Peer Review File

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Reviewer A

In the study entitled "DRD4 is differentially expressed in breast tumors 1 and breast cancer 2 stem cells: pharmacological implications" Rosas-Cruz et al, investigated the expression and function of Dopamine Receptors in breast cancer, particularly in the subpopulation of cancer stem cells. In a concise and well written introduction, the authors allow the reader to understand the rationale and originality of their systematic analysis of all five Dopamine Receptors in breast cancers. They concluded that DRD4 reduced expression or inhibition favors CSC-pool expansion in agreement with better clinical outcome of patients with tumors overexpressing this receptor. Even if some findings are clear, there are many points that need to be addressed. Overall this study is relevant in the field of breast cancer.

Major concerns

1/In figure 1A-B, the authors reported that DRD1 and DRD2 mRNA levels were reduced in breast tumors compared to normal tissue whereas DRD4 expression was significantly higher in tumor samples. Is there any difference between breast cancer molecular subtypes? The correlation if any with the expression of HER2 or ER and PR receptor should be analyzed, especially in the light of the results of survival analyses restricted to ER-negative breast cancer patients reported in Figure 1C-D.

Reply 1. *We are including the comparison of DRD1, DRD2, and DRD4 expression among breast cancer molecular subtypes (Figure 1C and page 6, lines 152-155). We have also analyzed the coexpression of DRD4 with ER, PR, and HER2 (Suppl. Figure 1 and page 6, lines 161-163), showing week to null correlation.*

2/The statistical test used is not always mentioned (e.g. Figure 2B and 2C, Figure 3B and 3C). This should be corrected.

Reply 2. *The statistical test employed have been listed in "methods" (page 5, lines 136-142) and added to each figure legend.*

3/ The parameters chosen by the author for their survival analyses seem cherry-picked. Why restricting their analysis to ER-negative patients when the DRs expression is increased in breast tumors that are 80% ER+? The authors should perform a systematic analysis with at least DRD1, DRD2 and DRD4 and use the same cohort, TCGA for instance, according to breast cancer molecular subtypes, particularly in Luminal A and triple negative breast cancers, for consistency with their following molecular analysis in breast cancer cell lines.

Reply 3. *Taking in consideration Reviewer´s comments, we have: i) focused our survival analysis on the TCGA cohort (which has the larger number of patients); ii) analyzed the survival by molecular subtype (no statistical differences found; data not shown); and iii) analyzed clinical outcome in ER- and ER+ patients. The results of the latter are presented in Figure 1 D-E and page 6, lines 157-160).*

4/ The effects of haloperidol, tendency to increase the MDA-MB-231 SORE6-GFP+ fraction and reduction in their cell migration suggest a tonic signaling of this receptor. Is there any dopamine- (or other agonist ?) released in the tumor micro-environment in patients and what about the culture conditions? Please explain

Reply 4. *We found no reports of the release of DRs ligands by breast cancer cells in culture. No changes to the manuscript were made.*

Minor points

1/ In the Kaplan-Meier analysis curves presented in Figure 1C-E, the number of patients included is not clear and should be stated. The number of patients at risk at each time point should also be specified as well as the type of statistical test used.

Reply. *As stated above, we are presenting new Kaplan-Meier curves obtained from the TCGA cohort in Figures 1D-E. The number of patients at risk has been included in the graphs and information of the statistical test employed incorporated to the corresponding figure legend.*

2/ The author conclusion "These results suggest that DRD1 expression promotes clinical progression whereas DDR4 expression favors better prognosis" should be rephrased. They presented only correlation od DRs expression level with patient's clinical outcome and no functional link can be inferred from this type of analysis.

Reply. *We have removed the phrase given that in the TCGA cohort no correlation between DRD1 expression and clinical outcome was found.*

3/ line 173 : Figure 1A should be replaced by Figure 2A.

Reply. *The mistake has been corrected (page 6, line 169)*

4/ There is poor consistency between the mRNA expression of DRDs in breast cancer cell lines and their protein expression: DRD2 mRNA was expressed in most the cell lines analyzed but could not be detected neither in MCF7 nor in MDA-MB-231 cells. DRD4 mRNA had a higher expression in MDA-MB-231 compared to MCF7, however, it seems to be detected similarly in both cell lines. Again, a systematic analysis of the content of total, membrane and intracellular pools of DRD1, 2 and 4 would be helpful.

Reply. *The discrepancy between mRNA and protein levels in cancer cells has been previously reported (see for example https://doi.org/10.1038/srep10775). In the case of the levels of DRD1 in MCF-7 cells, we demonstrated that the protein is expressed but fails to locate at the cell membrane (Suppl. Figure 2), which partially explains the discrepancy.*

5/ Please provide details of which receptor is modulated by the used drug. Define D1 or D2-like subfamily and particularly it is not clear in which lays DRD4? The statement "DRD4-targeting drugs Quinpirole and Haloperidol", line 211-212, should appear before.

Reply. *We have specified the selectivity of the compounds employed along the text (see for example page 9, line 235).*

6/ The experiments using the different drugs in Figure 5 should include a "vehicle" control.

Reply. *We have corrected Figure 5 to clearly state that the experimental condition labeled as "control" was actually "vehicle". This has been specified also in the corresponding Figure legend.*

7/ Data are often presented as average ± e.e.m. Please define "e.e.m".
Reply. *We have corrected text in Figure legends to "average ± SEM" and defined the abbreviation.*

Reviewer B

The title of the article does not fully reflect the content of the article. The authors analyzed the expression of five dopamine receptors (DRs) in breast tumors and breast cancer cell lines. But the results of the DRD1, DRD2, DRD3 and DD5 studies are not reflected in the title. Such a name belittles the results of the study, which is not acceptable. The article would be significantly improved if the authors reflected the features of the expression and effects of DRs in the title of the article.
Reply. *We appreciate the suggestion. Although we started studying all five DRs, the first results showed that only three of them are consistently expressed in breast tumors and cancer cell lines. Furthermore, only DRD1 and DRD4 proteins were detected on subpopulations of breast cancer cells. Thus, we have slightly modified the title to include DRD1.*

The chapter "Abstract" contains the necessary information for the reader. The authors provided background information on the expression of DRs, the purpose of the study is clear and interesting. The Abstract summarizes the methods and the main results. The conclusions are clear and consistent with the results of the study.
The presented keywords are necessary and reflect the research topic presented by the authors. However, this section does not reflect all medications (only haloperidol is presented), with the help of which the pharmacological effect on tumor cells and cancer stem cells (CSCs) was carried out. The article would be improved if the authors reflect the class of drugs in the "Keywords" section. This will improve the understanding of the results of the pharmacological section of the study.
Reply. *We have been included as keywords "Dopamine receptor antagonist" and "Dopamine receptor agonist" (page 3).*

In the "Introduction" section, the authors showed the degree of development of the problem and the duality of dopamine receptors in cancer. The connection between the previously published articles and the purpose of this study is visible, as well as the novelty of the study. The purpose of the study is clear. In addition, the "Introduction" section presents the main results of the study.
In the "Methods" section, it is quite fully and correctly presented how the analysis of gene expression was carried out. Cell lines, drugs and treatment methods, as well as CSCs identification and DRDs immuno-staining approaches, flow cytometry and wound healing analysis are described in the required volume and clearly. All information is extensive and necessary. The design of the study is clear. It is noted that in the section "Methods" the

authors make references to previously carried out work. Despite the positive impressions from the section "Methods", it should be noted that there is no information about the statistical analysis of the results. Please explain what is the reason for the absence of the subsection "Statistical analysis"?
Reply. *A subsection was created to describe the statistical analysis performed (page 5). The statistical methods employed are also listed in Figure legends.*

In addition, there is no information in the "Methods" section about the approval of the study by the ethical committees and supervisory boards. It is not clear whether the study was conducted in accordance with the ethical code of the World Medical Association (Helsinki Declaration). Please explain what is the reason for this state of affairs?
Reply. *We have included the corresponding information under "ethical statement" (pages 111-12, lines 285-291).*

In the "Results" section, all the results of the study are presented, while they are well illustrated with figures and tables. All the tasks planned by the authors have been completed. The results presented in the article are necessary, as it lays the foundation for the development of cancer treatment approaches. The drawings are legible, necessary and complement the content of the article.
In the "Discussion" section, using the literature, the authors discussed the research results. The submitted manuscript does not cause any concerns. The manuscript did not cause any ethical problems. All references to publications presented by the authors in the article are necessary and correct, made in the right style. I have no concerns about the similarity of this article with other articles published by the same authors.
Reply. *We thank all comments from Reviewer.*

Reviewer C
This manuscript shows some relationship between dopamine receptor subtypes and breast cancer and/or cancer stem cells. Although the participation of dopamine receptors in various types of cancer has been reported, more study is needed to answer the roles of dopamine receptor subtypes in development, progression, survival and acquisition of resistance of cancer.

This manuscript needs to answer the following questions and refer to some comments.
1) The authors say that reduced expression of DRD4 is related with increased risk for metastasis patients (Fig. 1E). Is it true? Fig. 1E shows that reduced expression of DRD4 was related with low metastatic probability values. Please explain.
Reply 1*. Following the suggestion of Reviewer A, we have removed the referred data and focused only in the analysis of TCGA data.*

2) How do you know the cellular location of DRD1 and DRD4. Although you are describing about in the text, your results do not show about it.

Reply 2. *We detected membrane proteins by immunostaining in live cells, whereas intracellular proteins were detected in in fixed and permeabilized cells. This information has been explained in "methods (page4, lines 122-124). We are now including the results of intracellular staining for DRD1, DRD2 and DRD4 in MCF-7 cells (Suppl. Figure 3).*

3) In the Abstract: DRD1-targeting drug? Many of them are just research agents, not drugs.
Reply 3. *We have substituted "drugs" for "compounds" in Abstract (pages 2-3) and along the text.*

4) Line 23: there are two 'in'. Remove one 'in'.
Reply 4. *The mistake has been corrected.*

5) Line 38: In MCF-7 and MDA-MB-231 In case of MCF-7 and MDA-MB-231
Reply 5. *Abstract has been modified (see point 10 below). As result, the quoted text was removed.*

6) Line 44: … or MDA-MB-231 cells 'is' irrelevant… The 'is' is too confirmative. "seems to be(?)" or "appears to be(?)" may be better.
Reply 6. *Suggested change has been made in "abstract" (page 3) and "conclusions" (page 11).*

7) Fig. 1 B: the font size of legends is too small (primary tumor, solid tissue normal)
Reply 7. *We have increased font size in Figure 1B.*

8) Fig. 2 legend: the first B) should be B-C)
Reply 8. *Figure 2 legend has been corrected.*

9) Fig. 4: what is e.e.m? Is it SEM (or sem)?
Reply 9. *We have corrected the abbreviature to SEM in all Figure legends.*

10) Pharmacological modulator: This term is ambiguous. In places possible, please indicate clearly (e.g., activators or inhibitors)
Reply 10. *We have specified that both, DRs agonists and antagonists were employed (see for example page 8, lines 198-200).*

11) Please rewrite Abstract (especially the Results and Conclusions). It is not easy to understand.
Reply 11. *We have carefully reviewed/ corrected Abstract to improve clarity.*