Author’s response to reviews

Title: Localization of uPAR and MMP-9 in lipid rafts is critical for migration, invasion and angiogenesis in human breast cancer cells.

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Author’s response to reviews:

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To
The Editor
BMC Cancer

RE: Revision of MS: 644493314151827 – Lipid Raft disruption by cholesterol depletion attenuates migration, invasion and angiogenesis by downregulation of uPAR and MMP-9 in breast carcinoma cells- Reg ”

Dear Editor:

We appreciate the reviewers’ valuable comments on our manuscript entitled, “Lipid Raft disruption by cholesterol depletion attenuates migration, invasion and angiogenesis by downregulation of uPAR and MMP-9 in breast carcinoma cells” submitted to BMC Cancer. We have now clarified several of the reviewers’ concerns in the revised manuscript and appreciate consideration of this manuscript for publication in BMC Cancer.

Answer to referees comments:

Associate Editor’s comments: “The authors need to reconsider the title of the manuscript given that now the data presented which indicate that the phenotypic effects of cholesterol depletion are mediated by downregulation of uPAR and/or MMP-9.

As suggested by the Editor, we have made changes in the manuscript title and have modified the same.

Reviewer # 1.
Major Points:

1. Fig. 2A and B upper panels, MMP-9 staining images: It is difficult to judge co-localization of MMP-9 with GM1, because the signals for MMP-9 are saturated in these images. The authors should provide better images to show their co-localization.

We agree with the reviewer and as suggested, better staining images have been shown to show the co-localization of MMP-9 and GM1. The new panel has been incorporated in Fig. 2A and Fig.2B.

2. Fig.1, 2, 4 and 5: The authors provide several bar graphs, in which relative values are represented in arbitrary units. It would be better to set the control value to 100% and then show the relative values. Additionally it is unclear how the relative values were calculated or the absolute values were normalized in these graphs. In particular, in Fig. 5A and C, the values for untreated should be set to 100% for each sample, because each antibody has different titer and therefore it is not reasonable to compare the signals between different antibodies.

As suggested by the reviewer, the control values for Figures 1, 2, 4 and 5 have been taken as 100% and the relative values calculated. The changes have been made in the figures and in the text accordingly.

Minor points:

1. Materials and Methods: The methods for cell culture and determination of cellular cholesterol levels are missing.

As suggested by the reviewer, the methods for cell culture in page 4 para 3 and determination of cellular cholesterol (page 5 para 3) have been incorporated in the text.

Reviewer # 2:

Major

1. Toxicity assay method is given and ‘non-toxic’ levels mentioned but data is not shown for methyl beta cyclodextrin toxicity, since many results could be explained by general toxicity I think this data should be shown.

We agree with the reviewer about the cellular toxicity at Methyl beta Cyclodextrin. But in our study we have used non toxic concentrations and as suggested by the reviewer, the data showing the cellular toxicity levels for different concentrations of M#CD is given in the form of a bar graph and is incorporated in Fig. 3 as Fig 3C.

2. Data is referred to without showing the data:

Results:
Para 2. Lysates from different time point ranging from 1-24 h (only 1 and 24h are shown)

As noted by the reviewer, the lysates were collected for 1hr and 24h time points only and not from different time points as mentioned in the text. The error is regretted and appropriate changes have been made in the text.

Para 4. Time kinetics are referred to for MMP-9 activity in MDA when only 1 and 24h are shown.

As observed by the reviewer, MMP-9 activity was done at 1h and 24h only. The time kinetics was not done as mentioned. The phrase 'Time kinetics' has been removed and changes have been made in the text.

Para 8. Total Akt and P13-K not shown as stated in text and fig. legend.

We fully appreciate the keen detail of the reviewer. As suggested both Total Akt and total P13-K levels have been shown in the Figure. 5A and 5B as mentioned in the text and Fig. legend.

3. There seem to be insufficient controls for western blots and zymograms

For example:

Fig 1 & 2 westerns D & E

2 westerns were shown of lysates from 2 time points 1 and 24h, GAPDH was used as a loading control but only 1 GAPDH blot was shown, was this the loading control from 1h or 24 h? Both need to be shown.

This is a very valid point suggested by the reviewer. As pointed out by the reviewer, GAPDH from both the time points have been incorporated in the Figure 1D & 1E and in Figure 2D & 2E.

4. The westerns are all cropped, there are no positive controls----- how do you know or how can readers judge if the antibodies are specific?

As suggested by the reviewer, the appropriate Mol.Wt for all the westerns have been mentioned and incorporated in the corresponding figures.

5. Zymos again no positive controls for uPA or MMP-9

Throughout the paper the MMP-9 zymo is used to infer that it is a measurement of MMP-9 inhibition. This is actually only a measurement of the level of MMP present not even its activity, since pro-MMP-9 will also generate a band of clearing on a zymo and there is no positive control to show where pro and active MMP-9 would run, no inference about MMP-9 activity is made.

We agree with the reviewer. As suggested, we have re-run the Zymo for MMP-9 and uPA activity with controls and show that there is inhibition of uPA and MMP-9 upon M#CD treatment. We have incorporated the appropriate changes in
Figures 1C and 2C and also at the corresponding places in Fig.legend and in the Results section.

6. Fig.2 Zymograms are not quantitative since do not have a linear readout and there are no loading controls.

As noted by the reviewer, Zymograms with loading controls have been incorporated in Fig.2C.

7. Presumably the best western blots are shown and the quality of some of the blots shown is not sufficient to perform the quantification that is presented,

Fig.2D 1h untreated there is a big non-specific blob

As suggested by the reviewer, the blot has been removed, and a better blot has been replaced in Fig.2D 1h time point.

Fig 8B lane 3- half the band is missing.

As suggested by the reviewer, we have made the appropriate changes in Figure 8B.

8. Para 4 percentages given in the text don’t match the figure.

As suggested by the reviewer, the appropriate changes have been made in the text.

Minor essential revisions.

1. Abbreviations are often used without defining e.g. MMP, uPAR, LR etc.

As mentioned, the abbreviations have been defined and are mentioned at the end of the manuscript.

2. “As previously shown” is used at the start of nearly every section in results without a reference or Figure number- it would aid clarity to add these.

As observed by the reviewer, the appropriate references and figure numbers in places where “As previously shown’ is written have been incorporated.

3. The clarity of the paper could be enhanced both:

- 1h and 24h time points used are presumably post the 1h treatment time? That isn’t stated.

As keenly noted by the reviewer, 1h and 24h time points represent 1h post treatment time. Mention of the above has been incorporated in the Material and Methods section.

4. Results par 9. Typo “with” missing

As suggested by the reviewer, the changes have been made in the text.
5. Figure and Figure legends only clarity:

Fig 2 has the wrong antibody –
As noted by the reviewer, the change has been made in the Figure legend.

6. Fig. 4A & B are in the wrong place at the beginning and in the middle which time point is shown for the RT-PCR? This is not real-time PCR data it is semi-quantitative RT-PCR change in legend and text.

As suggested by the reviewer, necessary corrections have made in the Fig. legend and text. The time point shown for RT-PCR is 8h time point.

Figure 4 Are these fractions or a pooled fraction – not clear from text, legend or figure.

These fractions are a pooled fraction. It has now been mentioned clearly in the text, figure legend and also in the figure.

Fig.6 Data should be presented in the order discussed to avoid confusion, legend confusing as jumps around.

As suggested by the reviewer, the data have been presented and legends have been rearranged in the order discussed.

We hope we have answered to all the queries to the satisfaction of the reviewers. If you have any questions or require additional information, please feel free to contact me.

Sincerely,

Jasti S. Rao, Ph.D.
Department Head and Program Director