that influences its RXR heteropartner, it is possible that the RXR-antibody that we used may no longer has access to its corresponding binding site; therefore, impeding immunoprecipitation [45].

DNA methylation and histone deacetylation promote a compacted chromatin conformation, resulting in the transcriptional inactivation of gene expression [46]. Specifically, ERα gene expression is modulated by cis-regulatory elements located upstream from the initiation site and methylation of these sequences resulting in a loss in the transcription of the ERα gene [47, 48]. Our observations showed a greater increase in ERα expression when calcitriol was combined with epigenetic modulators, pointing out a reciprocal relationship between calcitriol and epigenetic modulators. Moreover, the DNMT and HDAC enzymatic activities were downregulated by calcitriol. Taken together, these results indicate that calcitriol acts through epigenetic events in receptor reactivation in ER-negative breast cancer cells. We previously demonstrated that calcitriol induces ERα expression through a VDR dependent mechanism in ER-negative breast cancer cells, given that TEI-9647, a VDR antagonist, significantly prevented the stimulatory effect of calcitriol in the ERα gene expression [16]. In accordance with this, we performed Western blot analyses to determine if calcitriol affects VDR protein expression in the SUM-229PE cell line. The results showed that calcitriol (1×10^{-8} M) up-regulated VDR protein expression (data not shown). Like these results, calcitriol treatment increased VDR pro-

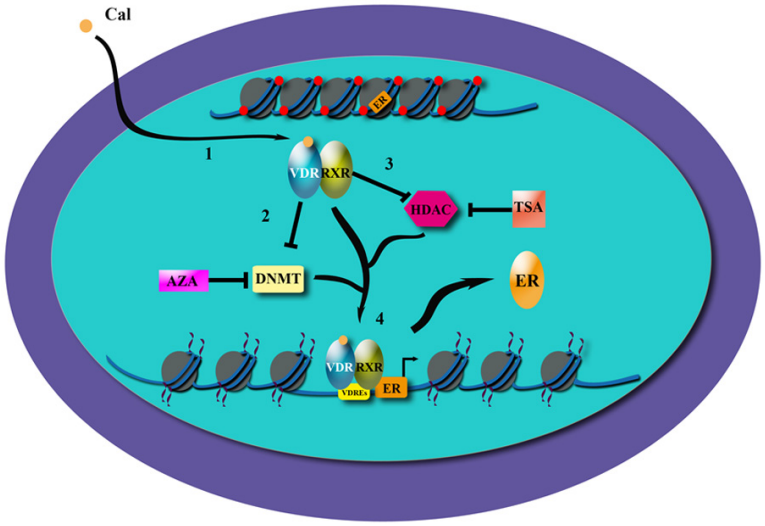

Figure 7. Graphic summary. Estrogen receptor (ER) α re-expression by calcitriol in ER-negative breast cancer cells is via direct transcriptional regulation and epigenetic modifications. (1) Calcitriol (Cal) binds to vitamin D receptor (VDR) and heterodimerizes with retinoid X receptor (RXR). This complex decreases (2) DNMT and (3) HDAC activity leading to change in open chromatin structure. Afterwards, (4) the activated VDR/RXR complex interacts with specific vitamin D response elements (VDREs) in ERα promoter region that result in ERα re-expression.

tein expression in the MCF-7 breast cancer cells with ER-positive phenotype [49]. The increment in the VDR protein's abundance could favor the binding of the VDR-RXR complex to VDREs in the ERα gene promoter region, as well as, the calcitriol effects in the regulation DNMT and HDAC enzymatic activity.

It should be noted that, to our knowledge, this study is the first to demonstrate the ability of calcitriol to induce the ERα expression through two pathways that consist in direct transcriptional regulation of VDR with putative VDREs in ERα gene promoter and the modulation of epigenetic-driven chromatin conformational events in ER-negative breast cancer cells (**Figure 7**). Since the loss of ER expression has also been related to mechanisms such as hyperactivation of the mitogen-activated protein kinase (MAPK) signaling pathway or increased expression of specific microRNAs [5, 8, 12, 48, 50]. We do not rule out that these mechanisms could be involved in ERα induction by calcitriol. In this regard, we showed that calcitriol treatment did not modify the MAPK activity in ERα-negative breast cancer cells, suggesting that ERα induction by calcitriol was independent of the MAPK pathway [16].

Concerning microRNAs regulation by calcitriol to modulate ERα expression, future experiments must be carried out. A possible explanation of how calcitriol induces ERα expression in ER-negative cells could be via its ability to induce cell differentiation and therefore affecting and improving the phenotype of cancer cells [51, 52].

These results are relevant because the presence of ERα correlates with increased disease-free survival and better prognosis; in addition, ERα-positive breast cancers respond appropriately to endocrine therapies [39, 53, 54]. Our findings could provide a novel therapeutic approach by using a natural compound alone or combined with epigenetic modulators in order to re-establish ERα expression and improve treatment and prognosis on ER-negative breast cancer.

Conclusion

Calcitriol induces ERα expression in ERα-negative breast cancer cells through the direct interaction of VDR-RXR complex with VDREs at the ERα gene promoter. It was also interesting to see that inhibition of HDAC and DNMT enzymatic activity, increased calcitriol abilities to restore the ERα positive tumor cells phenotype. These results might contribute to the development of new and promising therapeutic strategies for the treatment of ERα-negative breast cancer patients.

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Disclosure of conflict of interest

None.

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References

[1] Clarke R, Liu MC, Bouker KB, Gu Z, Lee RY, Zhu Y, Skaar TC, Gomez B, O'Brien K, Wang Y and Hilakivi-Clarke LA. Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling. Oncogene 2003; 22: 7316-7339.

[2] Lower EE, Glass EL, Bradley DA, Blau R and Heffelfinger S. Impact of metastatic estrogen receptor and progesterone receptor status on survival. Breast Cancer Res Treat 2005; 90: 65-70.

[3] Shao W and Brown M. Advances in estrogen receptor biology: prospects for improvements in targeted breast cancer therapy. Breast Cancer Res 2004; 6: 39-52.

[4] Gupta GK, Collier AL, Lee D, Hoefer RA, Zheleva V, Siewertsz van Reesema LL, Tang-Tan AM, Guye ML, Chang DZ, Winston JS, Samli B, Jansen RJ, Petricoin EF, Goetz MP, Bear HD and Tang AH. Perspectives on triple-negative breast cancer: current treatment strategies, unmet needs, and potential targets for future therapies. Cancers (Basel) 2020; 12: 2392.

[5] Bayliss J, Hilger A, Vishnu P, Diehl K and El-Ashry D. Reversal of the estrogen receptor negative phenotype in breast cancer and restoration of antiestrogen response. Clin Cancer Res 2007; 13: 7029-7036.

[6] Madak-Erdogan Z, Lupien M, Stossi F, Brown M and Katzenellenbogen BS. Genomic collaboration of estrogen receptor alpha and extracellular signal-regulated kinase 2 in regulating gene and proliferation programs. Mol Cell Biol 2011; 31: 226-236.

[7] Adams BD, Cowee DM and White BA. The role of miR-206 in the epidermal growth factor (EGF) induced repression of estrogen receptor-alpha (ERalpha) signaling and a luminal phenotype in MCF-7 breast cancer cells. Mol Endocrinol 2009; 23: 1215-1230.

[8] Muhammad N, Bhattacharya S, Steele R and Ray RB. Anti-miR-203 suppresses ER-positive breast cancer growth and stemness by targeting SOCS3. Oncotarget 2016; 7: 58595-58605.

[9] Yang X, Phillips DL, Ferguson AT, Nelson WG, Herman JG and Davidson NE. Synergistic activation of functional estrogen receptor (ER)-alpha by DNA methyltransferase and histone deacetylase inhibition in human ER-alpha-negative breast cancer cells. Cancer Res 2001; 61: 7025-7029.

[10] Bovenzi V and Momparler RL. Antineoplastic action of 5-aza-2'-deoxycytidine and histone deacetylase inhibitor and their effect on the expression of retinoic acid receptor beta and estrogen receptor alpha genes in breast carcinoma cells. Cancer Chemother Pharmacol 2001; 48: 71-76.

[11] Jang ER, Lim SJ, Lee ES, Jeong G, Kim TY, Bang YJ and Lee JS. The histone deacetylase inhibitor trichostatin A sensitizes estrogen receptor alpha-negative breast cancer cells to tamoxifen. Oncogene 2004; 23: 1724-1736.

[12] Garcia-Becerra R, Santos N, Diaz L and Camacho J. Mechanisms of resistance to endocrine therapy in breast cancer: focus on signaling pathways, miRNAs and genetically based resistance. Int J Mol Sci 2012; 14: 108-145.

[13] Li Y, Meeran SM, Patel SN, Chen H, Hardy TM and Tollefsbol TO. Epigenetic reactivation of estrogen receptor-alpha (ERalpha) by genistein enhances hormonal therapy sensitivity in ERalpha-negative breast cancer. Mol Cancer 2013; 12: 9.

[14] Gajulapalli VNR, Malisetty VL, Chitta SK and Manavathi B. Oestrogen receptor negativity in breast cancer: a cause or consequence? Biosci Rep 2016; 36: e00432.

[15] Giacinti L, Giacinti C, Gabellini C, Rizzuto E, Lopez M and Giordano A. Scriptaid effects on breast cancer cell lines. J Cell Physiol 2012; 227: 3426-3433.

[16] Santos-Martinez N, Diaz L, Ordaz-Rosado D, Garcia-Quiroz J, Barrera D, Avila E, Halhali A,

Medina-Franco H, Ibarra-Sanchez MJ, Esparza-Lopez J, Camacho J, Larrea F and Garcia-Becerra R. Calcitriol restores antiestrogen responsiveness in estrogen receptor negative breast cancer cells: a potential new therapeutic approach. BMC Cancer 2014; 14: 230.

[17] Yao S and Ambrosone CB. Associations between vitamin D deficiency and risk of aggressive breast cancer in African-American women. J Steroid Biochem Mol Biol 2013; 136: 337-341.

[18] Shirazi L, Almquist M, Borgquist S, Malm J and Manjer J. Serum vitamin D (25OHD3) levels and the risk of different subtypes of breast cancer: a nested case-control study. Breast 2016; 28: 184-190.

[19] Zinser GM, Suckow M and Welsh J. Vitamin D receptor (VDR) ablation alters carcinogen-induced tumorigenesis in mammary gland, epidermis and lymphoid tissues. J Steroid Biochem Mol Biol 2005; 97: 153-164.

[20] Campbell MJ and Trump DL. Vitamin D receptor signaling and cancer. Endocrinol Metab Clin North Am 2017; 46: 1009-1038.

[21] Segaert S and Bouillon R. Vitamin D and regulation of gene expression. Curr Opin Clin Nutr Metab Care 1998; 1: 347-354.

[22] Carlberg C. Mechanisms of nuclear signalling by vitamin D3. Interplay with retinoid and thyroid hormone signalling. Eur J Biochem 1995; 231: 517-527.

[23] Rhodes SJ, Chen R, DiMattia GE, Scully KM, Kalla KA, Lin SC, Yu VC and Rosenfeld MG. A tissue-specific enhancer confers Pit-1-dependent morphogen inducibility and autoregulation on the pit-1 gene. Genes Dev 1993; 7: 913-932.

[24] Fetahu IS, Hobaus J and Kallay E. Vitamin D and the epigenome. Front Physiol 2014; 5: 164.

[25] O'Brien KM, Sandler DP, Xu Z, Kinyamu HK, Taylor JA and Weinberg CR. Vitamin D, DNA methylation, and breast cancer. Breast Cancer Res 2018; 20: 70.

[26] Stefanska B, Salame P, Bednarek A and Fabianowska-Majewska K. Comparative effects of retinoic acid, vitamin D and resveratrol alone and in combination with adenosine analogues on methylation and expression of phosphatase and tensin homologue tumour suppressor gene in breast cancer cells. Br J Nutr 2012; 107: 781-790.

[27] Flouriot G, Griffin C, Kenealy M, Sonntag-Buck V and Gannon F. Differentially expressed messenger RNA isoforms of the human estrogen receptor-alpha gene are generated by alternative splicing and promoter usage. Mol Endocrinol 1998; 12: 1939-1954.

[28] Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M and Werner T. MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics 2005; 21: 2933-2942.

[29] Toell A, Polly P and Carlberg C. All natural DR3-type vitamin D response elements show a similar functionality in vitro. Biochem J 2000; 352: 301-309.

[30] Kos M, Reid G, Denger S and Gannon F. Minireview: genomic organization of the human ERalpha gene promoter region. Mol Endocrinol 2001; 15: 2057-2063.

[31] Thompson PD, Jurutka PW, Haussler CA, Whitfield GK and Haussler MR. Heterodimeric DNA binding by the vitamin D receptor and retinoid X receptors is enhanced by 1,25-dihydroxyvitamin D3 and inhibited by 9-cis-retinoic acid. Evidence for allosteric receptor interactions. J Biol Chem 1998; 273: 8483-8491.

[32] Yu J, Zayas J, Qin B and Wang L. Targeting DNA methylation for treating triple-negative breast cancer. Pharmacogenomics 2019; 20: 1151-1157.

[33] Wu Y, Alvarez M, Slamon DJ, Koeffler P and Vadgama JV. Caspase 8 and maspin are downregulated in breast cancer cells due to CpG site promoter methylation. BMC Cancer 2010; 10: 32.

[34] Carlberg C. The vitamin D(3) receptor in the context of the nuclear receptor superfamily : the central role of the retinoid X receptor. Endocrine 1996; 4: 91-105.

[35] Kato S. The function of vitamin D receptor in vitamin D action. J Biochem 2000; 127: 717-722.

[36] Deeb KK, Trump DL and Johnson CS. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. Nat Rev Cancer 2007; 7: 684-700.

[37] Krishnan AV and Feldman D. Mechanisms of the anti-cancer and anti-inflammatory actions of vitamin D. Annu Rev Pharmacol Toxicol 2011; 51: 311-336.

[38] Swami S, Krishnan AV and Feldman D. 1alpha,25-Dihydroxyvitamin D3 down-regulates estrogen receptor abundance and suppresses estrogen actions in MCF-7 human breast cancer cells. Clin Cancer Res 2000; 6: 3371-3379.

[39] Nadji M, Gomez-Fernandez C, Ganjei-Azar P and Morales AR. Immunohistochemistry of estrogen and progesterone receptors reconsidered: experience with 5,993 breast cancers. Am J Clin Pathol 2005; 123: 21-27.

[40] Swami S, Krishnan AV, Wang JY, Jensen K, Peng L, Albertelli MA and Feldman D. Inhibitory effects of calcitriol on the growth of MCF-7 breast cancer xenografts in nude mice: selective modulation of aromatase expression in vivo. Horm Cancer 2011; 2: 190-202.

[41] Krishnan AV, Swami S and Feldman D. Vitamin D and breast cancer: inhibition of estrogen synthesis and signaling. J Steroid Biochem Mol Biol 2010; 121: 343-348.
[42] Swami S, Krishnan AV, Peng L, Lundqvist J and Feldman D. Transrepression of the estrogen receptor promoter by calcitriol in human breast cancer cells via two negative vitamin D response elements. Endocr Relat Cancer 2013; 20: 565-577.
[43] Stoffers KL, Sorg BL, Seuter S, Rau O, Radmark O and Steinhilber D. Calcitriol upregulates open chromatin and elongation markers at functional vitamin D response elements in the distal part of the 5-lipoxygenase gene. J Mol Biol 2010; 395: 884-896.
[44] Cazares-Ordonez V, Gonzalez-Duarte RJ, Diaz L, Ishizawa M, Uno S, Ortiz V, Ordonez-Sanchez ML, Makishima M, Larrea F and Avila E. A cis-acting element in the promoter of human ether a go-go 1 potassium channel gene mediates repression by calcitriol in human cervical cancer cells. Biochem Cell Biol 2015; 93: 94-101.
[45] Haussler MR, Jurutka PW, Mizwicki M and Norman AW. Vitamin D receptor (VDR)-mediated actions of 1alpha,25(OH)(2)vitamin D(3): genomic and non-genomic mechanisms. Best Pract Res Clin Endocrinol Metab 2011; 25: 543-559.
[46] Kouzarides T. Chromatin modifications and their function. Cell 2007; 128: 693-705.
[47] Ottaviano YL, Issa JP, Parl FF, Smith HS, Baylin SB and Davidson NE. Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. Cancer Res 1994; 54: 2552-2555.
[48] Rahman MM, Brane AC and Tollefsbol TO. MicroRNAs and epigenetics strategies to reverse breast cancer. Cells 2019; 8: 1214.
[49] Zheng W, Duan B, Zhang Q, Ouyang L, Peng W, Qian F, Wang Y and Huang S. Vitamin D-induced vitamin D receptor expression induces tamoxifen sensitivity in MCF-7 stem cells via suppression of Wnt/beta-catenin signaling. Biosci Rep 2018; 38: BSR20180595.
[50] Oh AS, Lorant LA, Holloway JN, Miller DL, Kern FG and El-Ashry D. Hyperactivation of MAPK induces loss of ERalpha expression in breast cancer cells. Mol Endocrinol 2001; 15: 1344-1359.
[51] Gordon LA, Mulligan KT, Maxwell-Jones H, Adams M, Walker RA and Jones JL. Breast cell invasive potential relates to the myoepithelial phenotype. Int J Cancer 2003; 106: 8-16.
[52] Pendas-Franco N, Gonzalez-Sancho JM, Suarez Y, Aguilera O, Steinmeyer A, Gamallo C, Berciano MT, Lafarga M and Munoz A. Vitamin D regulates the phenotype of human breast cancer cells. Differentiation 2007; 75: 193-207.
[53] McGuire WL, Osborne CK, Clark GM and Knight WA 3rd. Steroid hormone receptors and carcinoma of the breast. Am J Physiol 1982; 243: E99-102.
[54] Clark GM and McGuire WL. Steroid receptors and other prognostic factors in primary breast cancer. Semin Oncol 1988; 15 Suppl 1: 20-25.