Author's response to reviews

Title: Cross-talk between alpha1D-adrenoceptors and transient receptor potential vanilloid type 1 triggers prostate cancer cell proliferation.

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Author's response to reviews: see over
Dear Editors,

we wish to resubmit the revised version of our manuscript MS: 6916364961292275 entitled “Cross-talk between alpha1D-adrenoceptors and transient receptor potential vanilloid type 1 triggers prostate cancer cell proliferation” by Maria Beatrice Morelli, Consuelo Amantini, Massimo Nabissi, Sonia Liberati, Claudio Cardinali, Valerio Farfariello, Daniele Tomassoni, Wilma Quaglia, Alessandro Piergentili, Alessandro Bonifazi, Fabio Del Bello, Matteo Santoni, Gabriele Mammana, Lucilla Servi, Alessandra Filosa, Angela Gismondi and Giorgio Santoni in BMC Cancer.

We answered to the reviewers' comments and added the additional information requested. Thanks to their suggestions our manuscript is now greatly improved.

We hope that the revised version of our manuscript can be now acceptable for publication in BMC Cancer.

With best regards,

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RESPONSES TO REVIEWERS' COMMENTS

REVIEWER #1

REVIEWER #1: "Figure 1: How can you explain that „just” 40% of DU145 and 50% of PC3 co-expressed both receptors? Both cell line are androgen insensitive. Did you use any androgen sensitive cell line for example LNCaP cells? The expression of α₁D-adrenoceptors and transient receptor potential vanilloid type 1 depend on the androgen sensitivity? Could you show the co-expression of α₁D-AR and TRPV1 in benign prostate hyperplasia (BPH) and advanced prostate cancer (PCa) tissues?"

Answer: PCa cell lines are extremely heterogeneous for the expression of different α₁-AR subtypes and TRP channels. The data presented on the co-expression of α₁D-AR and TRPV1 in the prostate cancer cell lines are the results of the different capability of the distinct α₁-AR subtypes to be co-expressed with the same or different TRP channels. Thus, in LNCaP cells, co-expression of α₁A-AR with different members of TRPC channels has been found; in addition, findings in our hand showed that in PC3 cells, TRPV1 are co-expressed with different α₁-AR subtypes (e.g., α₁D-ARs and α₁B-ARs).

As suggested by the reviewer, we have performed the double immunofluorescence and FACS analysis for the α₁D-AR and TRPV1 receptors using the androgens sensitive cell line LNCaP. We have found that the percentage of LNCaP co-expressing α₁D-AR and TRPV1 is similar to that observed in PC3 and DU145 cells. Thus, our results indicate that the expression of α₁D-adrenoceptors and transient receptor potential vanilloid type 1, in vitro, does not depend on the androgen sensitivity. Methods (pp. 7, 8), Results (pp. 15), Figure legends (pp. 31), Figure 1 have been accordingly modified.

Moreover, as required by the reviewer, we have carried out double immunofluorescence to show the co-expression of α₁D-AR and TRPV1 in benign prostate hyperplasia (BPH) and advanced prostate cancer (PCa) tissues. Data have been included in Figure 2 panel F.

Methods (pp. 9), Results (pp. 16), Figure legends (pp.31) and Figure 2 have been accordingly modified.

REVIEWER #1: " Figure 2: The bicalutamide is a non-steroidal anti-androgen and it can bind to androgen receptor (AR). The mRNA expression of α₁D-adrenoceptors and TRPV1 transient receptor
potential vanilloid type 1 decreased in neoadjuvant treated PCa. How can the authors explain this result? What is a connection among α₁D-adrenoceptors - transient receptor potential vanilloid type 1 and AR receptor?"

**Answer:** Androgen receptor (AR) expression and function are maintained in advanced prostate cancer and the growth of ablation-resistant prostate cancer cells remains AR-dependent. AR is a transcription factor that targets specific genes regulating their expression. Our data, indicating a down-regulation of α₁D adrenoceptor and TRPV1 mRNA levels as a consequence of AR inhibitor treatment, suggest that AR could be a transcription factor involved in the regulation of α₁D adrenoceptor and TRPV1 expression. In agreement with our findings, Jariwala and coworkers identified, by ChIP Display, TRPV1 as a novel target gene of AR (Jariwala et al., 2007). In addition microarray analysis, performed in primary advanced prostate cancer specimens obtained from patients receiving no therapy or receiving three months of goserelin plus flutamide androgen ablation therapy, showed that the androgen deprivation treatment significantly decreases the expression of TRPV1 (Jariwala et al., 2007). This reasoning is also included in the Discussion section page 19.

**REVIEWER #1:** "Figure 6: The authors show that the PLC-PKC-ERK signal pathways play a role the NA induced cell proliferation of PC3 cells. They register that inhibition of these signals (alone) reduce the cell growth. These signals are parallel or successive? What happen if you use these inhibitors combination (Chelerythrine, PD98059, U73122) with NA?"

**Answer:** our data show that the PLC-PKC-ERK activation are very fast and all three signals occur 3-5 min after the NA stimulation. Our data suggest that PLC signal is successive to TRPV1 activation; PKC signals seem to be successive to PLC activation induced by TRPV1.

As suggested by the reviewer we have performed the cell viability assay using the combination of the three inhibitors. Results (pp.18) and Figure 6 have been accordingly modified.

**REVIEWER #1:** "Figure 7: I suppose the author used the transient gene silencing. How did you check the double gene silencing? I think you have 3 population of cells i) si α₁D--adrenoceptor, ii) siTRPV1 and iii) si α₁D-AR/siTRPV1. How many percent of cells is double gene silenced? Did you check for example by FACS or immunocytochemistry?"

**Answer:** as requested by the reviewer, we have checked by FACS analysis the expression of α₁D-AR and/or TRPV1 in siα₁D-AR/siTRPV1 PC3 cells, demonstrating a total abrogation of PC3 subpopulation co-expressing α₁D-AR and TRPV1 proteins. Methods (pp.8), Figure legends (pp.35) and Additional Figure 2 have been accordingly modified.
REVIEWER #1: "Figure 1 A and B: I don’t think that the authors have to show the negative control here (GARB-PE – RAG-FITC). If they want to show it they can in additional file."
Answer: as suggested by the reviewer, the negative control have been removed from Figure 1.

REVIEWER #1: "Figure 4: You should write the molecular weight of ERK and p38 (Fig. 4B) and the molecular weight of the protein standard (Fig. 4C) at the western blot figures."
Answer: as suggested by the reviewer, we have included the molecular weight in Figure 4.

REVIEWER #1: "Figure 5: same comment as at Fig. 4"
Answer: as suggested by the reviewer, we have included the molecular weight in Figure 5.

REVIEWER #1: "Figure 4: I think the p38 is a correct form and not the „P38‟."
Answer: as suggested by the reviewer, we have replaced P38 with the correct form p38 in Figure 4 and in the manuscript.

REVIEWER #2:

REVIEWER #2: "The differential frequency of TRPV1 and α₁D-AR receptor expression suggested a heterogeneous population of prostate cancer cell lines. Please provide the mean specific fluorescence intensity and the number of experiments performed. Double staining of both receptors would have been more appropriate using flow cytometry and antibodies labelled with distinct fluorescence."
Answer: As reported in the Figure Legend 1, data shown are representative of one out of three separate experiments. The percentage of co-expressing cells obtained in the three experiments are: in PC3 cells 52.4, 47.5 and 54.7 and in DU145 cells 40.7, 43.5 and 39.8. The flow cytometric analysis of these experiments was performed by double staining using distinct fluorescence. In brief, TRPV1 was labelled by using anti-TRPV1 Ab followed by Fluorescein isothiocyanate-conjugated secondary Ab (FITC, green fluorescence) and α₁D-AR with anti-α₁D-AR Ab followed by Phycoerythrin-conjugated secondary Ab (PE, red fluorescence). Details of the procedure are included in the Methods section.

REVIEWER #2: " The quality of figure 1 should be enhanced."
**Answer:** as requested by the reviewer quality of Figure 1 has been enhanced. Now Figure 1 has a resolution of 600 dpi and the 24-bit RGB color mode.

**REVIEWER #2:** "Please provide further information regarding the prostate cancer lesions analysed."

**Answer:** as suggested by the reviewer, we have revised Additional Table 1 containing information about patients and prostate cancer lesions analyzed. Additional Table 1 includes age of patients, preoperative PSA levels, clinical stage, pathologic stage, positive surgical margin, nodal stage, pathologic Gleason, extra-prostatic extension and information about number of patients receiving neo-adjuvant androgen deprivation therapy.

**REVIEWER #2:** "The time course analyses in Figure 4 showed an altered pERK activation. Please discuss why P38 expression is not altered."

**Answer:** several findings demonstrated that $\alpha_1$-AR subtypes are able to activate or inhibit different members of the mitogen-activated protein (MAP) kinase family such as ERK, JNK and p38 and the modulation of MAPK pathways is subtype specific (Hein P et al. 2007). Herein we found that p38 was phosphorylated at basal level, and that NA through TRPV1 and $\alpha_{1D}$-AR cross-talk induces ERK phosphorylation. These findings are in accordance with previous results obtained in $\alpha_{1D}$-AR-transfected PC12 cells where the specific stimulation of $\alpha_{1D}$-AR induces ERK but not p38 phosphorylation (Zhong et al. 1999). The higher expression of $\alpha_{1D}$-AR subtypes in PC3 cells compared with the other $\alpha_1$-AR subtypes (Quaglia et al. 2005), could be responsible for the NA-induced effects.

**REVIEWER #2:** "Please give information about the percentage of inhibition of both receptors by siRNA. The discussion should be shortened."

**Answer:** as suggested by the reviewer, we have checked the double gene silencing by FACS analysis demonstrating a total abrogation of PC3 subpopulation co-expressing $\alpha_{1D}$-AR and TRPV1 proteins in $\text{sia}_{1D}$-AR/siTRPV1 PC3 cells. The result has been included in Additional Figure 2. Methods (pp.8), Figure legends (pp.35) and Additional Figure 2 have been accordingly modified. Moreover, as requested by the reviewer, the Discussion has been shortened.

**REVIEWER #2:** "There exist a number of typos throughout the manuscript."

**Answer:** as suggested by the reviewer the manuscript has been checked for typos.