Author's response to reviews

Title: Altered human breast cancer cell features and increased chemosensitivity mediated by adipose-tissue derived mesenchymal stromal cells

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Version: 2 Date: 26 September 2013

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

On behalf of all co-authors I would like to submit revised version of our research paper entitled “Altered features and increased chemosensitivity of human breast cancer cells mediated by adipose tissue-derived mesenchymal stromal cells” for consideration to be published in the journal BMC Cancer. The manuscript was edited according to the reviewers’ recommendations and we believe that all the issues were sufficiently resolved. We have documented multiple effects between breast cancer cell and mesenchymal stromal cells which result in the biological effects with a relevance to the clinical situation.

All the co-authors of the manuscript state the material is original research, has not been previously published and has not been submitted for publication elsewhere while under consideration.

Conflict of interest statement

The authors declare no conflict of interest.

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Detailed response to the reviewer comments.

Major Compulsory Revisions:

Q: Figure 1C only 3 of the target genes have been statistically considered. All genes should be similarly analyzed with data being presented; it is unclear why a gel is shown when real-time RT-PCR is being conducted for another aspect of the study;
A: The data from the figure 1C were quantitatively evaluated and all the target genes were analyzed by real-time RT-PCR to demonstrate significant effects.

Q: Figure 1D-besides representative images, quantitative data should also be included either in the figure of text of results;
A: We have evaluated the mammosphere cultures quantitatively by viability luminescent assay and confirmed that both inhibitors slightly inhibit the tumor cell proliferation but the difference is not significant between the standard culture and the MSC-CM supplemented mammosphere culture.

Q: Figure 1E-statistics need to be included for this data set;
A: The data set in previous Fig 1E was analyzed statistically. Figure was moved and in the edited manuscript version it was labeled Fig. 2A. The theoretical additive value for the amount of secreted cytokine was calculated and compared to the value observed in the direct coculture of the cells to distinguish between additive and synergistic effects in the tumor/stromal cell cocultures.

Q: Figure 2 A & B require statistics, and it is unclear why a gel is shown when real-time RT-PCR has been performed for other aspects of the study.
A: Data in former Fig 2A-B were analyzed statistically. Significance values were included in the figures designated Fig 2C-D in the edited version of the manuscript. Figures were re-ordered to better follow the sequential steps of the analysis. The former picture 2C was moved to 2B and it has to be done by qualitative PCR analysis. We intended to show also the negative result for the several analyzed target genes to stress that there are target genes which do not change in the response to MSC-CM exposure in the tumor cells. Moreover, the target amplicons were designed to span exon-intron regions in order to exclude gDNA false positivity, and therefore there are many of them longer than 250bp which is not suitable for the quantitative real-time PCR analysis. We are persuaded that this qualitative analysis design is suitable to
demonstrate sufficiently the point of induction of gene expression by MSC-CM. It is not possible to quantify the relative amount of the gene expression for the targets which are not expressed in the parental reference cells by delta-delta-Ct method. (Denominator in the figure must not be zero).

Q: Figure 3A should display the collective data set rather than just 1 result (or, at a minimum, 3 representative results to permit statistics);

A: In the figure 3A we have exported the live-cell imaging Incucyte data into excel software to perform the analysis of the pooled experiment as shown in the edited figure 3A.

**Minor Essential Revisions:**
- There needs to be a better description of the various inhibitors as well as the concentrations tested in the text;
- The inhibitor characteristics and the concentrations used for the treatments were included in the text of the manuscript to better describe the course of the study.
- Concentrations of the various chemotherapeutic agents need to be included in the text;
- The drug concentrations were included in the text and described also in the figure legends.
- Many of the figures are lacking critical details (i.e., axis labels are missing, asterisks are included in one figure with no mention of the meaning, many of the graphs and/or legends are missing p-values, asterisks, etc.)

Figures were carefully edited to fill in all the missing labels and details in order to make the manuscript suitable for the publication.
- Were any positive controls included for the mouse studies (i.e., your MSCs contributing to the progression of MDA-MB231, as reported previously). If not, you cannot be certain whether there wasn't a technical problem with the MSCs.

We have been working in the field of MSC since 2005 and many studies including interactions of MSC and tumor cells were conducted and published. We are quite confident that our experimental strategy did not include any technical problems with MSC. We have sufficient expertise in conducting these types of studies in vivo and we have previously reported several experimental outcomes to demonstrate the
capability of MSC to support the tumor growth (Kucerova et al., Mol Cancer 2010). The studies on the interactions with MDA231, SKOV3 and other cell lines are subject of our ongoing studies (unpublished data) fully confirm the expected outcomes as reported in literature and excluded the technical hindrance in the experiment.

**Discretionary Revisions:**

- In light of the negative in vivo data, perhaps mention of future studies are needed to test in vivo whether MSCs alter chemosensitivity in vivo (demonstrating this would make for a much more impactful study);

We are fully aware of the impact of the in vivo tumor model. That was actually the initial motivation for the experiments and a reason behind the extensive testing of the potential capability to support the SKBR3 tumor growth. We have been aiming at a development of a novel model for the evaluation of antitumor treatments in the in vivo tumor xenotransplant model. However, we were not able to achieve tumor growth and we think that the interaction of MSC with SKBR3 is really insufficient to increase the tumorigenicity of these low tumorigenic cells in vivo. The commentary was included in the discussion section.

- Mention that adipose MSC are derived from the abdomen and not breast tissue; perhaps include in the discussion some comments and literature citations as to whether the source of adipose MSCs matters when considering mammary cancer progression.

Several novel citations regarding this issue were included in the discussion section. It remains a matter of an intense research and evaluation to dissect whether the MSC isolated from the different tissues share the same properties and what differences might be useful for their use in the cell therapy approaches.

- The discussion is very long and reads more like a review; the manuscript would be strengthened if this section was more focused on how the data presented contributes to the field.

Discussion was substantially edited, shortened, focused on the subject of the novel findings which add the knowledge to the field by showing that the AT-MSC and BM-MSC exert very similar effects on the breast tumor cells in a multilevel signaling.
-Similarly, the introduction quite extensively cites published literature but as a listing of 1-sentence descriptions of each. An integration of this literature leading up to the hypothesis and goals of this study would strengthen the paper.

We have edited this part of the manuscript, shortened the introduction, reformulated the statements and summarized the data that led to the study goals and investigation.

**Reviewer's report:**

The manuscript by Kucerova at el entitled “Altered human breast cancer cell features and increased chemosensitivity mediated by adipose tissue derived mesenchymal stem cells” describes the effects of either co-culture or MSC-CM on the growth and chemosensitivity of a breast cancer cell. Although the findings are interesting, however, it is very hard to follow. The following issues need to be addressed:

1. The AT-MSCs should be characterized, what is their differentiation potential?

   The standard characterization of the AT-MSC has been already published in our initial study (Kucerova et al., Cancer Research 2007) and it has been routinely performed. We did not feel the need to include it as a part of the study with the respect to the space limitations. The immunophenotype and the differentiation outcomes were included in the supplementary figure S1.

2. The methods need to be described better, for example, it is not clear how and based of what standard method the authors have measured the confluency of culture.

   The methods were edited to remain concise but clearly described at the same time. More details were supplemented where necessary. Many of the approaches were already used and described in detail in our previous publications; therefore we tried to keep the section compressed with the appropriate references to comply with the journals restrictions on the length of the manuscripts. We think that by a question about the confluence, the reviewer is asking for the explanation of the live-cell imaging system. The principle of the assay is that each time-point and each phase-contrast image with high resolution is analyzed by the proprietary software package included in the Incucyte platform. It is based on the overlay of the confluence masks
as refined by the user-edited processing definitions which are applied on each image and the confluence of the cultures is determined.

3. The use of CytD in chemoresistance experiments should be described. CytD was used as a negative control in the migration assays to block the polymerization of actin and control the absence of any spontaneous wound closure during the 72 hrs incubation period. We have realized that this control belongs to the assay setup and does not bring any relevant experimental data, therefore the line was omitted from the graph for the better clarity of the experimental outcome showing increased tumor cell migration stimulated by MSC-CM.

4. Data in figure 3 are paradoxical. In proliferation studies how the higher confluency of SKBR3-MSC-CM and co-culture are correlated with tumor cell proliferation inhibition. The data mentioned might look paradoxical at the first look. However, it has been detected that MSC-CM increased tumor cell confluence by flattening the MSC-CM exposed SKBR3 cells shifting them towards mesenchymal morphology, altering their adhesion and thereby increasing the cell confluence. On the other hand, unaffected SKBR3 cell have rounded morphology, tend to grow in multilayered cellular clusters with low adherence to the plastic support and tendency to detach easily. This leads to the relatively lower confluence with denser cell clusters of the cell cultures. However, the proliferation of the cells was higher, there were more cells and we could see the correlation of the proliferation inhibition with the increasing number of MSC within the cocultures and the increasing proportion of MSC-CM admixed to the standard culture medium. Our data only stress the need for the confirmation and verification of the findings by several independent methods.

5. There are several grammatical and descriptive errors throughout the manuscript. A revision is recommended.
We have edited manuscript text extensively. Based on the recommendation of the reviewer some parts of the text were changed and shortened to better stress the goal of the study, experimental strategies and the outcomes. We believe that we have corrected the grammatical and descriptive errors and therefore we hope that the reviewers can consider the edited version of the manuscript suitable for publication.