Author's response to reviews

Title: Differences in integrin expression and signaling within human breast cancer cells

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Author's response to reviews: see over
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Editorial Board
BMC Cancer

Dear Editor,

We are submitting a revised manuscript entitled: **Differences in integrin expression and signaling within human breast cancer cells**, to BMC Cancer for consideration of publication.

In our study, we used three breast cancer cell lines to examine a number of cellular factors affecting integrin signaling and have noted major phenotypic variations between the cancer lines that may account for some of the heterogeneity of breast cancer. These differences include expression of integrins, formation of integrin-related structures, expression of integrin co-receptors, and cell agonist-induce and cell adhesion-induced intracellular signaling via MAPK and Src pathways.

We have addressed issues raised by the two reviewers and an itemized listing is provided below.

Major Compulsory Revisions:

1. For example, the first experiment, where the authors looked for integrins expression, they treated cells with PMA for 2h, whithout any coating. Could it be that the different coatings would alter integrin’s expression, and that could explain the alteration seen in cell-adhesion and in phenotypic alterations detected by F-actin staining?
   - performed new experiments of adhered versus suspended cells +/- PMA looking at integrin expression and in results section commented on specific changes in aVb3 expression.

2. The authors show that 2h PMA treatment did not enhanced integrin expression. Did they tried increased times?
   - Comment added about increased time in results section

3. In the cell-adhesion assay, the authors have unstimulated and PMA treated cells. These unstimulated are DMSO treated cells? Why is not refered in the text?
   - Yes, they have been copied from the figure legend and added to the text to make this point clearer.
4. In cell adhesion assays, the authors speculate that the lack in upregulation of adhesion, in PMA treated cells compared with unstimulated cells, was a result of the nonstimulated cells expressing activated integrins which negated any further increase with PMA treatment. To support of that speculation, they observed that less than 5% of nontreated GM1500 cells adhered to Fg, and the cell adhesion increased up to four-fold following PMA treatment (data not shown). With those results they say that they can conclude that these breast cancer cells and the transformed Hek-293 cells expressed activated integrin. However, in the opinion of this reviewer, this experiment doesn’t show that. For them to prove that unstimulated cells show increased integrin activation, they should block integrin-extracellular matrix (ECM) interaction, by using an anti-integrin antibody, and see if cell adhesion was diminished. --- As suggested we performed new experiments using anti-integrin antibodies and showed that cell adhesion was diminished.

5. The agonist-induced signaling was quite confusing. Before even starting looking for the expression of integrin-signaling molecules after PMA stimulation, in FN-coated cells, the authors should characterize the pattern of expression of integrins in these conditions. - This was already done and was made clearer in the text. We also performed new experiments and added the data in the results section showing that integrin expression does not change upon PMA stimulation or upon adhesion.

6. The authors, by the experiments that they show, cannot conclude that uPAR expression is not causal for upregulated cell adherence. For this conclusion, they should Knock-down or inhibit specifically uPAR in MCF-7 and see if cell-adhesion was compromised. - Modified this statement in results and discussion section.

Minor essential revisions:
7. Bcl2 and pErk2 appear as a parachute in the study, without a clean link to the rest of the study. - Modified this section for better connection

8. The names of cell lines should be complete (for example – MDA-435 is MDA-MB-435). --- Corrected to MDA-MB-435 and MDA-MB-231

9. Materials and methods should be better described, such as the antibody clones should be mentioned. --- added

10. The type of collagen is not described. --- added

11. Company and type of Isotype-matched monoclonal antibodies should be referred. --- added

12. The number of replicates and independent experiments should be mentioned in each specific assay. --- added
13. Initially, the authors studied the expression of integrins, in several cell lines, by flow cytometry. As represented in figure 1A, they see differences in the pattern of integrin expression between the different cell lines. To simplify and to facilitate the analysis of the results, the histograms of the four cell lines for a specific integrin, should be placed in a unique histogram. The same, should be done, in figure 1B, in what corresponds to the DMSO treatment versus PMA.
--- Data presented is best way to show differences between samples and from controls.

14. Western blot figures, for p-Src (Y416) should be repeated, since the authors claim that there is an increase in p-Src for MDA-MB-435 cell line, after PMA treatment, and this increase is not clear at all. As so, all Western blot, should be quantified and a quantitative representation of the bands should be shown.
--- New experiment performed and some data added to figure

15. Discussion is a little bit confusing and should be reformulated.
--- Modified

16. There is more recent bibliography on integrin signaling in breast cancer that should be referred.
--- added

Major Compulsory Revisions
17. The authors must explain why they use 150nM PMA as the concentration of choice. Do they have experimentise and concluded that this is the most effective for the particular experiment??
--- Old experiments added as new figure 1.

18. How do they know that just 1 hr is enough for the cells to settle onto the coverslips and "feel at home". It has been well established that individual cells need some time to attach onto a substrate and establish their normal shape, metabolism. Is just one hr enough to allow the cells to relax and tightly bind on the substrate?? The authors should provide more information related to the procedure
--- More details provided in text of results section

19. In discussion authors must discuss PMA addition in relation to literature [e.g. Panagopoulou E (2005)] and discuss PMA effect on stress fiber perturbation.
--- Examined and text of results and discussion added.

Please find attached a copy of the manuscript highlighting changes made.

Sincerely,

Thomas A. Haas, Ph.D.