Author's response to reviews

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

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Version: 4 Date: 21 January 2014

Author's response to reviews: see over
Author's response to reviews

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

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Version: 2 Date: 20 January 2014

Author's response to reviews: see over
**Reviewer’s report**

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

**Version:** 1  **Date:** 5 October 2013

**Reviewer:** Filippo Acconcia

**Reviewer’s report:**

In this paper the Authors studied the effect of calcitriol and one vitamin D analogue for their ability to restore ERalpha expression in ERalpha negative breast cancers and breast cancer cell lines. In this way, the Authors propose these ‘novel’ ERalpha positive breast cancer cells become sensitive to anti-estrogen therapies.

Although the idea proposed in this manuscript is intriguing I have many concerns that preclude me to positively judge this work.

**Major Compulsory Revisions:**

A) It is not clear why one should treat ERalpha negative breast cancer with molecules that will re-express ERalpha and thus, in theory, restore estradiol signalling in breast cancer. As a matter of fact, one could argue that ERalpha positive tumours are indeed treated with drugs that induce ERalpha degradation (i.e., 4OH-tamoxifen; fulvestran). Thus a therapy that would give the opportunity to cancer cells to acquire a selective advantage in cell growth because of the ERalpha presence and than to treat them with anti-estrogens appears to be a little convoluted. Moreover, if ones put this work into a clinical perspective, women that undergo surgical removal of the breast tumour still have their ovaries functional and circulating estradiol levels. Thus, one may expect that re-expressing ERalpha in the remaining cells would have a detrimental impact for the patient, who will further undergo treatment with many drugs (i.e., calcitriol and anti-estrogens) and consequently many possible side effects.

The Authors should clarify these points addressing them with a specific experimental design that would highlight benefits of such potential therapeutic approach.

We deeply thank the reviewer comments and suggestions aimed at improving our manuscript. We understand the concern of the reviewer regarding the re-expression of the ERα in previously negative ERα-cells. Herein we will expose the reasons why this, we believe, is a positive effect of calcitriol that allows for anticancer treatment.
Estrogens are mitogenic in ER-positive cells and anti-estrogens are an efficient adjuvant therapy for these tumors. However, on the other hand, the fact that estrogens and their receptors protect against cancer cell invasiveness through distinct mechanisms in experimental models may explain why the presence of ERα is associated with well-differentiated and less invasive tumors (Critical Reviews in Oncology/Hematology, 51 (1):55–67, 2004). Indeed, the re-expression of the ERα is a sign of differentiation, as opposed to de-differentiation, which is the characteristic process involved in carcinogenesis. One of the most prominent anticancer effects of calcitriol is its pro-differentiating action, which we believe is what is happening in our breast cancer cells. Acquiring the ERα is in fact a positive feature, since ERα is associated with a more favorable prognosis in breast cancers (Critical Reviews in Oncology/Hematology, 51 (1):55–67, 2004).

In our study, calcitriol induced ERα re-expression allowing treating the cells with anti-estrogens, which act through the ER in order to reduce growth. In fact, after calcitriol exposure the cells were sensitive to hormonal therapy. Moreover, we demonstrated in this study that not only the antiestrogens inhibited proliferation, but incubations in the presence of estradiol, as the natural ER ligand, did not promote growth, as could have naturally been expected. The possible explanation of this fact might involve the well-known and deeply characterized antiproliferative and pro-apoptotic effects of calcitriol acting through its receptor, the VDR. Thus, the final result of the treatment with calcitriol plus antiestrogens is a win-win situation for cancer therapy, benefiting from the antiestrogenic effects of tamoxifen/ICI through the ERα, as well as the antineoplastic effects of calcitriol, acting through the VDR.

Furthermore, re-expression of a functional ERα has previously been used as a good strategy to render ERα-negative cells (difficult to treat and more aggressive) sensitive and responsive to the antiproliferative effects of ER antagonists (Cancer Res, 66:6370, 2006, doi: 10.1158/0008-5472.CAN-06-0402; Molecular and Cellular Endocrinology 314: (1) 17–22, 2010). From a clinical point of view, there is a critical need to improve treatment of women bearing breast cancers that lack the expression of hormone receptors, since for them, only cytotoxic chemotherapy is applicable. Therefore, we believe that the strategy of converting ER-negative tumors to ER-positive tumors, using a natural antineoplastic agent, namely calcitriol, could provide a new avenue for management of patients with endocrine-resistant breast cancers; however, as the referee pointed out, further studies highlighting the benefits of the data presented herein should be performed.

B) - I found the presented data too preliminary. In this regard, my main problem is with the conclusion that calcitriol restores ERalpha signalling and functionality. In details:

1: More than one ERalpha negative breast cancer cell line is required to make a general conclusion in order to exclude that it is a type-specific effect.
In this regard, seven different cell lines were tested for ERα regulation by calcitriol; which make our study by itself no preliminary. As depicted in figure 1A of the original manuscript (Fig. 2A in the revised version), this study included: cultured tumor-derived cells from five patients with ERα-negative breast tumors (ranged between 5 and 9 in the Scarff-Bloom-Richardson system score) as well as the ERα-negative SUM-229PE established cell line and the ERα-positive cell line BT-474. The results clearly showed that in all ERα negative cell lines (6 in total) calcitriol significantly induced ERα gene expression. Moreover, calcitriol stimulated ERα mRNA transcription in a concentration dependent manner, as depicted in figure 1B of the original manuscript (Fig. 2B in the revised version).

2: In figure 4, the authors did not measure cell growth in response to estrogens because they used a test that assays the cellular metabolic activity. Can the Author explain why re-expression of ERalpha does not lead to an increase in cell metabolism or in ‘cell growth’? The Authors should perform growth curves analysis as well as BrdU incorporation to directly connect with cell proliferation.

Regarding the question of why re-expression of ERα does not lead to an increase in cell growth, and although we do not have a clear answer, we believe that due to the possible well-known and deeply characterized VDR-dependent antiproliferative and pro-apoptotic effects of calcitriol they may counterbalance the mitogenic effects of estradiol by arresting the cells in G0/G1 phase of the cell cycle. This effect is associated with inhibition of genes related to proliferation as shown in this and other studies from our laboratory (Figure 1A, 1B and 1C). In support to this observation, and as depicted in figure 5C in the revised version, estradiol increased, as expected, cell growth in non-calcitriol treated ERα-positive cells.
Calcitriol inhibited cell proliferation and expression of genes involved in cell growth. A) Cultured breast tumor-derived cells were incubated in the presence of different calcitriol concentrations or its vehicle (V) during 0, 3 or 6 days, and cell growth was assayed by the XTT colorimetric method. Calcitriol significantly inhibited cell proliferation in a concentration-dependent manner. Results are expressed as the mean ± S.D. (n=6); different letters indicate statistical significance (P<0.05). Similar results were obtained using cells derived from different biopsies (Exp Cell Res, 316(3):433-42, 2010). B) Ki-67 and Eag1 are transcriptionally downregulated by calcitriol in breast cancer cells. The gene expression of Ki-67 (dark circle) and Eag1 (white circle) was downregulated by calcitriol in cultured breast tumor-derived cells (B) and SUM-229PE cells (C). Relative Ki-67 and Eag1 mRNA levels were obtained by normalizing against GAPDH mRNA expression. Vehicle values were set to one. N= at least 3, *P<0.05 vs control (PLoS ONE, 7(9): e45063, 2012).

In figure 3, one gene is not enough to make the statement of re-acquisition of estradiol sensitivity and ERalpha re-activation. pS2/Tiff (presenelin-1) and cathepsin D could be additional candidates. ERalpha phosphorylation in the serine residue 118 is a better marker of ERalpha transcriptional activation. Furthermore, the same experiments should be performed in the presence of VDR inhibitor and siRNA for VDR to demonstrate that the effect of calcitriol on re-activation of ERalpha is via VDR.

Following the recommendation of the reviewer, we used the following primers to further study the transcriptional functionality of the re-expressed ERα: catctttctttctacctgagca / gtctgtgccacccagcat for Homo sapiens cathepsin D (CTSD), (NM_001909.4), and cccctggtgcttc tatccta / gatccctgcagaagtgtctaaaa for Homo sapiens trefoil factor 1 (TFF1), (NM_003225.2). These two genes are estrogen-responsive genes. Breast cancer cells with ERα-negative phenotype were
incubated during 48 hr in the presence of calcitriol and further treated with estradiol or ICI-182,780. Afterwards, RNA was extracted and qPCR analysis was performed. The results showed that, unexpectedly, calcitriol per se significantly stimulated the expression of these genes (Figure 2A and B, respectively) in a similar manner as with other ERα-dependent gene: the progesterone receptor (PR) (Figure 2C). This interesting observation, which deserves further investigation, rendered us unable to observe any significant stimulation by estradiol. It was therefore important to us the fact that estradiol specifically stimulated PRL transcription only in calcitriol-treated cells (Fig 2D).

We did use a VDR inhibitor in this study; the results showed that TEI-9647 significantly inhibited calcitriol upregulation of ERα gene expression (figure 2C in the revised version).

![Graphs showing gene expression](image)

**Figure 2.** Calcitriol stimulated the expression of CTSD, TFF1 and PR, but not PRL. Breast cancer cells were incubated in the absence (black bars) or presence of calcitriol 1X10⁻₈ M (white bars) for 48 hr. Subsequently, cells were coincubated with or without calcitriol plus estradiol (E2, 1x10⁻⁸ M), ICI-182,780 (ICI, 1x10⁻⁶ M) or vehicle (C) for 24 hr. A) CTSD, B) TFF1, C) PR and D) PRL gene expression were determined by qPCR. Results are shown as the mean ± S.D. vs GAPDH mRNA normalized ratio. *P≤0.05 vs. C black bar.
4-In figure 1 and 2 VDR knockdown is required.

As recommended by the reviewer, additional experiments were performed in order to silence the VDR in both cultured breast tumor-derived cells and SUM229PE cell line. Unfortunately, the cells were not transfectable even using different transfecting reagents [PolyFect (QIAGEN Inc.), FuGENE (Roche Applied Science), Lipofectamine LTX with PLUS Reagent (Invitrogen)]. As in the previous query, a VDR antagonist was used to overcome these transfections difficulties. The results agreed with the concept that ERα expression by calcitriol was a VDR-dependent mechanism.

5-In general, standard deviations are very high to make any conclusion. Additional experiments should be performed to have a higher number of data to analyze.

Indeed the SDs are high; however, the sample size used allowed us to observe significant differences as depicted in the graphics of the revised version.

Minor essential revision:
1-Supplemental figures should be put in the main text.

As suggested by the reviewer, supplemental figures 1 is now included in the main text of the revised version of the manuscript. The other supplemental figures were eliminated considering that enough information is available for the purpose of the main objective of the study.

2-In figure 2 standard deviations and p values in densitometric analyses should be inserted.

The changes required by the reviewer have been made in new figure 3 of the revised version as recommended.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests:

I declare that I have no competing interests.
Reviewer’s report

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

**Version:** 1 Date: 13 October 2013

**Reviewer:** Antimo Migliaccio

**Reviewer’s report:**

The paper by Santos Martinez et al. shows that calcitriol induces expression of ER# in ER-negative breast cancer cells, thereby restoring the hormone-sensitive phenotype and anti-estrogen responsiveness. The experiments presented in the paper show that upon calcitriol treatment anti-estrogens down regulate cell growth of ER negative cancer cells and CCND1 and EAG1 potassium channel expression. This action is mediated by the Vitamin D receptor (VDR), as it is abolished by a VDR antagonist. This suggests a potential use of Vitamin D in the treatment of the hormone resistant breast cancer.

This is a potentially very interesting report as it address the question of the rescue of hormone-responsiveness of hormone independent cancers, which are characterized by a poor prognosis and reduced disease-free survival. The experiments shown in this paper undoubtedly demonstrate that calcitriol restores, at least partially, the hormone-dependence of ER cells, but say very little about the mechanism by which this occurs.

The main pitfall of this paper is the lack of mechanistic insight of the findings. Therefore I think that:

1- A study of ER promoter to identify sites for VDR could greatly improve the paper impact.

We thanks for the valuable referee’s comment. Indeed we have made a detailed *in silico* analysis using the MathInspector software (Bioinformatics, 21:2933-2942, 2005). With this analysis several vitamin D response elements were identified, and are included herein:
Therefore, we know added a new paragraph in the Discussion section addressing this important issue (page 14, line 16 of the revised version).

2- As the Authors hypothesize that the up regulation of ERα by calcitriol is probably mediated by MAPK, a MAPK assay, as well as analysis of other proteins possibly involved in the MAPK pathway (e.g...Src, Ras ,Mek) to investigate whether their activity is related to calcitriol action.

As recommended by the reviewer, a MAPK assay was conducted in ERα-negative breast cancer cells (SUM-229PE). Cells were incubated in presence of medium, ethanol, and calcitriol during 15, 30 min and 4 hr, and western blot analyses were performed. As seen in Figure 1, below, the western blot did not show significant differences among treatments, suggesting that ERα induction by calcitriol was independent of the MAPK pathway. Therefore, this suggestion was omitted from the discussion section, as seen in the revised manuscript. As discussed in the previous referee’s question, the possible mechanism involved in the regulation of the ERα by calcitriol might be the nuclear pathway via binding of the activated VDR to the vitamin D-responsive elements in the promoter region of ERα.
Figure 1. Modulation of MAPK ERK1 and ERK2 by calcitriol in ERα-negative breast cancer cells. A) SUM-229PE cells were incubated with medium (-), ethanol (C) and calcitriol (Cal, 1X10^-7 M) for 15, 30 min and 4 hr, and western blot analysis was performed. Integrated densities of phospho-p44 MAPK and phospho-p42 MAPK are shown in panels (B) and (C), respectively. Data were normalized by γ tubulin protein expression. Each bar represents the results of three independent experiments.

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:**

I declare that I have no competing interests.
Reviewer's report

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

**Version:** 1  **Date:** 16 October 2013

**Reviewer:** Diego Sisci

**Reviewer's report:**

In this manuscript, the authors demonstrate that Calcitriol restores the antiestrogen responsiveness of estrogen receptor negative breast cancer cells. They demonstrate that Calcitriol induces the expression of a functional ER in ER negative breast cancer cells and that it is mediated by vitamin D receptor. Functionally, ER expression reduces cell proliferation in response to antiestrogens treatment by decreasing CCND1 and EAG1 expression.

**Major comments**

1) The results are convincing and confirm the hypothesis; however, the involvement of VDR is only demonstrated by using an antagonist of the receptor, that is not sufficient, experiments overexpressing and/or silencing the receptor are required.

We deeply thank the reviewer comments and suggestions aimed at improving our manuscript. As recommended by the reviewer, we have done the majority of these studies in the past; however, cells were not transfectable even using different transfecting reagents [PolyFect (QIAGEN Inc.), FuGENE (Roche Applied Science), Lipofectamine LTX with PLUS Reagent (Invitrogen)] (see Figure 1 included herein). This technical difficulty was overcome by the use of a well characterized the VDR antagonist (J Biol Chem, 274(23):16392-16399, 1999; Mol Endocrinol, 14(11):1788-1796, 2000). As shown in figure 2C in the revised version, TEI-9647 inhibited calcitriol upregulation of ERα gene expression.
2) The regulation of PRL, CCND1 and EAG1 expression have been used as cell response to ER expression and function, this is not correct since both protein are regulated by ER through a not canonic mechanism. It would be better to use PS2 or Cathepsin D that respond to ER through an ERE.

Following the recommendation of the reviewer, we used the following primers to further study the transcriptional functionality of the re-expressed ERα: catcttctctttcatctgagca / gtctgtgccacccagcat for Homo sapiens cathepsin D (CTSD), (NM_001909.4), and cccctggtgcttctatccta / gatcctgcagagtgtctaaaa for Homo sapiens trefoil factor 1 (TFF1), (NM_003225.2). These two genes are estrogen-responsive genes. Breast cancer cells with ERα-negative phenotype were incubated during 48 hr in the presence of calcitriol and further treated with estradiol or ICI-182,780. Afterwards, RNA was extracted and qPCR analysis was performed. The results showed that, unexpectedly, calcitriol per se significantly stimulated the expression of CTSD and TFF1 (Figure 2A and B, respectively) in a similar manner as previous results from our laboratory using another ERα-dependent gene: the progesterone receptor (PR) (Figure 2C). We believe that, as a consequence of this, no further stimulation of gene expression could be observed after treating the cells with estradiol, as depicted in graphic 2 included herein. This is the main reason why we
selected other genes that respond to ER signalization, such as prolactin (PRL), which is included in the revised version (Figure 4) and figure 2D, herein. The promoter region of the PRL gene has been shown to contain DNA sequences that support the direct interaction of estrogen receptors with DNA; i.e. a functional estrogen responsive element. It is by this direct ER/DNA interaction that estrogen is thought to modulate expression of PRL (Mol Cell Endocrinol 281:9-18, 2008; Prolactin in the Immune System, chapter 4, DOI: 10.5772/53538, http://www.intechopen.com/books/prolactin/prolactin-in-the-immune-system). Therefore, the fact that prolactin was significantly stimulated by estradiol in ERα-negative cells only after being pre-treated with calcitriol, together with the fact that this was prevented by the ER antagonist ICI -182,780 (Figure 2D), suggests that this secosteroid was responsible of rendering ERα-negative cells sensitive to estradiol by inducing the expression of a functional ERα. It is noteworthy to mention that PRL was not regulated per se by calcitriol, as depicted in control (vehicle-treated) and calcitriol-treated breast cancer cells (Figure 2D).

Figure 2. Calcitriol stimulated the expression of CTSD, TFF1 and PR. Breast cancer cells were incubated in the absence (black bars) or presence of calcitriol 1X10^-8 M (white bars) for 48 hr. Subsequently, cells were coincubated with or without calcitriol plus estradiol (E2, 1x10^-8 M), ICI-182,780 (ICI, 1x10^-6 M) or vehicle (C) for 24 hr. A) CTSD, B) TFF1, C) PR and D) PRL gene expression
was determined by qPCR. Results are shown as the mean ± S.D. vs GAPDH mRNA normalized ratio. *P≤0.05 vs. C, black bar.

Minor comments
1) In fig.4B both E2 and ICI/E2 samples are reported significantly different from untreated, but considering the SD reported it seems to be a mistake.

The reviewer is right if the comparison was performed between black and white bars. However, our comparisons were done among the same groups (black bars or white bars, respectively). This point has been already corrected in Figure 5 in the revised version.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Not suitable for publication unless extensively edited

Before submitting this new version, the written English of the manuscript was carefully revised and corrected when necessary.

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests