ERRα/VDR Axis Promotes Calcitriol Degradation and Estrogen Signaling Activation and Correlates with Poor Prognosis in Basal-like Breast Cancer Patients.

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Abstract

Background

Vitamin D is used to reduce cancer risk and improve the outcome of cancer patients, but the VDR pathway needs to be functionally intact to ensure the biological effects of circulating calcitriol. Besides ERα, another nuclear receptor, ERRα, has recently been shown to interfere with the VDR pathway, but its role in the cytotoxicity and transactivation activity of calcitriol is completely unknown in breast cancer (BC).

Methods

We investigated the function of ERRα on BC cell proliferation and calcitriol cytotoxicity and transactivation activity by silencing ERRα expression. We then performed a colony formation assay and cell cycle analysis to assess cell proliferation, and western blot and RT-PCR to investigate underlying mechanisms of cytotoxicity and VDR genomic action. Immunofluorescence was used to investigate VDR and ERRα cellular distribution. Bioinformatics analyses were performed to uncover the interaction network of VDR and ERRα. The translational significance of both bioinformatics and in vitro results were studied in the TCGA-BRCA (BReast CAncer) cohort.

Results

ERRα functionally supported the proliferation of BC cell lines and acted as a calcitriol-induced co-activator of the VDR complex. As such, ERRα deregulated the calcitriol/VDR genomic action by enhancing CYP24A1 and both ESR1 and aromatase (CYP19A1) expression in calcitriol-treated cells. In contrast, ERRα functionally supported calcitriol cytotoxicity by enhancing calcitriol- induced G0/G1 phase cell cycle arrest and by affecting the expression of cyclin D1 and p21/Waf[1]. The interactome analysis suggested PPARGC1A and PELP-1 were key players in genomic actions of the calcitriol/VDR/ERRα axis. Evaluation of patients' outcome in the TCGA dataset definitely showed the translational significance of VDR/ERRα biological effects, highlighting that VDR-CYP24A1-ESRRA overexpression correlates with poor prognosis in basal-like breast cancer setting.

Conclusions

Collectively, our findings identified a novel VDR/ERRα axis in breast cancer through which ERRα promotes the corruption of VDR genomic action and drives worsening of prognosis in BC patients.

Background

Breast Cancer (BC) still remains a deadly disease despite the significant advances in treatment strategies [1]. Hence, the molecular mechanisms of cancer progression need to be further explored and potential biomarkers identified to improve diagnosis and the prognostic classification of breast cancers.

Recently, a large body of epidemiological studies has highlighted a strong association between vitamin D deficiency and increased risk of breast cancer development as well as worse outcome [2, 3]. Therefore, much attention has been directed to using vitamin D to reduce cancer risk and improve the prognosis and outcome of breast cancer patients [4]. In addition to its classic role in regulating mineral homeostasis and bone metabolism, vitamin D is known to exert several anti-proliferative and pro-differentiating effects through its derivative, the steroid hormone calcitriol, in a wide range of tumors including breast cancer. The anticancer activity of calcitriol is mostly mediated via genomic actions through binding to the vitamin D receptor (VDR) and activation of the VDR and retinoid X receptor (RXR) heterodimeric complex, which in turn recruits co-factors on vitamin D response elements (VDREs) to induce the expression of target genes [5]. Numerous studies have highlighted that high expression levels of VDR in breast cancer tissues are associated with favorable tumor-related prognostic factors and a decreased risk of breast cancer death [6–9]. The antitumor effects of the vitamin D pathway also depend on the levels of the CYP24A1 catalytic enzyme that maintains the levels of circulating calcitriol stable through its conversion to inactive metabolites [10]. Nevertheless, the significance of CYP24A1 expression level as an independent prognostic factor in breast cancer is still a matter of debate [11–13]. The mechanisms by which the calcitriol/VDR axis promotes protective actions from breast cancer are numerous [14], though interference with estrogen receptor (ER) signaling and with aromatase enzyme (CYP19A1) activity [15] has been frequently described. Recent studies have reported that calcitriol can inhibit proliferation of ER-negative cell lines [16, 17] and have shown that calcitriol induces the expression of functional ERα in such cells, thus suggesting that the growth-suppressive action of calcitriol is not solely mediated through the ER pathway in breast cancer. Because of its functional kinship with ERα, much attention has been focused over the past decade on ERRα (estrogen-related receptor alpha) as an important biomarker in ER-negative breast cancer [18]. ERRα is a constitutively active nuclear receptor, still lacking a natural ligand, which controls the expression of genes involved in oxidative phosphorylation, lipid metabolism and the tricarboxylic acid cycle. Growing evidence suggests that ERRα plays a central role in coordinating oncometabolic programs that fuel cancer cell proliferation, migration and metastasis [19], apart from being an important component of proliferative signaling networks [20]. High levels of ERRα expression are associated with a poor prognosis in breast cancer [21] while several reports have described ERRα as a predictive biomarker of response to endocrine therapy in the same setting [22–24]. Recent studies have described a novel cross-talk between ERRα and the vitamin D pathway in diabetes [25]. Astninski et al., indeed, demonstrated that the induction of CYP24A1 by fasting was regulated through a peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α)-ERRα-dependent mechanism, showing, for the first time, a role for ERRα in the suppression of vitamin D signaling. Among interactors of VDR, Battaglia et al. [26] highlighted the role of Lysine-specific demethylase 1A (LSD-1), also known as KDM1A, in the corruption of VDR activity in prostate cancer, and Carnesecchi et al. [27, 28] reported a close interaction between ERRα and LSD-1 to regulate each other, mostly in aggressive cancers. Collectively, these findings prompted us to evaluate the function of ERRα in the corruption of the VDR signaling network in breast cancer in vitro and through a bioinformatics approach to explore the relevant interactions underlying the biological behavior of ERRα. Our findings have demonstrated that ERRα serves the cytotoxic activity of calcitriol while acting as a co-activator for VDR to boost the expression of CYP24A1 and trigger that of ERα and aromatase. More importantly, starting from the hypothesis that ERRα

overexpression may induce drastic changes in VDR genomic actions, our bioinformatics analysis revealed that simultaneous ERRα/VDR/CYP24A1 overexpression is significantly correlated with shorter survival in patients.

Materials And Methods

Cell cultures

Human breast cancer MCF7 cell line was purchased from ATCC. SUM149PT cells were purchased from the JCRB Cell Bank. The MDA-MB-231 breast cancer cell line and the bona fide normal breast cell line MCF 10A were generously gifted to us by Prof. Stephan Reshkin, Dipartimento di Bioscienze, Biotecnologie E Biofarmaceutica - University of Bari. The cell lines were stored in liquid nitrogen at very early passages before use. All the cell lines were cultured as reported in Additional file 1.

ERRα Knockdown procedure

For transient small interfering RNA (siRNA) transfection, the cells were transfected using the siPORT-NeoFX Transfection Reagent (ThermoFisher). The siPORT-NeoFX agent was diluted at 1:20 in the OPTI-MEM medium (ThermoFisher) and mixed to the ERRα siRNA (s4830) and Silencer® Select Negative Control siRNA (4390843) to allow transfection complex formation (siRNA 5 nM); the mixture was then dispensed into six-well plates containing the cell suspension. Transfected cells were incubated in cell culture condition ready for assay. All the cells were tested for ERRα downexpression and siRNA was considered efficient when the ERRα expression was inhibited by at least 60%-70% compared to the Select Negative Control siRNA as shown in figure S1 (Supplementary Figure S1).

Statistical analyses

Gene expression data, namely delta-delta Ct values, were compared through an analysis of variance model (“aov” function). The fitted model was then analyzed through a post-hoc test (Tukey Honest Significant Differences, “TukeyHSD” function) to know which pairwise comparison was significant. The “stats” R package was used (R v3.5) and p-values were considered to be significant when $p \leq 0.05$.

Interactome analysis

An extended network was built through BioGRID data using PSICQUIC (Proteomics-Standard-Initiative-Common-QUery-InterfaCe) for the retrieval of interaction data to identify the interactors of VDR and ESRRA. VDR and ESRRA were then queried and Pathlinker was used to identify the shortest path network. All the steps were performed in Cytoscape v.3.7.1. Moreover, to obtain a directed network through the Cluepedia + Cluego app, the subnetwork was enriched with information derived from the STRING database.

Pathway cross-talk analysis in TCGA BReast CAncer dataset

RNA-Seq FPKM, survival data and molecular subtype information were retrieved with the TCGAbiolinks package (2.13.6) [30]. The StarBioTrek package (1.10.0) was used to perform pathway crosstalk analysis [31]. In particular, Biocarta pathway information was integrated with PHint (PHysical interaction) network data. Basal cases were dichotomized for ESRRA expression through the “dichotomize” function of the Binda package (1.0.3). The survival curves were obtained and the log-rank test was performed with the Survival R package (3.1.8). All the analyses were carried out in the R 3.6 environment.

The detailed methods for cell culture conditions, treatments, colony formation assay, RNA extraction and qRT-PCR, immunofluorescence, cell cycle analysis by flow cytometry and cell target modulation by western blotting have been described in Additional file 1.

Results

ERRα, VDR and RXR basal expression in tested breast cancer cells

We first evaluated the expression levels of ERRα, VDR and RXR transcripts in the MCF7, MDA-MB-231 and SUM149PT cells based on our hypothesis that these biomarkers may affect the response to calcitriol. The real time evaluations, performed by using bona fide normal MCF 10A cells as a reference, showed that a higher ERRα mRNA level was found in SUM149PT cells compared to MCF7 and MDA-MB-231 (though it did not reach the significant p value, $p = 0.08$); (Fig. 1a). The VDR transcript levels were lower in the MDA-MB-231 ($p = 0.004$) and MCF7 ($p = 0.05$) cells than in the SUM149PT (Fig. 1b) cell line; while no significant difference was found among the three cell lines in the basal RXRα mRNA levels (Fig. 1c). Since our focus was on the calcitriol degrading enzyme and estrogen signaling, we determined the basal expression levels of the CYP24A1, ESR1 and CYP19A1 transcripts (Supplementary Figure S2). Collectively, these data pointed out that the SUM149PT cell line showed the highest expression levels of both the VDR and ERRα transcript, while there was no significant difference regarding the CYP24A1 and CYP19A1 expression levels. As expected, unlike MCF7, which is an ER + luminal A breast cancer model, both MDA-MB-231 and SUM149PT displayed barely detectable levels of ESR1 since they represent triple negative breast cancer models. [32].

Genomic effects of the calcitriol/VDR axis: focus on the calcitriol degradation enzyme, CYP24A1 and the estrogen pathway

Next, to explore the genomic action of VDR, we challenged the cells with 100 nM calcitriol, which is the concentration generally used to study the effects of VDR activation [33]. We found that CYP24A1 transcript expression rapidly increased after 4 h of calcitriol treatment in SUM149PT cells (> 500 fold and > 50 fold over the vehicle-treated cells; $p = 0.004$) (Fig. 2a) and further increased after 24 h of treatment (> 10000 fold and > 1000 fold over the vehicle-treated cells;

p = 0.004) in MCF7 cells (Fig. 2b). CYP24A1 transcript expression increased to a lesser extent in MDA-MB-231 cells than in the SUM149PT and MCF7 cell lines. It was about 2 fold greater than in the vehicle-treated cells (p = 0.01) after 4 h of calcitriol (Fig. 2a), and up to 3 fold greater than in the vehicle-treated cells (p = 0.22) after 24 h of treatment (Fig. 2b). Given that Santoz-Martinez et al. reported that 100 nM calcitriol induced the expression of a functional ERα in the MDA-MB-231cell line [17], and we hypothesized a functional interaction between VDR and ERRα that may activate estrogen signaling, we also determined the effect of calcitriol on the expression of ESR1 and CYP19A1 transcripts. We found that 100 nM calcitriol induced a time-dependent stimulation of ESR1. ESR1 transcript levels were more than 1 fold higher in SUM149PT cells than in the vehicle-treated cells (p = 0.03) by 4 h, and more than 3 fold higher than in the vehicle-treated cells (p = 0.02) (>) (Fig. 2c) after 24 h of calcitriol treatment (>) (Fig. 2d), while a transient stimulation of ESR1 transcript occurred only after 4 h (p = 0.01) in MDA-MB-231 cells (Fig. 2c). Calcitriol did not significantly modulate ESR1 gene expression in MCF7 cells (Fig. 2c-d). A slight but significant induction of CYP19A1 transcription (p = 0.03) occurred by 4 h (Fig. 2e) in the MDA-MB-231 cells, but it was no longer detectable after 24 h of treatment (Fig. 2f). Aromatase transcript levels increased in the MCF7 cells (> 1 fold higher than in the vehicle-treated cells p = 0.04) and to a much greater extent in the SUM149PT cells by 24 h (> 14 fold higher than in the vehicle-treated cells p = 0.007) (Fig. 2f). Collectively, our findings demonstrated that in the SUM149PT cell line calcitriol strongly induced the expression of its degrading enzyme (CYP24A1) as well as of key estrogen signaling biomarkers. We thus chose the SUM149PT cell line to assess the role of ERRα in the biological behavior of VDR in a representative model of triple-negative, inflammatory breast cancer falling within the most aggressive Basal-like subtype of BC (BLBC), and the MCF7 cell line for the same purpose in a Luminal A breast cancer model that is less invasive and aggressive.

ERRα loss of function abrogated VDR genomic action on CYP24A1, ESR1 and CYP19A1, but activated that on KDM1A

To investigate the biological function of ERRα on calcitriol/VDR genomic action, MCF7 and SUM149PT cell lines were treated with 100 nm calcitriol, after the cells had been transfected with siRNA targeting ERRα or with negative control (NC). Knockdown of ERRα restored the basal expression of CYP24A1 in both SUM149PT (p = 0.0003) and MCF7 (p = 0.01), thus completely abrogating the effect of calcitriol on its degrading enzyme (Fig. 3a). Remarkably, ESR1 expression also decreased and returned to its basal level in SUM149PT cells (p = 0.0008) (Fig. 3b), and the same happened to CYP19A1 transcript in both MCF7 (p = 0.009) and SUM149PT cell lines (p = 0.03) (Fig. 3c). These results suggest that ERRα was a crucial coactivator for the VDR transcription complex to carry on a program leading to calcitriol degradation and activation of estrogen signaling. Of note is that this phenomenon occurred to a higher extent in the basal-like model than in the luminal A model. Recently, Battaglia et al. reported that LSD-1 mediated the epigenetic corruption of vitamin D signaling in prostate cancer [26], and Carnesecchi et al. reported a close interaction between ERRα and LSD1 to regulate each other, mostly in cancer cell invasive behavior [27, 28]. In particular, LSD-1 was involved in the maintenance of ERRα protein stability, while the ERRα protein induced LSD-1 to erase repressive marks in vitro, thereby promoting the transcriptional activation of genes involved in the invasion of the extracellular matrix. Hence, we explored the effect of ERRα silencing on KDM1A expression upon calcitriol treatment to gain insights into the functional interaction of ERRα and KDM1A in VDR signaling in BC. Interestingly, ERRα silencing did not alter KDM1A expression in either cell line (Supplementary Figure S3) while calcitriol treatment significantly upregulated the mRNA expression of KDM1A only in transfected SUM149PT cells (Fig. 3d).

Effect of ERRα knockdown on cell clonality, calcitriol cytotoxicity and underlying mechanisms

To assess whether ERRα influenced tumor cell proliferation and sensitivity to calcitriol, we first tested the effect of single treatments –either calcitriol or ERRα knockdown– on cell clonality and then we tested the effect of the combined treatment. The results of colony formation assays indicated that i) calcitriol induced a concentration-dependent reduction of the numbers and size of colonies in both cell lines, with MCF7 cells being the most sensitive to calcitriol (data reported as supplementary material (Supplementary Figure S4) ii) ERRα knockdown significantly reduced colony formation in both cell lines (Fig. 4a-4d) iii) by contrast, ERRα silencing abrogated calcitriol cytotoxicity in SUM149PT cells and strongly reduced it in the MCF7 cell line. Calcitriol reduced colony formation in MCF7 much less than in non-silenced cells (si-NC + calcitriol) (Fig. 4a-4d). Since estrogens preferentially induce cyclin D1 to trigger breast cancer proliferation while p21 is transcriptionally regulated by ERRα to remove constraints in tumor progression [34], we evaluated the function of ERRα in the expression of these targets and in VDR protein expression to explore the potential regulatory mechanism of sensitivity to calcitriol. We found that calcitriol induced an increase in VDR protein expression in both cell lines in ERRα-silenced cells and in ERRα-expressing cells (transfected with si-NC), meaning that VDR activation occurred [35] irrespective of ERRα expression. However, by comparison, calcitriol reduced cyclin D1 expression in si-NC-MCF7 (control) cells to a much greater extent than in si-ERRα-MCF7 cells, while no effect was observed on p21 expression in both. By contrast, calcitriol increased p21 expression in si-NC-SUM149PT cells much more than in si-ERRα-SUM149PT cells, while no variation was found for cyclin D1 expression (Fig. 4c-4f). Accordingly, the data on gene expression showed that p21 was regulated by ERRα in SUM149PT cells, as ERRα silencing significantly upregulated p21 in the SUM149PT cell line and not in MCF7 cells (Fig. 4g). Target modulation was reflected at the level of cell cycle progression. Calcitriol caused G0/G1 phase cell cycle arrest in both SUM149PT and MCF7 cells while combination with ERRα-targeting treatment abrogated the effect of calcitriol on the cell cycle in both cell lines (Fig. 4b-4e). Collectively, the results indicated that ERRα supported proliferation in both cancer models. Our findings suggested that, although a preferential involvement of ERRα conveyed sensitivity to calcitriol in SUM149PT cells while ERα did so in MCF7 cells, ERRα was crucial for the tumor-suppressive ability of calcitriol in both tumor models, which is in line with the ability of ERRα and ERα to interfere and collaborate each other as demonstrated by their co-regulation of several common target genes [36].

VDR and ERRα cellular localization in MCF7 and SUM149PT cells after calcitriol treatment

To further address a VDR and ERRα interaction, we performed immunofluorescence analysis to visualize the cellular distribution of VDR and ERRα in calcitriol-treated cells versus vehicle-treated cells. As shown in Fig. 5a, time-dependent nuclear accumulation of VDR and ERRα was observed in SUM149PT cells, in which both nuclear receptors basically co-localized after treatment with calcitriol. The MCF7 cell line showed a higher basal ERRα expression in the nucleus, unlike VDR. Upon calcitriol treatment both ERRα and VDR increased in the nucleus (Fig. 5b). Consistent with data we reported before, immunofluorescence results suggest that VDR and ERRα interact and that their interaction is ligand-dependent in SUM149PT cells and ligand-enhanced in MCF7 cells. To examine whether this was a result of a direct interaction we performed a bioinformatics analysis.

Interactome analysis of VDR/ESRRA axis

An interactome analysis was set-up. Through BioGRID, we built an extended network to query VDR, ESRRA, ESR1, BRCA1 and KDM1A as main interacting protein hubs. Such a choice was based on ESRRA interacting proteins emerged by our study and by recent report showing a direct interaction between ESRRA and BRCA1 in BRCA1-mutated carriers [37], which is a setting represented by the SUM149PT cell line in our experiments, while others have demonstrated a direct interaction between ESRRA and KDM1A [28, 27]. The subnetwork, identified from the whole database (Fig. 6a), evidenced a cluster of 31 interacting proteins and was further analyzed through the STRING interaction database. This further analysis allowed to better explore the BioGRID subnetwork, highlighting the specific types of interactions connecting the nodes (Fig. 6b). Of note, PPARGC1A played a central role in the directed network, connecting VDR and ESRRA.

From biological network to pathway cross-talk

The impact of our in vitro results was studied in the TCGA-BRCA cohort. Cases were selected according to molecular subtypes in order to reflect the setting of cell lines, namely Basal-like (SUM149PT) and Luminal A (MCF7). After the selection step, the in silico cohort included 567 patients with Luminal A BC and 194 patients with basal-like BC.

In order to assess the pathway activity related to the VDR/ESRRA axis, the FPKM values of the KDM1A, BRCA1 and PPARGC1A genes were also included in the crosstalk analysis, given the roles they proved to have in our in vitro experiments and according to our interactome results. The StarBioTrek package was used because it is more informative than enrichment analysis in providing information on pathways and their relative cross-talk integrating networks and gene expression data. We found “control of gene expression by vitamin d receptor”/“regulation of pgc-1a” and “pelp1 modulation of estrogen receptor activity”/“control of gene expression by vitamin d receptor” crosstalks with AUC values of 0.55 and 0.52, respectively, using Biocarta pathway data integrated with PHint network information. We thus tried to identify the biological role of ESRRA by dichotomizing the basal-like subset for its expression. Interestingly, we detected the same crosstalks as in the previous comparison with AUC values of 0.67 and 0.66, respectively in the ESRRA overexpressing group. Such a result is promising because ESRRA stratification is able to biologically discriminate basal cases with more elevated AUC values than a basal-like versus Luminal A group analysis.

Translational significance of ESSRA/VDR axis and survival in TCGA dataset

Literature data and our in vitro and in silico results left the prognostic value of the VDR-CYP24A1-ESRRA axis open to question. Overall survival data of basal-like patients were downloaded and the patients were stratified into two groups according to whether VDR-CYP24A1-ESRRA simultaneous overexpression was present or not. The Kaplan-Meier curves (Fig. 6c) and log-rank test showed that patients overexpressing VDR-CYP24A1-ESRRA genes had a significantly worse survival than the other group (p-value = 0.017), clearly indicating a prognostic value of such a biomarker signature for basal-like breast cancer.

Discussion

To the best of our knowledge, no evidence has been reported on the interplay between VDR signaling and ERRα in breast cancer. In this study, by hypothesizing a convergence of signaling, we uncovered a novel ERRα/VDR axis through which ERRα promoted a putative mechanism of vitamin D deficiency and corruption of VDR genomic action by activating estrogen signaling in breast cancer cell lines. Here, ERRα was identified as a calcitriol-induced co-activator of the VDR complex and as a regulator of calcitriol/VDR antitumor action in both ER-positive and ER-negative breast cancer models. Functionally, ERRα sustained the proliferation of BC cell lines and up-regulated the expression of CYP24A1 (the enzyme that catalyzes calcitriol degradation), ESR1 and CYP19A1 in calcitriol-treated cells. In contrast, ERRα functionally supported calcitriol-dependent inhibition of clonogenic cell survival. Although the latter appears to be a controversial result, it may be explained if we hypothesize potential points of ERRα-ERα crosstalk. There is growing evidence that calcitriol promotes breast cancer-protective actions in ERα-positive tumors, mostly because it constrains estrogen signaling effects [4]. We found that calcitriol reduced the clonogenic survival of both MCF7 and SUM149PT cells, while inducing ESR1 expression in the SUM149PT cell line. Therefore, we can speculate that an ERα-dependent activity of ERRα mediated the antiproliferative function of calcitriol in both cell lines. Since estrogens preferentially induce cyclin D1 to trigger breast cancer proliferation while p21 is transciptionally regulated by ERRα to remove constraints on tumor progression [34], we evaluated the effect of ERRα on the expression of such targets. Through loss of function experiments we demonstrated that ERRα abrogated calcitriol-induced upregulation of p21 in SUM149PT cells and strongly reduced calcitriol-induced downregulation of cyclin D1 in MCF7 cells. Such target modulation was also reflected in cell cycle progression and clonogenic survival, further supporting the notion that ERRα-ERα crosstalk regulated sensitivity to calcitriol in both cancer models, while ERRα caused deregulation of VDR genomic action mostly in the basal-like model. After a well-known ERRα regulator, KDM1A, [30] was recently observed to be involved in the corruption of vitamin D signaling in prostate cancer, we assessed the ERRα-KDM1A connection in the VDR pathway of BC. We found that KDM1A expression was upregulated by silencing ERRα in calcitriol-treated SUM149PT cells, basically suggesting that the ERRα–containing complex represses KDM1A transcription in the presence of active VDR. Since KDM1A is also involved in maintaining ERRα protein stability [27], we can speculate that KDM1A upregulation by calcitriol may have compensated the loss of ERRα by sustaining ERRα expression to promote ERRα-dependent deregulation of the VDR pathway. The bioinformatics analysis we carried out provided evidence of an interacting network in the ERRα/VDR axis, thereby strengthening our hypothesis regarding the connections between ERRα and ESR1 and between ERRα and KDM1A. In line with the pivotal role of PGC-1α as a key regulator of metabolic

reprogramming in advanced cancer [38; 39], PGC-1α emerged as a central mediator in the directed network connecting VDR and ESRRA, thus supporting the notion that a PGC-1α /ERRα-containing complex drives a program that alters vitamin D metabolism in advanced breast cancer. Furthermore, since a high ERRα expression has been associated with tumor aggressiveness [19], we performed a pathway cross-talk analysis that measured the activity of pathways and their relationships to provide evidence of the biological effects triggered by ERRα overexpression. The analysis showed a crosstalk between “control of gene expression by VDR” and the “regulation pathway of PGC-1α” highlighting the existence of an interaction between the VDR/ERRα axis and PGC-1α-dependent metabolic function. A connection was also detected between “control of gene expression by VDR” and the “PELP1 modulation of estrogen receptor activity”, indicating crosstalk between the VDR/ERRα and PELP1/ERα pathways in patients.

PGC-1α is a coactivator of VDR [40] and a regulator of ERRα [41; 19], while PELP1 is a coactivator of ERα and it is involved in epigenetic modifications of the aromatase promoter through interactions with ERRα and KDM1A [42; 43] to induce *in situ* estrogen synthesis. We thus hypothesized a model of ERRα/PGC-1α/VDR-mediated gene regulation in which ERRα acts as a VDR coactivator and as the protein connecting VDR and estrogen signaling to induce estrogen activation, perhaps by modulating the demethylating activity of KDM1A through interaction with PELP1. (Fig. 7). Since the best record in terms of pathways cross-talk was achieved in the BLBC setting and, collectively, our findings supported the view that i) ERRα deregulated VDR function mostly when it was highly expressed in the BLBC setting and ii) calcitriol induced an increase in VDR and CYP24A1 expression in both in vitro models, we assessed the prognostic significance of a simultaneous overexpression of ERRα, VDR and the target gene CYP24A1 in BLBC. This approach pointed out the translational potential of such a signature by showing that overexpression of all three biomarkers definitely defined a poor prognosis in BLBC patients and may be correlated with a reduction in circulating calcitriol.

Conclusions

Our findings pointed out that i) ERRα plays a role in vitamin D metabolism and sensitivity in breast cancer, ii) the ERRα/VDR axis is at the crossroads of estrogen signaling activation, and iii) the simultaneous overexpression of ERRα, VDR and CYP24A1 is correlated with poor prognosis in basal-like breast cancer.

Collectively, our results confirm ERRα as a master regulator of onco-metabolic and proliferating signals in breast cancer, and provide insights into the molecular mechanisms underpinning VDR genomic and anti-tumor action in advanced breast cancer. ERRα may lead to a defective vitamin D pathway, which, as suggested by Feldman et al. [4], would make vitamin D administration less effective or even harmful in this setting.

Abbreviations

BC: Breast Cancer; VDR: vitamin D receptor; RXR: retinoid X receptor; VDREs: vitamin D response elements; ERα/ESR1: estrogen receptor alpha; ERRα/ESRRA (estrogen-related receptor alpha) ; siRNA: small interfering RNA; PSICQUIC: Proteomics-Standard-Initiative-Common-QUery-InterfaCe; BLBC: Basal-like subtype of BC; NC: negative control.

Declarations

- Ethics approval and consent to participate: not applicable
- Consent for publication: not applicable
- Availability of data and materials:

The datasets analysed during the current study were generated by the TCGA Research Network [https://www.cancer.gov/tcga.] and are available in the GDC data portal repository [https://portal.gdc.cancer.gov/projects?filters=%7B%22content%22%3A%5B%7B%22op%22%3A%22in%22%2C%22content%22%3A%7B%22field%22%3A%22projects.program.name%22%2C%22value
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Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

KD and LP designed, performed the experiments and wrote the manuscript; SDS performed all statistical and bioinformatic analyses; RDF and SS performed western blot and immunofluorescence experiments and assisted in figure preparation; BP and RL editing of the manuscript; AA and SS supervised research,

reviewed the manuscript and were involved in funding acquisition. All authors read and approved the final manuscript.

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Figures

Fig.1

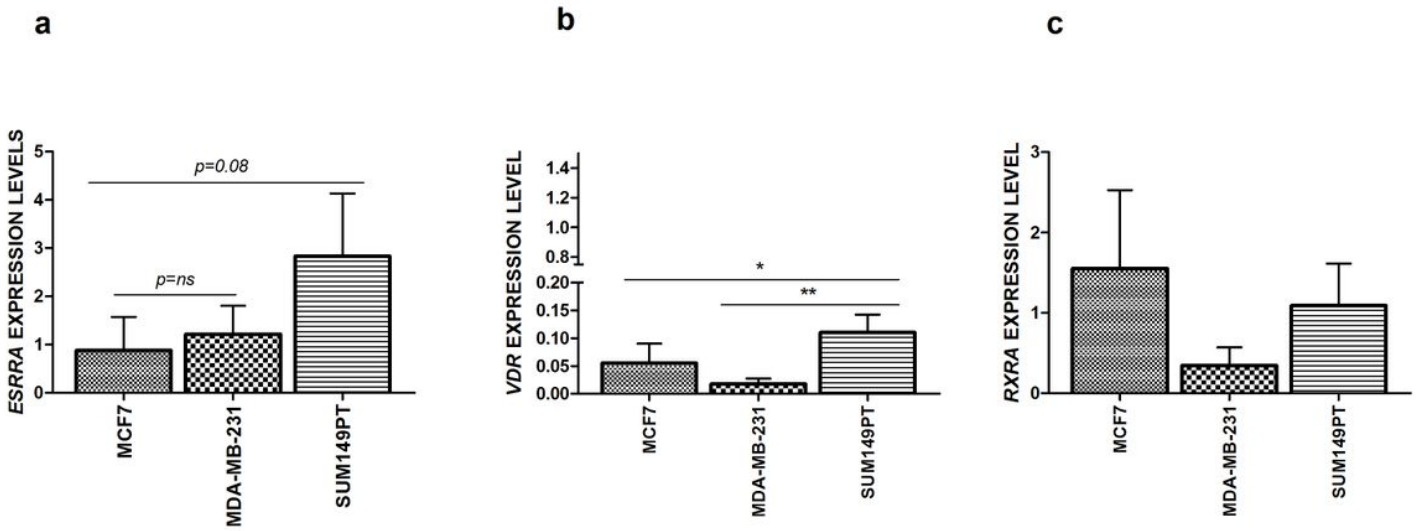

Figure 1

The basal levels of the three nuclear receptors transcripts are different in MCF7, MDA-MB-321 and SUM149PT breast cancer cells. a ESSRA gene expression b VDR gene expression and c RXRA gene expression was measured by qRT-PCR. Data was normalized to the levels of RN18S1 mRNA expression and presented as 2-△△CT. Data are representative of three independent experiments performed in duplicate and represents the mean ± SD; *: p≤0.05; **: p≤0.001 vs MCF 10A cells.

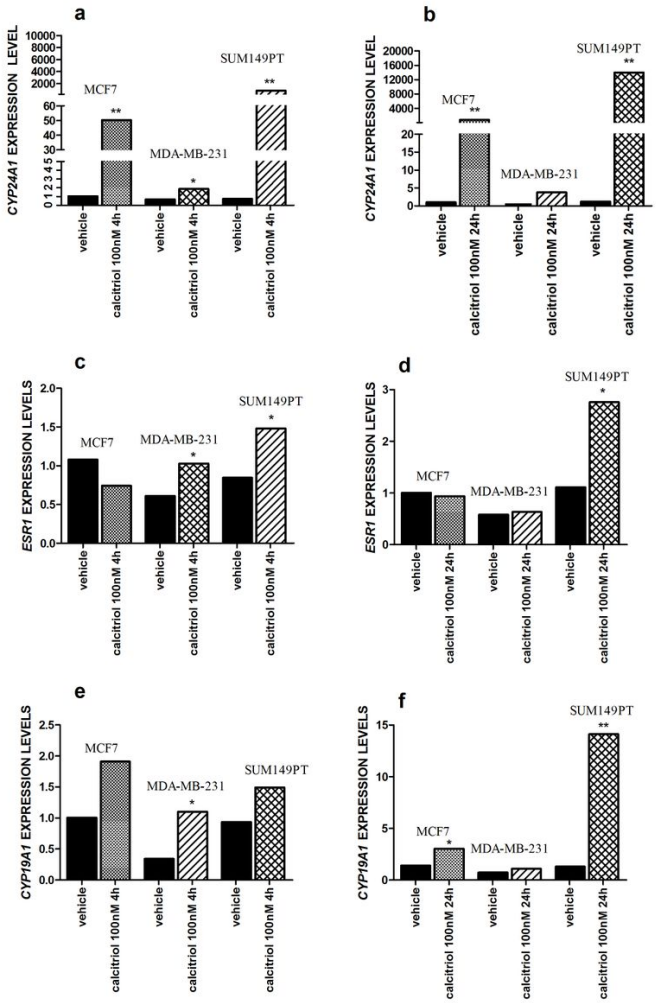

Figure 2

Calcitriol treatment induces the expression of genes involved in vitamin D metabolism and estrogen signaling in a time- and cell-depend manner. CYP24A1 fold change levels in MCF7, MDA-MB-321 and SUM149PT breast cancer cells by a 4h and b by 24h of calcitriol treatment; c ESR1 mRNA levels in MCF7, MDA-MB-321 and SUM149PT cells by 4h of calcitriol and d by 24h of calcitriol; CYP19A1 gene expression in MCF7, MDA-MB-321 and SUM149PT breast cancer cells e by 4h of calcitriol and f by 24h of calcitriol. Data was normalized to the levels of RN18S1 mRNA expression and presented as 2-△△CT. Data are representative of three independent experiments performed in duplicate and represents the median; *: p≤0.05; **: p≤0.001 vs vehicle cells.

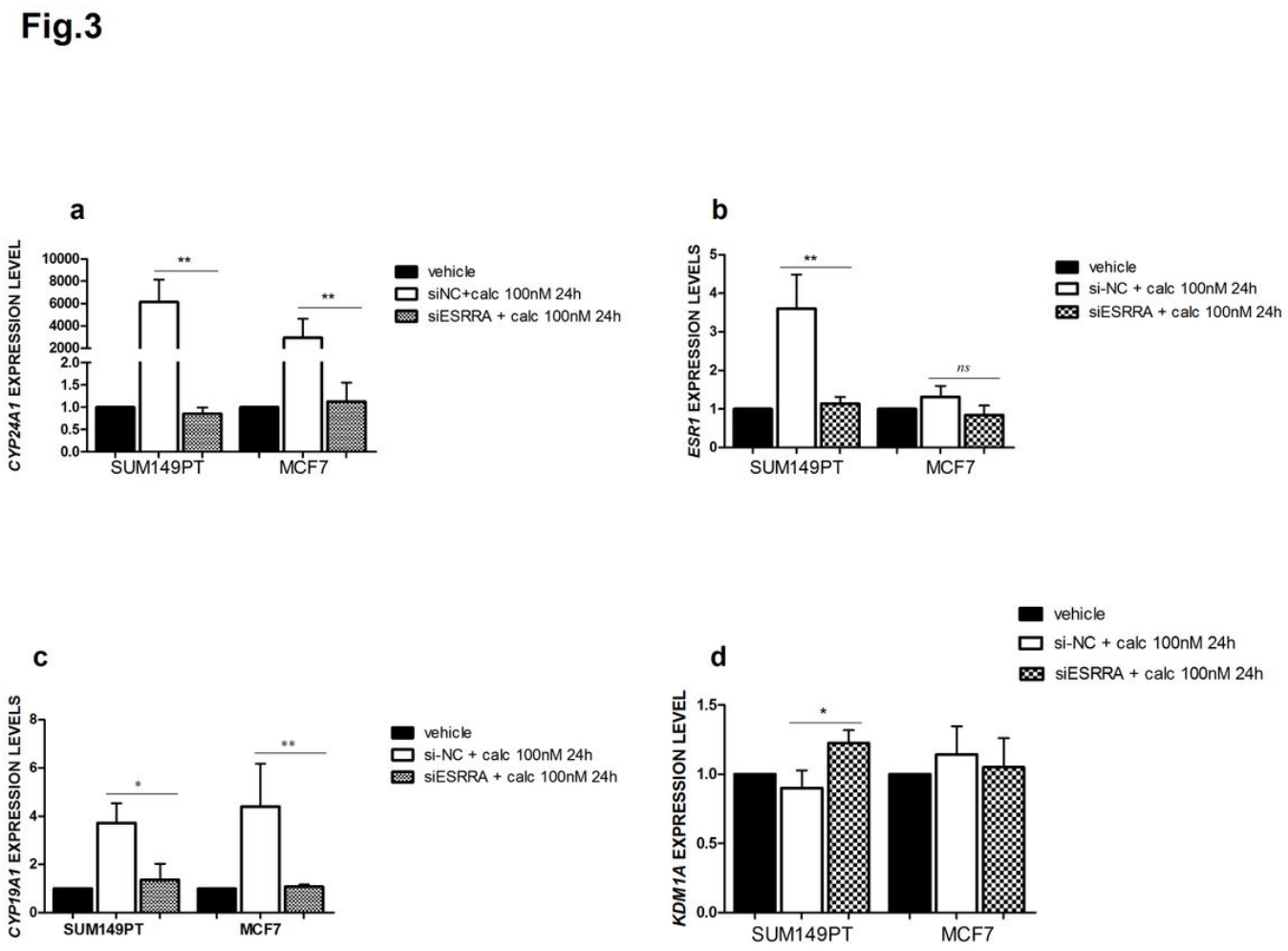

Figure 3

Effects of ERRα knockdown on VDR genomic action in MCF7 and SUM149PT breast cancer cells. Analysis for a CYP24A1, b ESR1, c CYP19A1 and e KDM1A mRNA in MCF7 and SUM149PT cells undergoing calcitriol treatment and si-ERRα. Moreover, d the impact of ERRα knockdown on KDM1 transcript was showed. Data was normalized to the levels of RN18S1 mRNA expression and presented as 2-△△CT. Data are representative of three independent experiments performed in duplicate and represents the mean ± SD; *: p≤0.05; **: p≤0.001 vs vehicle cells and calcitriol- empty vector cells or only empty vector cells.

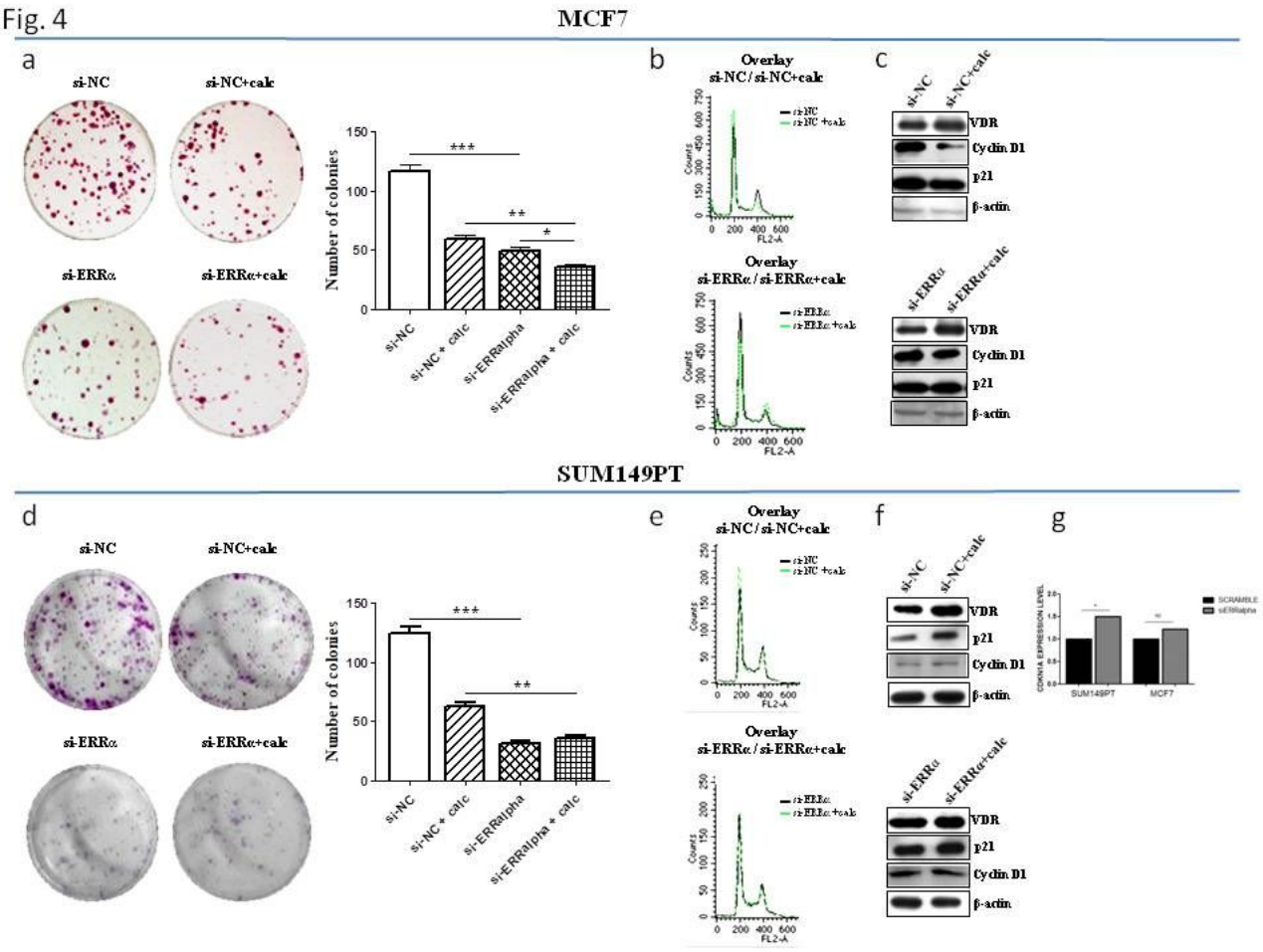

Figure 4

ERRα supports cells proliferation and serves calcitriol antitumor action. a Left, clonogenic survival assays in MCF7 cells and d SUM149PT cells treated with calcitriol IC50 concentration or/and 5 nM NC/si-ERRα. Right, statistics of the number of colonies is shown. Data are representative of three independent experiments performed in duplicate and represent the mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. b The overlay of the cell cycle analysis in MCF7 and e in SUM149PT cells treated with 100nM calcitriol or/and 5 nM NC/si-ERRα, assessed by FCM. c Western blot analysis showing the expression of cyclin D1 and p21 after treatment with 100nM calcitriol or/and 5 nM NC/si-ERRα in MCF7 and in f SUM149PT cell lines. g CDKN1A mRNA levels in MCF7 and SUM149PT cells undergoing ERRα silencing. Data is representative of three independent experiments performed in duplicate and represents the median value. $p \leq 0.05$ vs NC-ERRα. Ns indicated no significance.

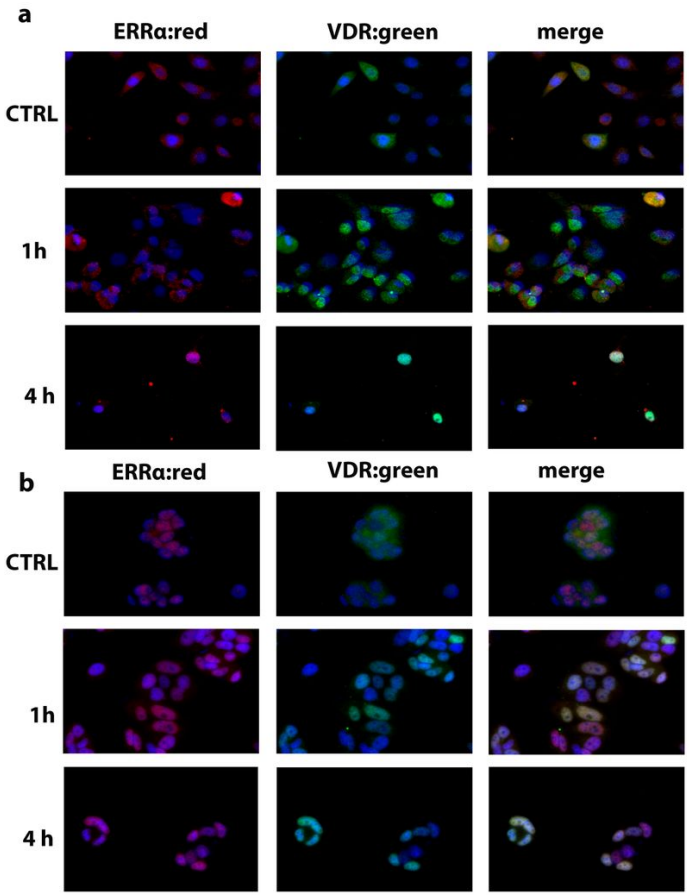

Figure 5

Immunofluorescence images showing the colocalization of ERRα and VDR. Images of a MCF7 cells and b SUM149PT cells after 1h and 4 h of calcitriol treatment. n = 3 independent experiments.

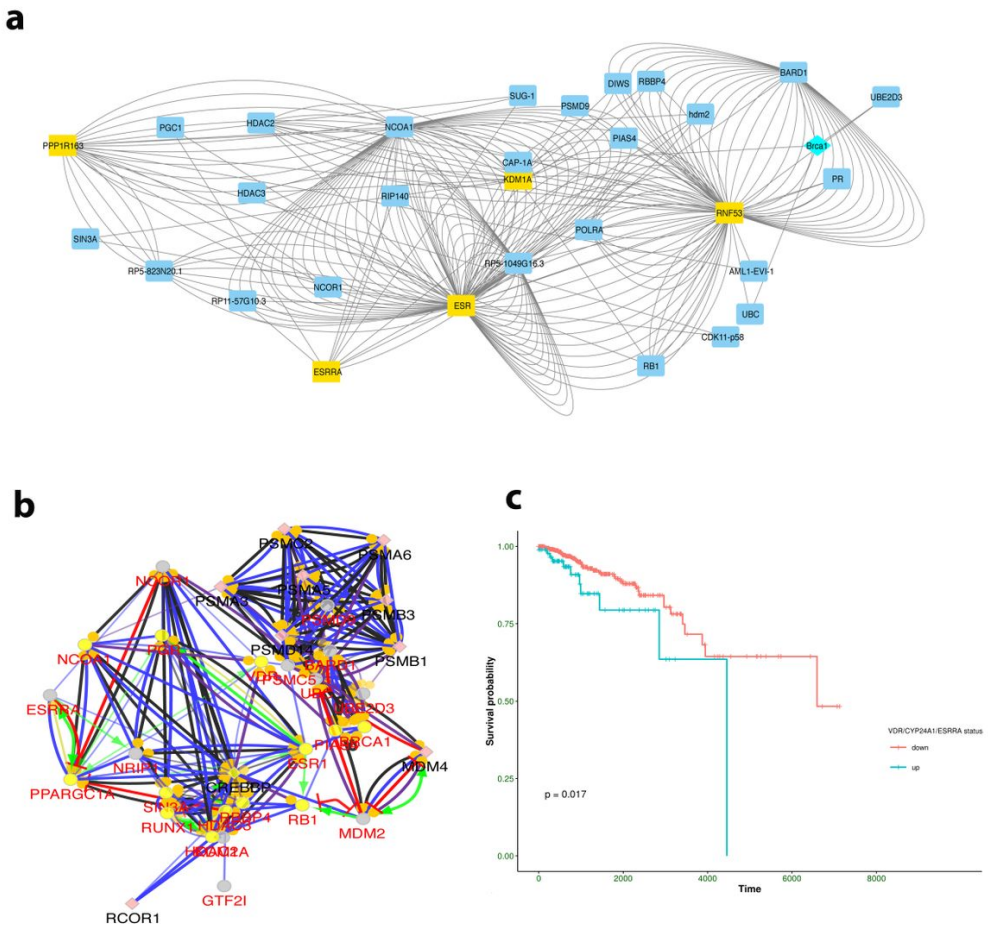

Figure 6

Interactome analysis and survival in TCGA dataset. a. BioGRID network including known interactors of VDR and ESRRA; b. STRING-based network enriched with the types of interactions linking nodes; c. Kaplan-Meier curves and log-rank test comparing overall survival of TCGA cases with simultaneous overexpression of VDR-CYP24A1-ESRRA (blue curve) and those without (red curve).

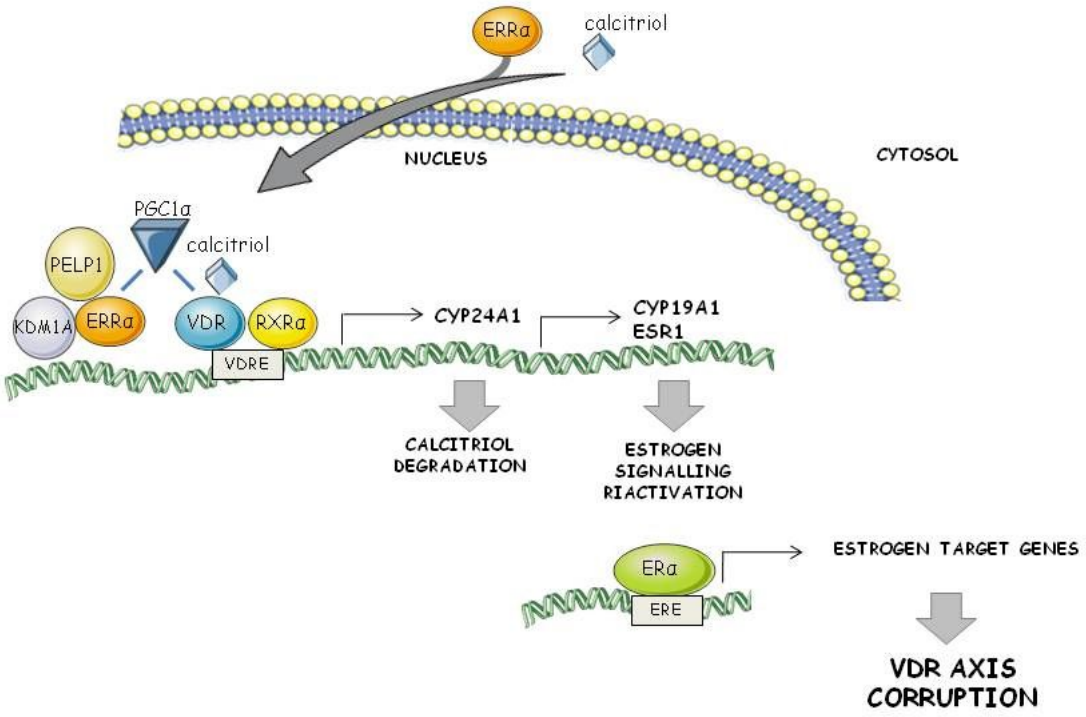

Figure 7

Graphical representation of the complex ERRα/PGC1α/VDR mediating gene expression regulation in loci in which ERRα acts as coactivator of VDR. Calcitriol promotes the translocation of ERRα from cytosol to nucleus. We thus hypothesize a model of mediated gene regulation in which PGC1α plays a key role by coupling VDR with ERRα. The latter NR (nuclear receptor) acts as either coactivator of VDR and as connecting protein between VDR and estrogen signaling, by interacting with PELP1 and KDM1. This transcriptional complex ERRα/PGC-1α/VDR boosts the expression of CYP24A1 and induces the activation of ESR1 and aromatase expression.

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