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

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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: 4 Date: 10 February 2014

Reviewer: Diego Sisci

Reviewer's report:

The authors answered to all my questions.

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

Quality of written English: Acceptable

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
Reviewer's report

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

Version: 4 Date: 13 February 2014

Reviewer: Antimo Migliaccio

Reviewer's report:

The paper has been significantly improved and most of my concerns have been addressed.

Anyway, I think that:

1- a ChiP analysis of interaction of calcitriol receptor with some of vitamin D response elements, although not mandatory, would greatly improve the impact of their data.

We thank the valuable referee’s suggestions. In fact, we are very interested to perform in the future Chip analyses in order to evaluate the interaction of calcitriol receptor with vitamin D response elements as a potential mechanism to explain ERα induction.

2- As concerns the role of rapid signaling I note that there is some stimulation of MAPK activity already in 15 min, ashcan be detected by blots. I suggest that the Authors discuss this issue and explain why they believe that it is negligible.

The changes of MAPK in Western blots, as pointed out by the referee, did not show significant differences, which explained why we considered these changes as negligible. However, we believe that in the case that it would be observed, the most probably explanation would have been due to calcitriol induces apoptosis by a mechanism involving selective caspase-dependent MEK cleavage and up-regulation of MEKK-1 (McGuire et al., J Biol Chem 2001). This has been discussed in the new version of the manuscript.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

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’. 
Reviewer’s report

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

**Version:** 4  **Date:** 3 February 2014

**Reviewer:** Filippo Acconcia

Reviewer’s report:

Major compulsory revision.

The manuscript presented by the Authors is improved after the revision. Nonetheless, I am still not convinced that the conclusion by which calcitriol determines ERalpha re-activation in negative breast and for this reason becomes targettable by anti-estrogen cancer is supported by the data:

We deeply thank the reviewer for the interest and time taken to improve our manuscript. Following are the corresponding answers to the queries.

1- Estradiol does not lead to cell proliferation.

As the reviewer pointed out, it is well known that estradiol exhibits proliferative effects and therefore stimulates tumor growth in breast cancer (Doisneau-Sixou et al., Endocr Relat Cancer 2003; Dickson et al., Endocr Rev 1995). However, in the present study, the presence of estradiol did not result in increased proliferation when cells were pretreated with calcitriol. It is possible that the antiproliferative effects of calcitriol counteracted the proliferative actions of estradiol, observation which deserves to be further investigated. This possibility is supported by Krishnan et al. (Krishnan et al., Journal of Steroid Biochemistry and Molecular Biology 2010) who have described the potential therapeutic benefits of vitamin D in the treatment of ER-positive breast cancer. In addition, it has been shown that calcitriol inhibits the growth of MCF cells that are ER-positive (Swami et al., Clin Cancer Res 2000).

Indeed, the mechanisms involved in calcitriol anticancer effects include cell cycle arrest and stimulation of apoptosis. Because of this, our hypothesis to explain the lack of mitogenic activity by estradiol through the newly expressed ERα is that the pre-treatment with calcitriol during 48 h, as performed in our study, primed the cells to an antiproliferative state, which further prevented estradiol (which was added only after these 48 h) to induce a mitogenic activity. Therefore, priming would be the process occurring when cells are pretreated with calcitriol causing them to differentiate and change its proliferative program in a way that no subsequent estradiol mitogenic stimulus could make them proliferate. In other words, herein we propose the concept that
calcitriol acts as priming factor in the context of breast cancer progression; promoting a switch from growth driven by steroid hormones to “growth-arrest” by blocking the signals for cell cycle progression. In support of this consideration, herein there are some important observations on the mechanisms of calcitriol antineoplastic activity, which might explain breast cancer cells-priming towards an antiproliferative/proapoptotic state:


b. Calcitriol induces cleavage of caspase 3, polyadenyl ribose 6 phosphate (PARP), and the growth-promoting/prosurvival signaling molecule mitogen-activated protein kinase (MEK) in a caspase-dependent manner (McGuire et al., J Biol Chem 2001).

c. Calcitriol inhibits the phosphorylation and expression of Akt, a kinase regulating an important cell survival pathway (Bernardi et al., Clin Cancer Res 2001).

d. The expression of Bcl-2, an important anti-apoptotic protein, is down-regulated by calcitriol (James et al., Pharmacol 1998).

e. Calcitriol treatment may lead to a reduction in insulin-like growth factor-I (IGF-I) signaling. This effect is due to an increase in IGF-I binding proteins, which capture the growth factor keeping it away from the receptor. Also, calcitriol exerts direct effects on the IGF-I receptor levels (reviewed in Colston et al., Endocr Relat Cancer 2002).

Interestingly, our results agreed with Bayliss et al., who showed that the agonistic effect of estradiol upon proliferation was not observed in cells where ERα was reexpressed or when ER-negative breast cancer cells were transfected with the ER gene in which estradiol inhibited rather than stimulated cell growth (Bayliss et al. Clin Cancer Res 2007; Jiang et al., Natl Cancer Inst 1992; Garcia et al., Proc Natl Acad Sci USA 1992). This has been discussed in the new version of the manuscript.

2- Estradiol does not determine the activation of its classical target genes

As previously mentioned, and following the recommendation of the reviewer, we studied the transcriptional functionality of the re-expressed ERα by investigating the regulation of two estrogen-responsive genes: cathepsin D (CTSD) and trefoil factor 1 (TFF1). The results showed that calcitriol per se significantly stimulated the expression of CTSD and TFF1. This observation makes, by its own, a difficult condition to discriminate the activity of ER in calcitriol treated cells. However, we have shown the ability of newly calcitriol-induced ERα to express PRL in these cells. In addition to the previously exposed, we believe that estradiol did not activate its classic target genes CTSD and TFF1 because calcitriol might be interfering with these signalization pathways at
some point (by crosstalk with some of the mechanisms enumerated in the previous question), while unlocks the resistance to tamoxifen and ICI in ER-negative breast cancer cells. This has been included in the Results section page 12, line 12 of the new version of the manuscript.

3- Estradiol-dependent activation of Ser118 phosphorylation has not been performed

Phosphorylation of serine residues is important for ER-mediated transcription and probably the most important site in ERα is serine 118 (Chen et al., Breast Cancer Res Treat 2010); indeed, E2 stimulation results in ERα serine-118 phosphorylation. The suggestion of the reviewer to get insights into this process in our calcitriol-treated cells is indeed very interesting; however, it was not possible for us to perform these studies at the present time. Nevertheless, this is a matter that clearly deserves to be further investigated.

In my opinion, the explanation given by the Authors are not satisfactory. Is it possible that the effects observed depend on calcitriol binding to ERalpha?

The possibility that the effects observed depend on calcitriol binding to ERα promoter are indeed strongly feasible, considering this, we made a detailed in silico analysis using the MatInspector software (Bioinformatics, 21:2933-2942, 2005). With this analysis several vitamin D response elements were identified. This point was included in the discussion section.

The explanation given by the Authors that ERalpha re-expression is a win-win strategy to combat negative breast cancer is to me still too preliminary. Indeed, Authors should also consider that it is now clear that aggressive breast tumours, including those classified as ERalpha negative on the basis of the presence in the nucleus of the ERalpha, are indeed ERalpha positive: as a matter of fact the same nuclear ERalpha associates with signalling intermediates in the cytoplasm of isolated breast cancer cells as well as in biopsy of breast cancer patients and association with these signalling molecules are detrimental in terms of disease-free survival (EMBO Mol Med. 2012 Nov;4(11):1200-13. doi: 10.1002/emmm.201201615).

This is an interesting observation made by the reviewer; however, we do not think that the rescue of the ER alpha activated a rapid non-genomic signaling mechanism through Src-PI3K-Akt pathway. In fact, it is known that calcitriol by itself inhibits phosphorylation and expression of Akt, a crucial downstream target of the complex ERα/Src/PI3K and the kinase that regulates an important cell survival pathway in breast cancer cells (Bernardi et al., Clin Cancer Res 2001).
Moreover, in the response the statement that ‘estrogens and their receptors protect against cancer cell invasiveness through distinct mechanisms’ is not convincing.
Indeed, there are overwhelming evidence that indicate how estradiol is a proliferative and a migratory hormone for ERalpha positive breast cancer (Please, see the work of Dr Tommaso Simoncini, Dr Ellis Levin, Dr Rakesh Kumar to mention only some important Authors in the field). Furthermore, estradiol is a complete mitogen for breast cancer cells (Please see all the work of Dr Auricchio) and therapies for breast cancer patients target estradiol synthesis (aromatase inhibitors) or estrogen receptor alpha expression (e.g., tamoxifen; Faslodec).

We agree with the reviewer that estrogens and their receptors do not protect against cancer cell invasiveness. As the reviewer mentions, it is well known that estradiol via its receptor promotes growth, migration and invasion of ER-positive breast cancer cells and different therapeutic approaches are aimed at reducing estrogen levels or to block signaling through ERα. Indeed, patients with ER-negative breast cancer fail to respond adequately to antiestrogen therapy. In order to use hormonal therapy other authors using different strategies have been able to restore ERα expression in ERα-negative breast cancer (Yuanyuan et al., Molecular Cancer, 2010 and 2013; Bayliss et al. Clin Cancer Res 2007). Interestingly, our results showed that calcitriol is an additional agent able to restore ERα expression in ERα-negative breast cancer, which suggest an important role of this secosteroid hormone. Therefore, the use of calcitriol in combination with aromatase inhibitors or ER antagonists may be considered as a new therapeutic strategy for both ERα-negative and the triple-negative breast cancer, which deserve further investigation. As the reviewer pointed out, it is well known that estradiol exhibits proliferative effects and therefore stimulates tumor growth in breast cancer (Auricchio et al., Steroids 2010). However, in the present study, the presence of estradiol did not result in increased proliferation when cells were pretreated with calcitriol. It is possible that the antiproliferative effects of calcitriol counteracted the proliferative actions of estradiol, observation which deserves to be further investigated. This possibility is supported by Krishnan et al (J Steroid Biochem and Mol Biol 2010) who have described the potential therapeutic benefits of vitamin D in the treatment of ER-positive breast cancer. In addition, it has been shown that calcitriol inhibits the growth of MCF cells and promotes differentiation of several cell types (Swami et al., Clin Cancer Res 2000; Pendas-Franco et al., Differentiation 2007; Palmer et al., J Cell Biol 2001) and inhibits aromatase expression in both ER-positive and ER-negative breast cancer cells (Krishnan et al., Endocrinology 2010) avoiding the estrogen dependent signaling pathway. This has been discussed in the new version of the manuscript.

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

**Quality of written English:** Acceptable

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