Calcitriol restores antiestrogen responsiveness in estrogen receptor negative breast cancer cells: A potential new therapeutic approach

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Abstract

**Background:** Approximately 30% of breast tumors do not express the estrogen receptor (ER) α, which is necessary for endocrine therapy approaches. Studies are ongoing in order to restore ERα expression in ERα-negative breast cancer. The aim of the present study was to determine if calcitriol induces ERα expression in ER-negative breast cancer cells, thus restoring antiestrogen responses.

**Methods:** Cultured cells derived from ERα-negative breast tumors and an ERα-negative breast cancer cell line (SUM-229PE) were treated with calcitriol and ERα expression was assessed by real time PCR and western blots. The ERα functionality was evaluated by prolactin gene expression analysis. In addition, the effects of antiestrogens were assessed by growth assay using the XTT method. Gene expression of cyclin D1 (CCND1), and Ether-à-go-go 1 (EAG1) was also evaluated in cells treated with calcitriol alone or in combination with estradiol or ICI-182,780. Statistical analyses were determined by one-way ANOVA.

**Results:** Calcitriol was able to induce the expression of a functional ERα in ER-negative breast cancer cells. This effect was mediated through the vitamin D receptor (VDR), since it was abrogated by a VDR antagonist. Interestingly, the calcitriol-induced ERα restored the response to antiestrogens by inhibiting cell proliferation. In addition, calcitriol-treated cells in the presence of ICI-182,780 resulted in a significant reduction of two important cell proliferation regulators CCND1 and EAG1.

**Conclusions:** Calcitriol induced the expression of ERα and restored the response to antiestrogens in ERα-negative breast cancer cells. The combined treatment with calcitriol and antiestrogens could represent a new therapeutic strategy in ERα-negative breast cancer patients.

**Keywords:** Estrogen receptor, Breast cancer, Hormonal therapy, Calcitriol, VDR

**Background**

Breast cancer is a heterogeneous disease, encompassing a number of distinct biological entities that are associated with a variety of pathological and clinical features [1]. The gene expression profile of breast cancer allows to classify this disease in five groups, two of them estrogen receptor (ER)-positive (luminal A and B) and three ER-negative (normal breast-like, human epidermal growth factor receptor- 2 (HER2) and basal-like) [2]. Approximately 30% of all breast tumors do not express ER, a protein with both prognostic and predictive values. Indeed, the presence of ERα correlates with increased disease-free survival and better prognosis. Importantly, ERα-positive breast cancers respond appropriately to endocrine therapies [3-5]. Tamoxifen is the most common and effective therapy in pre- and postmenopausal patients affected with ER-positive tumors, since a long-term use of this compound increases disease-free survival and reduces
tumor recurrence [6,7]. Unfortunately, up to 50% of patients bearing ERα-positive primary tumors lose receptor expression in recurrent tumors, and about one third of metastatic tumors develop resistance to tamoxifen and lose ERα expression [8]. The lack of ER expression has been linked to epigenetic mechanisms or to others such as hyperactivation of the mitogen-activated protein kinase (MAPK) signaling pathway or increased expression of specific microRNAs [9-11]. In fact, knockdown of specific microRNAs or inhibition of MAPK activity is followed by restoration of a functional ERα in ER-negative breast cancer cells [9,10]. These findings indicate that the ERα-negative phenotype could be reverted for therapeutic purposes.

Calcitriol, the most active metabolite of vitamin D, elicits significant antiproliferative activity in breast cancer cells by several vitamin D receptor (VDR) mediated mechanisms including regulation of growth arrest, cell differentiation, migration, invasion and apoptosis [12-14]. Epidemiological studies have demonstrated an association between low levels of calcidiol, the precursor of calcitriol, and increased risk of developing breast cancer [15]. Moreover, low levels of calcitriol are associated with disease progression and high incidence of ER-negative and triple-negative breast tumors [16,17], while VDR-positive breast cancer patients had significantly longer disease-free survival than those with VDR-negative tumors [18]. Indeed, VDR knock-out mice are more likely to develop ER- and progesterone receptor (PR)-negative mammary tumors as compared with their wild type littermates [17], highlighting calcitriol prodifferentiating properties. Our laboratory and other groups have demonstrated the potent antiproliferative activity of calcitriol in cells derived from biopsies or in established cell lines from breast cancer [19-21]. Additionally, other studies have demonstrated the antiproliferative effects of vitamin D compounds in ER-responsive human breast cancer cells through downregulation of ER and disruption of estrogen dependent signaling pathways [20,22,23]. However, calcitriol also inhibited proliferation in ER-negative cell lines, suggesting that growth inhibition induced by calcitriol is not solely mediated through the ER [12]. In this regard, ERα regulation studies in several human breast cancer cell lines showed that calcitriol treatment decreased or did not modify ER expression [20,22-24]. In contrast, in an ER-negative breast cancer cell line calcitriol increased estrogen binding proteins [24].

In order to increase our knowledge concerning the participation of calcitriol in ER regulation, the aim of the present study was to investigate if this hormone induces a functional ER and consequently could restore the antiproliferative effects of antiestrogens in ER-negative breast cancer cells.

### Methods

#### Reagents

Estradiol (E2), 4-hydroxytamoxifen and calcipotriol (MC 903) were purchased from Sigma (St. Louis, MO, USA). Cell culture medium was obtained from Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Hyclone Laboratories Inc. (Logan, UT, USA) and the antiestrogen ICI-182,780 (Fulvestrant) from Zeneca Pharmaceuticals (Wilmington, DE, USA). Gefitinib (Iressa, ZD1839) was kindly provided by AstraZeneca (Wilmington, DE, USA). U0126 was from Millipore (MA, USA). Trizol and the oligonucleotides for real time polymerase chain reaction (qPCR) were from Invitrogen (CA, USA). The VDR antagonist (23S)-25-dehydro-1-hydroxyvitamin D3-26,23-lactone (TEI-9647) and 1a,25-dihydroxycholecalciferol (calcitriol) were kindly donated from Teijin Pharma Limited (Tokyo, Japan) and Hoffmann-La Roche Ltd. (Basel, Switzerland), respectively.

#### Human tissues

The protocol was approved by the Institutional Review Board “Comité Institucional de Investigación Biomédica en Humanos (No. 1967, 2009)” of the “Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (Mexico City). Before mammary biopsies donation, all participating patients signed an informed consent. Biopsies were obtained from patients with ER-negative breast cancer. The samples were harvested and processed as described previously [19]. A total of 5 independent cultured specimens were used for this study. The ER-negative SUM-229PE (Asterand, San Francisco, CA) and the ER-positive BT-474 (ATCC) and MCF-7 (ATCC) established cell lines were also studied.

#### Cell culture

Primary tumor cultures were derived from biopsies of breast cancer patients as described previously [19,25]. The cells were cultured in DMEM-HG medium supplemented with 5% heat-inactivated-FBS, 100 U/ml penicillin, 100 μg/ml streptomycin; and incubated in 5% CO2 at 37°C. After approximately 8 passages cells were characterized by western blot and immunocytochemistry. Established cell lines were maintained according to indications from suppliers. All experimental procedures were performed in DMEM-F12 medium supplemented with 5% charcoal-stripped-heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.
**Immunocytochemistry**

Cultured cells were grown on glass coverslips and fixed in 96% ethanol. Antigen retrieval was done by autoclaving in EDTA decloaker 5x solution (pH 8.4-8.7, Biocare Medical, CA, USA) during 10 min. Slides were blocked with immunodetector peroxidase blocker (Bio SB, CA, USA) and incubated with ERα (1:250, Bio SB) [26] and VDR antibodies (1:100, Santa Cruz Biotechnology Inc, CA, USA) [27]. After washing, the slides were sequentially incubated with Immune-Detector Biotin-Link and Immuno-Detector HRP label (Bio SB) during 10 min each. Staining was completed with DAB and 0.04% H₂O₂.

**Western blots**

Cells were incubated in the presence of calcitriol (1X10⁻⁸ M and 1X10⁻⁷ M), MAPK inhibitors (U0126; 10 μM, Gefitinib; 0.8 μM) or the vehicle alone during 72 hr. Afterwards, whole-cell protein lysates were prepared using lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, pH 7.5) in the presence of a protease inhibitor cocktail. Protein concentrations were determined using the Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA, USA). The proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk and incubated overnight at 4°C in the presence of mouse anti-ERα (1:200, Santa Cruz) [28]. The membranes were washed and incubated with goat anti-mouse HRP-conjugated secondary antibody (1:2000, Santa Cruz). For visualization, membranes were processed with BM chemiluminescence blotting substrate (Roche Applied Science, IN, USA). For normalization, blots were stripped in boiling stripping buffer (2% w/v SDS, 62.5 mM Tris-HCl pH 6.8, 100 mM 2- mercapto-ethanol) for 30 min at 50°C and sequentially incubated with mouse anti-GAPDH (1:1000, Millipore) [29] and anti-mouse-HRP (1:10000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Densitometric analysis of resulting bands was performed by using ImageJ software (NIH, USA).

**Cell proliferation assay**

The cells were seeded in 96-well tissue culture plates at a density of 500-1000 cells/well by sextuplicate. After incubating for 24 hr, cells were incubated in the presence or absence of calcitriol (1X10⁻⁸ M) during 48 hr. Afterwards, culture medium was removed and incubations with E₂ (1X10⁻⁸ M), as an ER agonist, or tamoxifen (1X10⁻⁸ M) and ICI-182,780 (1X10⁻⁶ M), as ER antagonists, or their combination were performed in the absence or presence of calcitriol. Plates were incubated at 37°C for 6 days and cell viability was determined by using the colorimetric XTT Assay Kit (Roche) according to manufacturer’s instructions. After 4 hr incubation, absorbance at 492 nm was measured in a microplate reader (BioTek, Winooski, VT, USA).

**Real time RT-PCR**

For ERα gene expression analysis the cells were incubated in the presence of different calcitriol concentrations or the vehicle alone (0.1% ethanol) during 24 hr. In order to establish the participation of the VDR on calcitriol effects upon the ERα, the VDR antagonist TIE-9647 (1X10⁻⁶ M) was co-incubated with calcitriol in some experiments. Gene expression analyses of prolactin (PRL), cyclin D1 (CCND1) and the potassium channel Ether-à-go-go (EAG1) were also performed. For this, the cells were treated with calcitriol (1X10⁻⁹ M) during 48 hr. Afterwards, E₂ (1X10⁻⁹ M) or ICI-182,780 (1X10⁻⁶ M) were added to the culture media and the incubations proceeded for additional 24 hr. Next, RNA was extracted with Trizol reagent and then subjected to reverse transcription using the transcriptor RT system. Real-time PCR was carried out using the LightCycler 2.0 from Roche (Roche Diagnostics, Mannheim, Germany), according to the following protocol: activation of Taq DNA polymerase and DNA denaturation at 95°C for 10 min, proceeded 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, CCTTCTTCAAGAGAAGTATTCAAAGG; ERα-R, GTTTTTATCAAATGTTGCACTGG; EAG1-F, CCTGGAGGTTAGTCCAAGAGT; EAG1-R, CCA AACACGTCTCTCCTTTTC; CCND1-F, GAAATGTCG CCACTTG; CCND1-R, GACCTCTCTCCTCGACTTCT; PRL-F, AAAGGATCAGCATTGGAAAG; PRL-R, GCCAG GAGCAGGTTTGC. The gene expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured by real-time PCR and normalised to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using geometric mean of two control genes (beta-actin and 18S rRNA). The expression level of ERα was determined relative to the expression of GAPDH. The expression of ERα was significantly regulated by calcitriol, with a maximum increase of 10-fold at 1X10⁻⁷ M.

**Statistical analyses**

Data are expressed as the mean ± standard deviation (S.D.). Statistical analyses were determined by one-way ANOVA followed by the Holm-Sidak method, using a specialized software package (SigmaStat, Jandel Scientific). Differences were considered significant at P ≤ 0.05.

**Results**

Calcitriol induced ERα expression through a VDR-dependent mechanism in ER-negative breast cancer cells

Biopsies from five patients with ER-negative breast cancer were obtained and used for cell culturing. These biopsies had a diagnosis of invasive ductal carcinoma and ranged between 5 and 9 in the Scarff-Bloom-Richardson.
system score. All cultured breast tumor-derived cells were positive for VDR and further confirmed to be negative for ERα (Figure 1). In addition, the ER-negative SUM-229PE and ER-positive BT-474 established cell lines were also studied. All cell lines were incubated in the presence of calcitriol (1X10^{-7} M) during 24 hr and ERα gene expression was assessed by qPCR. As shown in Figure 2A, calcitriol significantly induced ERα mRNA expression in all tumor-derived cultured cells and SUM-229PE cells. In contrast, calcitriol downregulated ERα mRNA levels in BT-474 as it has been previously reported [30].

As shown in Figure 2B, calcitriol significantly increased ERα mRNA in a dose dependent manner with an EC_{50} of 9.8X10^{-9} M. This effect was specifically mediated through the VDR, since the VDR antagonist TEI-9647 significantly abolished the stimulatory effect of calcitriol upon ERα gene expression. The presence of the VDR antagonist by itself did not modify ERα gene expression (Figure 2C).

In order to assess if calcitriol induced ERα protein expression, the SUM-229PE cell line was incubated in the presence of calcitriol and western blot analyses were performed. Figure 3 shows the results of cells incubated with two calcitriol concentrations (1X10^{-8} and 1X10^{-7} M) during 72 hr. The presence of a 66 KDa band corresponding to ERα, as judged by the positive control in MCF-7 cells, was observed in calcitriol-treated cells. Moreover, a higher calcitriol concentration further increased the relative abundance of ERα as shown in Figure 3. Inhibitors of the MAPK signaling pathway (U0126 and Gefitinib) were used as controls of ERα induction [10].

**Calcitriol induced a functional ERα**

In order to determine the functionality of the ERα induced by calcitriol, we evaluated the effects of E2 and the antiestrogen ICI-182,780 on the expression of PRL, cathepsin D (CTSD) and trefoil factor 1 (TFF1) as examples of estrogen inducible genes [31]. Breast tumor-derived cells were cultured first in the presence or absence of calcitriol (1X10^{-8} M) during 48 hr and subsequently incubated in the presence of E2 (1X10^{-8} M) or ICI-182,780 (1X10^{-6} M) with or without calcitriol for 24 hr (Figure 4). In the absence of calcitriol (black bars), E2 and ICI-182,780 did not modify PRL mRNA; however, in calcitriol-treated cells (white bars), E2 significantly

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**Figure 1** Immunocytochemical analysis of ERα and VDR in primary and established breast cancer cells. Representative images of cultured tumor-derived (A-C, SUM-229PE (D-F) and BT-474 (G-I) cells are shown. Tumor-derived (A) and SUM-229PE (D) cells were negative for ERα, while BT-474 was ERα positive (G). All cells were positive for VDR (B, E and H) in the cytoplasmic, nuclear and perinuclear regions (brown staining). Negative controls were carried out in the absence of primary antibody for each cell line (C, F and I). Representative pictures are displayed (20 ×).
upregulated PRL expression. The presence of the antiestrogen alone did not change PRL gene expression. These data suggest that the calcitriol-induced ERα is a fully-transcriptionally active receptor. Interestingly, calcitriol per se significantly stimulated the expression of both CTSD and TFF1 genes, which may explain why E2 was not able to further increase gene expression (data not shown).

Calcitriol restored the antiestrogenic response in ERα-negative breast cancer cells

In order to assess whether the calcitriol-induced ERα was sensitive to the antiproliferative effects of the antiestrogens in ERα-negative breast cancer cells, growth assays were performed. Breast cancer cells were incubated in the presence of calcitriol (1X10^{-8} M) or the vehicle alone for 48 hr. Afterwards, cells were incubated with ER agonist (1X10^{-8} M), antagonists (1X10^{-6} M) or the combination of E2 plus antagonists during 6 days. The results demonstrated that in the absence of calcitriol (black bars), none of the compounds affected cell growth in both cultured breast tumor-derived cells (Figure 5A) and the SUM-229PE cell line (Figure 5B). Interestingly, in calcitriol-treated tumor-derived cells (white bars), antiestrogens alone or in combination with E2 significantly inhibited cell proliferation as compared with control cells.
(C, white bar). The presence of E2 at the dose of 1X10^{-8} M did not modify cell growth (Figure 5A); however, higher E2 concentrations (1X10^{-7} M) significantly inhibited cell growth (data not shown). Similar results were observed in SUM-229PE cells, but tamoxifen alone or in combination with E2 did not affect cell growth (Figure 5B). MCF-7 cells were used as control of the inhibitory effect of the antiestrogens via ERα (Figure 5C). As depicted, E2 significantly increased cell proliferation in cells not treated with calcitriol; however, this effect was not observed in those cells cultured in the presence of calcitriol, most likely due to its antiproliferative activity. As expected, antiestrogens and their combination with E2 significantly inhibited cell growth in both treated and not-treated calcitriol cells.

**Antiestrogen treatment downregulated CCND1 and EAG1 gene expression in calcitriol-treated breast cancer cells**

One of the molecular mechanisms by which antiestrogens inhibit cell proliferation is by decreasing CCND1 expression and blockage of cell cycle progression via the ER [32,33]. Thus, we studied the effects of ICI-182,780 and E2 on CCND1 expression in calcitriol-treated ERα-negative breast tumor-derived cells. As shown in Figure 6A, only in calcitriol-treated cells the presence of ICI-182,780 (1X10^{-6} M) but not E2 downregulated CCND1 gene expression.

In breast cancer cell lines the inhibition of EAG1 potassium channel expression is accompanied by a significant reduction of cell proliferation [19,34]. Therefore, we evaluated the effects of an agonist or antagonist of the calcitriol-induced ER on EAG1 expression. As shown in

**Figure 5 ERα induction restored the response to antiestrogens in ER-negative breast cancer cells.** A) Cultured breast tumor-derived cells, B) SUM-229PE and C) MCF-7 were incubated in the absence (black bars) or presence of calcitriol 1X10^{-8} M (white bars) for 48 h. Afterwards, cells were coincubated without (black bars) or with calcitriol (white bars) plus estradiol (E2, 1X10^{-8} M), tamoxifen (Tx, 1X10^{-6} M), ICI-182,780 (ICI, 1X10^{-6} M), ethanol (C), or combination of antagonists with E2 for 6 days. Cell growth assays by the XTT colorimetric method were performed. Bars represent the mean ± S.D. Data were normalized to 100% using the activity of vehicle-treated cells. Results are representative from two independent experiments performed in sextuplicates.* P ≤ 0.05 vs. control for each group (black bars vs black control or white bars vs white control).
Figure 6B, neither E₂ nor ICI-182,780 altered EAG1 gene expression in non-calcitriol treated cells (black bars); however, when compared with cells in the presence of calcitriol, the antiestroge n, in contrast to E₂ alone, significantly decreased EAG1 mRNA levels (white bars).

Calcipotriol, a vitamin D analogue, increased ERα expression
Calcipotriol, a synthetic low calcemic vitamin D analogue, has been considered a potent stimulator of cell differentiation and inhibitor of cell proliferation in cancer cells [35]. Figure 7 shows a comparison between different concentrations of calcipotriol and calcitriol (1X10⁻¹⁰ to 1X10⁻⁶ M) upon ERα gene expression in SUM-229PE. As depicted, both compounds increased ERα gene expression in a concentration-dependent manner with similar EC₅₀ values (2.74X10⁻⁸ M and 2.21X10⁻⁸ M, for calcipotriol and calcitriol, respectively).

Discussion
In breast cancer, the presence of the ERα is considered as a good indicator of disease-free survival and prognosis since patients with ERα-positive tumors are candidates for hormonal therapy [3,4,6]. In contrast, tumors lacking this receptor have the poorest clinical prognosis [36]. In this study we demonstrated the ability of calcitriol to induce the expression of ERα in both primary and established ERα-negative breast cancer cell lines. This effect was mediated by a VDR-dependent mechanism. In addition, our results demonstrated a fully active calcitriol-induced ER by its ability to increase PRL gene expression. Interestingly, pretreatment of ER-negative breast tumor-derived cells with calcitriol and the further incubation with this secosteroid in combination with tamoxifen or ICI-182,780 resulted in a significantly lower cell growth proliferation.

It is noteworthy to mention that, to our knowledge, this study is the first to demonstrate the ability of calcitriol to induce the expression of a functional ERα in both primary and established ERα-negative breast cancer cells, which we think is of biological importance given its potential for future treatment strategies to improve prognosis in ERα-negative breast cancer patients.

Since it has been observed that MAPK inhibitors increase ERs protein in ER-negative breast tumor cells [10], we hypothesized that the upregulation of ERs by calcitriol could be the result of decreased MAPK activity. Although, in this study we could not demonstrate any change in this kinase in the presence of calcitriol. An alternative, mechanism by which calcitriol via its receptor induced
ERα expression might be at the level of promoter-driven transcriptional regulation. Therefore, in order to identify putative vitamin D response elements we performed an in silico analysis with the MatInspector software [37] using a sequence derived from the human chromosome 6, which contains the promoter region of ERα [38]. The results from this analysis showed the presence of several putative vitamin D response elements of the DR3 and DR4 types, supporting the idea of a direct transcriptional regulation of ER promoter by calcitriol.

The observation that tamoxifen and ICI-182,780 inhibited cell growth in calcitriol-treated ER-negative breast tumor-derived cells indicated the induction of a functionally active ERα. However, cell growth inhibition by tamoxifen was not observed in the case of calcitriol-treated ER-negative SUM-229PE cells. This finding might be explained as a receptor resistance–like condition resulting probably from the hyperactivation of the MAPK signaling pathway due to overexpression of EGFR or HER2 as has been previously observed in breast cancer cells [10].

It is well known that E2 exhibits proliferative effects and therefore stimulates tumor growth in breast cancer [39,40]. However, in the present study, the presence of E2 did not result in increased proliferation of cells pretreated with calcitriol. It is possible that the lack of mitogenic activity of E2 through the newly expressed ERα was due to a priming antiproliferative effect of calcitriol, thus preventing the expected estradiol-mediated effects on cell proliferation. This observation agreed with those of Bayliss et al., [10] who showed that E2 did not increase proliferation in cells where the ERα was reexpressed by MAPK inhibitors, including in those studies in ER-negative breast cancer cells transfected with the ER [41].

In this study, the ability of antiestrogens to inhibit cell growth in an estradiol-depleted condition might require further investigation; however, some effects of these compounds on the mitogenic activity of growth factors, in the absence of estrogens have been already demonstrated in breast cancer [33,42]. In this regard, one of the most common regulators known to be altered and overexpressed in various cancers including breast is CCND1, which functions as mitogenic sensor and allosteric activator of cyclin-dependent kinase (CDK)4/6 [43]. It is known that the inhibitory actions of antiestrogens on breast cancer are in part exerted through the downregulation of CCND1 [33]. In this study, the results showing that ICI-182,780 significantly decreased CCND1 mRNA only in calcitriol-treated cells, indicated that these compounds may affect cell cycle regulation as has already been shown in ER-positive breast tumors [33]. Furthermore, the demonstration of a significant inhibition of EAG1 gene expression by ICI-182,780 in calcitriol-treated cells, suggested that the antiproliferative effects of these compounds involve a number of regulatory mechanisms which are under the control of ERα activation.

These results suggest that calcitriol in combination with ICI-182,780, through downregulation of EAG1 and CCND1 affect cell proliferation and tumor progression [34,44].

There are several markers associated with tumor aggressiveness. Among these, myoepithelial markers, which are preferentially expressed in ER-negative breast cancer, suggest that the loss of the steroid receptor is related to the degree of cellular dedifferentiation occurring in these tumors [45]. It is known that calcitriol promotes differentiation of several tumor cell types, including human breast and colon cancers [14,46]. This process involves the action of calcitriol on a number of events, such as the induction of adhesion proteins (E-cadherin, claudin, occludin) or by interfering with some intracellular signaling pathways, such as the Wnt/b-catenin signaling [14,46]. Our results revealed that calcitriol induced ERα gene and protein expression suggesting that calcitriol affects the phenotype of ERα-negative breast cancer cells by reverting cellular mechanisms associated with a more aggressive behavior and poor prognosis.

The development of numerous vitamin D analogues and intermittent calcitriol dosing have allowed substantial dose-escalation and reduced calcemic effects [47,48]. Calcipotriol, a synthetic vitamin D analogue with a significantly lower calcemic effect, is also known as a potent antiproliferative compound and an inducer of cell differentiation [35]. In this study, the demonstration that calcipotriol was also able to upregulate ERα gene expression in an ER-negative breast cancer cell line, suggest that treatment options in breast cancer patients might also include vitamin D analogues with reduced side calcemic effects.

Our results suggest that the use of calcitriol in combination with aromatase inhibitors or ER antagonists might be considered in the future as a new strategy for the treatment of ERα-negative breast cancer, including the triple-negative subtypes.

**Conclusions**

The results presented herein clearly demonstrated the ability of calcitriol and its synthetic analog calcipotriol to upregulate ERα expression in a subset of ER-negative breast cancer cells. These results may offer a therapeutic alternative, particularly in those patients affected with ER-negative tumors by sensitizing them to hormone therapy, with the aim at improving disease prognosis.

**Abbreviations**

CTSD: Cathepsin D; CCND1: Cyclin D1; E2: Estradiol; EAG1: Ether-à-go-go 1; EC50: Stimulatory concentration; EGFR: Human epidermal growth factor receptor-1; ER: Estrogen receptor; FBS: Fetal bovine serum; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HER2: Human epidermal growth factor receptor-2; MAPK: Mitogen-activated protein kinase; PR: Progesterone receptor; PRL: Prolactin; qPCR: Real time polymerase chain reaction; RT: Reverse transcription; SD: Standard deviation; TFF1: Trefoil factor 1; VDR: Vitamin D receptor.
Competing interests
The authors declare that they have no competing interests.

Authors' contributions
RGB and LD were involved in the conception, design and coordination of the study as well as in data analysis; interpretation of results, actively participated in all experimental procedures, and were involved in drafting the manuscript. NSM was in charge of all experimental procedures, participated in data analysis and interpretation, as well as in drafting the manuscript. DOR, JGQ, DB, MJIS and JEL participated in the experimental procedures and revised critically the content of the manuscript. HMF provided biological samples, carried out the clinical data collection and retrieved patients signed informed-consent forms. EA, AH and JC contributed in the interpretation of data and critically revised the manuscript for important intellectual content. FL participated in the interpretation of data, made substantive intellectual contribution to the study and drafting the manuscript. All authors read and approved the final manuscript.

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References
1. Simpson PT, Reis-Filho JS, Gale T, Lakhan S. Molecular evolution of breast cancer. J Pathol 2005, 205(2):248–254.
2. Sorlie T, Perou CM, Tibshirani R, Aas T, Geideron S, Johnson H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lonning P, Borresen-Dale AL, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D. Comparative analysis of breast cancer gene expression patterns. Proc Natl Acad Sci U S A 2001, 98(10):10869–10874.
3. Clark GM, McGuire WL. Steroid receptors and other prognostic factors in primary breast cancer. Semin Oncol 1988, 15(2 Suppl 1):20–25.
4. McGuire WL, Osborne CK, Clark GM, Knight WA 3rd. Steroid hormone receptors and carcinoma of the breast. Am J Physiol 1982, 243(2):E99–E102.
5. Nadj M, Gomez-Fernandez C, Ganji-Harz P, Morales AR. Immunohistochemistry of estrogen and progesterone receptors reconsidered: experience with 5,993 breast cancers. J Clin Pathol 2005, 58(12):121–127.
6. Powles TJ, Ashley S, Tidy A, Smith JE, Dowsett M. Twenty-year follow-up of the Royal Marsden randomized, double-blind tamoxifen breast cancer prevention trial. J Natl Cancer Inst 2007, 99(4):283–290.
7. Clarke MJ. WITHDRAWN: Tamoxifen for early breast cancer. Cochrane Database Syst Rev 2008, 4, CD000486.
8. Johnston SR. Acquired tamoxifen resistance in human breast cancer—potential mechanisms and clinical implications. Anticancer Drugs 1997, 8(10):911–930.
9. Zhao JJ, Lin J, Yang H, Kong W, He L, Ma X, Coppola D, Cheng QJ. MicroRNA-221/222 negatively regulates estrogen receptor alpha and is associated with tamoxifen resistance in breast cancer. J Biol Chem 2008, 283(45):31079–31086.
10. Bayliss J, Hilger A, Vithura P, Diehl K, El-Ashy D. Reversal of the estrogen receptor negative phenotype in breast cancer and restoration of antitumor response. Clin Cancer Res 2007, 13(23):7029–7036.
11. Oh AS, Lorant LA, Holloway JN, Miller DL, Kern FG, El-Ashy D. Hyperactivation of MAPK induces loss of Eta1/2 expression in breast cancer cells. Mol Endocrinol 2001, 15(8):1344–1359.
12. Fille RS, Sledge GW Jr, Proctor C. Effects of vitamin D3 on proliferation of cancer cells in vitro. Cancer Lett 1997, 120(1):65–69.
13. Simboli-Campbell M, Nanavez CJ, Tenwiosum M, Welsh J. 1,25-Dihydroxyvitamin D3 induces morphological and biochemical markers of apoptosis in MCF-7 breast cancer cells. J Steroid Biochem Mol Biol 1996, 58(4):367–376.
14. Pendas-Franco N, Gonzalez-Sancho JM, Suarez Y, Aguilara O, Steimney A, Gamallo C, Berciano MT, Lafarga M, Munoz A. Vitamin D regulates the phenotype of human breast cancer cells. Differentiation 2007, 75(3):193–207.
15. Janowsky EC, Lester GE, Weinberg CR, Millikan RC, Schildkraut JM, Garrett PA, Hulka BS. Association between low levels of 1,25-dihydroxyvitamin D and breast cancer risk. Public Health Nutr 1999, 2(3):283–291.
16. Mauer EB, Walls J, Howell A, Davies M, Ratcliffe WB, Bunded NJ. Serum 1,25-dihydroxyvitamin D may be related inversely to disease activity in breast cancer patients with bone metastases. J Clin Endocrinol Metab 1997, 82(1):118–122.
17. Yao S, Ambrosone CB. Associations between vitamin D deficiency and risk of aggressive breast cancer in African-American women. J Steroid Biochem Mol Biol 2012, 136:337–341.
18. Paty P, McClelland RA, Wilson P, Greene GL, Hauksler MR, Pike MC, Colston K, Easton D, Coombes RC. Immunochemical determination of estrogen receptor, progesterone receptor, and 1,25-dihydroxyvitamin D3 receptor in breast cancer and relationship to prognosis. Cancer Res 1991, 51(11):239–244.
19. García-Becerra R, Díaz L, Camacho, J. Barra, D., Ordaz-Rosado D, Morales A, Ortiz CS, Avila E, Barango G, Arellácas M, Hafali A, Larrea F. Calcitriol inhibits Ether-a-go-go potassium channel expression and cell proliferation in human breast cancer cells. Exp Cell Res 2010.
20. Swami S, Krishnan AV, 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(8):3371–3379.
21. García-Quirce J, García-Becerra R, Barra D, Santos N, Avila E, Ordaz-Rosado D, Rivas-Suarez M, Hafali A, Rodríguez P, Gamboa-Dominguez A, Medina-Franco H, Camacho J, Larrea F, Diaz L. Estrenolame synergizes calcitriol antiproliferative activity by inhibiting CYP24A1 and upregulating VDR: a novel approach for breast cancer therapy. PLoS One 2012, 7(9):e45003.
22. Simboli-Campbell M, Nanavez CJ, van Weelken K, Timweniisow M, Welsh J. Comparative effects of 1,25(OH)2D3 and EB1089 on cell cycle kinetics and apoptosis in MCF-7 breast cancer cells. Breast Cancer Res Treat 1997, 47(2):311–41.
23. Stoica A, Sacerda M, Fakhro A, Solomon HB, Fenster BD, Martin MB. Regulation of estrogen receptor-alpha gene expression by 1, 25-dihydroxyvitamin D in MCF-7 cells. J Cell Biochem 1999, 75(4):640–651.
24. Davoodi F, Brenner RV, Evans SR, Schumaker LM, Shabahang M, Nauta RJ, Buras RA. Modulation of vitamin D receptor and estrogen receptor by 1,25(OH)2-vitamin D3 in T-47D human breast cancer cells. J Steroid Biochem Mol Biol 1995, 54(3–4):147–153.
25. I. U., Bustos V, Miner J, Paulo A, Meng ZH, Zlotnicki CG, Ljung BM, Darker SH. Propagation of genetically altered tumor cells derived from fine-needle aspirates of primary breast carcinoma. Cancer Res 1998, 58(23):5271–5274.
26. Tesch M, Shawwa A, Henderson R. Immunohistochemical determination of estrogen and progesterone receptor status in breast cancer. Am J Clin Pathol 1993, 99:118–123.
27. Mauer U, Jehan F, Englert C, Hubinger G, Weidmann E, DeLuca HF, Bergmann L. The Wilm’s tumor gene product (WT1) modulates the response to 1,25-dihydroxyvitamin D3 by induction of the vitamin D receptor. Biol Chem 2001, 276(7):3727–3732.
28. Lappano R, Recchia AG, De Francesco EM, Angelone T, Cera MC, Picard D, Magiullini M. The cholesterol metabolite 25-hydroxycholesterol activates estrogen receptor alpha-mediated signaling in cancer cells and in cardiomyocytes. PLoS one 2011, 6(1):e16631.
29. Almeras L, Eyles D, Benech P, Lafitte D, Villard C, Patatian A, Bourcay J, Mackay-Sim A, McGrath J, Ferron F. Developmental vitamin D deficiency alters brain protein expression in the adult rat: implications for neuropyschiatric disorders. Proteomics 2007, 7(5):769–780.
30. Hussain-Hakimjee EA, Mehta RG. Regulation of steroid receptor expression by 1alpha-hydroxyvitamin D3 in hormone-responsive breast cancer cells. Anticancer Res 2009, 29(6):3555–3561.

31. Duan R, Ginzburg E, Vonderhaar BK. Estrogen stimulates transcription from the human prolactin distal promoter through AP1 and estrogen responsive elements in T47D human breast cancer cells. Mol Cell Endocrinol 2008, 281(1–2):19–28.

32. Musgrove EA, Hamilton JA, Lee CS, Sweeney KJ, Watts CK, Sutherland RL. Growth factor, steroid, and steroid antagonist regulation of cyclin gene expression associated with changes in T47D human breast cancer cell cycle progression. Mol Cell Biol 1993, 13(6):3577–3587.

33. Watts CK, Sweeney KJ, Warthers A, Musgrove EA, Sutherland RL. Antiestrogen regulation of cell cycle progression and cyclin D1 gene expression in MCF-7 human breast cancer cells. Breast Cancer Res Treat 1994, 31(1):95–105.

34. Pardo LA, Suhmer W. Eeg1 as a cancer target. Expert Opin Ther Targets 2008, 12(7):837–843.

35. Bindereup L, Bramm E. Effects of a novel vitamin D analogue MC903 on cell proliferation and differentiation in vitro and on calcium metabolism in vivo. Biochem Pharmacol 1988, 37(5):889–895.

36. Osborne CK, Yochmowitz MG, Knight WA 3rd, McGuire WL. The value of estrogen and progesterone receptors in the treatment of breast cancer. Cancer 1980, 46(12 Suppl):2884–2888.

37. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayeletin M, Werner T. MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics 2005, 21(13):2933–2942.

38. Kos M, Reid G, Denger S, Gannon F. Minireview: genomic organization of the human ERalpha gene promoter region. Mol Endocrinol 2001, 15(12):2057–2063.

39. Dotsis-Souif S, Serigo-CM, Carroll JS, Hu R, Musgrove EA, Sutherland RL. Estrogen and antiestrogen regulation of cell cycle progression in breast cancer cells. Endocr Relat Canc 2003, 10(2):179–186.

40. Dickson RB, Lippman ME. Growth factors in breast cancer. Endocrine reviews 1995, 16(5):559–589.

41. Jiang SY, Jordan VC. Growth regulation of estrogen receptor-negative breast cancer cells transfected with complementary DNAs for estrogen receptor. J Natl Cancer Inst 1992, 84(8):580–591.

42. Vignon F, Bouton MM, Rochefort H. Antiestrogens inhibit the mitogenic effect of growth factors on breast cancer cells in the total absence of estrogens. Biochem Biophys Res Commun 1987, 146(3):1502–1508.

43. Palmer HG, Gonzalez-Sanchez JM, Espada J, Berciano MT, Puig I, Baulida J, Quintanilla M, Caño A, de Herreros AG, Lafarga M, Munoz A. Vitamin D (3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of beta-catenin signaling. J Cell Biol 2001, 154(2):369–387.

44. Beer TM, Munar M, Henner WD. A Phase I trial of pulse calcitriol in patients with refractory malignancies: pulse dosing permits substantial dose escalation. Cancer 2001, 91(12):2451–2459.

45. Masuda S, Jones G. Promise of vitamin D analogues in the treatment of hyperproliferative conditions. Mol Cancer Ther 2006, 5(4):797–808.

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