Author's response to reviews

Title: Amyloid-beta Precursor Protein Promotes Cell Proliferation and Motility of Advanced Breast Cancer

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Author's response to reviews: see over
Dear Dr. Solera,

Thank you for the comments regarding our manuscript #1806927553127073, entitled “Amyloid-beta precursor protein promotes cell proliferation and motility of advanced breast cancer”. We have carefully read and addressed the reviewers’ comments and the critiques and responses are outlined below point-by-point.

**Reviewer:** Kuniko Horie-Inoue  
**Major points**

1. They performed loss-of-function study of APP but not gain-of-function study in breast cancer cells. To confirm their findings in regard to APP knockdown, it is required to investigate the effect of APP overexpression on the biology of breast cancer cells.

   - As suggested by the reviewer, the functional assays with both loss- and gain-of-function experiments would be great complementary studies to support the biological findings in general. However, in our study the gain-of-function experiment is inappropriate to pursue due to the nature of APP gene. As we described in the manuscript, APP has no oncogenic enzyme activity that can force normal breast epithelial cells to become cancerous cells (i.e., acquiring highly proliferative or invasive phenotype) with a single gene amplification or overexpression. Thus, the overexpression of APP in aggressive breast cancer cells (M-IV or MDA-MB-231) would have similar biological responses compared to parental cells since those cells already have high enough APP expression for their proliferative and invasive ability. Thus, we believe that the loss-of-functional study is the optimal experimental approach to prove the functional role of APP in the context of aggressive breast cancer cells. In this regard, the recent Nature publication (Goodarzi et al., 2014) that the reviewer mentioned also utilized only the loss-of-function study by knocking down multiple genes that are highly expressed in the respective cell context.

2. Because they showed that APP was differentially expressed in M-II, -III, -IV cells compared to M-I cells, most of the functional studies for APP should be performed in at least 2 cell lines with different APP expression (e.g., M-IV versus M-I), rather than in MDA-MB-231 cells alone.
In response to the reviewer’s comment, we have performed additional experiments and added the data demonstrating increased p27 expression, but not p21, in both M-I and M-IV cells which were transfected with either shAPP-5 or shAPP-7 (Fig. 2). Consistent with our conclusion from the MDA-MB-231 cell study, the basal level of p27 expression in M-I cells appeared markedly higher than M-IV cells in which approximately 50% knockdown of APP was enough to result in increased p27 expression.

3. Most of the functional experiments were performed by shAPP-7 alone in MDA-MB-231 cells. The similar experiments for Figures 1, 2, & 5 need to be conducted by shAPP-5 or by more than 2 siRNAs against APP in at least 2 cell lines.

- As described above, we have added new data from two different cells (M-I and M-IV) with either shAPP-5 or shAPP-7 transfection. Unlike MDA-MB-231 cells, the M-I or M-IV cells have no migration/invasion ability both in wound healing assay and in matrigel assay. Thus, we used MDA-MB-231 cells in the functional assays.

Minor points
1. shAPP-5 seems to be less efficient than shAPP-7 in regard to the effect of APP knockdown on APP protein expression (Figure 2A). Why the percentage of apoptotic cells in shAPP-5 was higher in cells treated with shAPP-5 than in those with shAPP-7? If their efficiency can be easily altered with different multiplicity of infection, they should monitor the concentrations of shAPP lentiviral particles such as through the integration of a reported gene (e.g., GFP) in the viral vector into the transfected cells.

- One potential factor affecting the biological consequences is the differential targeting region for each shRNA. Specifically, the sequence of shAPP-5 is designed to target 3’-UTR of APP mRNA, while shAPP-7 targets the protein coding region. How this might cause such biological consequence will be addressed in a future study. However, we believe that the biochemical, molecular and functional data presented in the manuscript support the conclusion that APP regulates breast cancer cell proliferation, survival, and migration.

2. For cell growth assay, MTT assay should be performed in a time-dependent manner, such as day 1 to day 7 after the transfection.

- We counted the actual cell number on day 2 and day 4 in Figure 1D which provide the insight how APP affect cell growth over time.

3. shAPP-7 significantly reduced the growth of MDA-MB-231 cells in conventional culture system (Figure 1E) and in xenograft study (Figure 4D), although the inhibitory effect of shAPP-7 on the cell growth was not so remarkable in 3D Matrigel on-top assay in Figure 4B. Please explain the reasons for the data.
- We understand the reviewer’s concerns. This phenomenon could be explained by the adaptive nature of cancer cells after knockdown of APP. We tried to perform our designed experiments with the knockdown cells less than 3 passages, but the cells with high efficient of APP knockdown often died or displayed retarded growth during the cultivation in puromycin-contained medium, which may have resulted in selection of cell population with moderate APP knockdown. Thus, the breast cancer cell growth could be slightly affected by the cell passage status.

4. A recent report by Goodarzi et al. (Nature 2014, doi: 10.1038/nature13466) showed that APP is rather a metastasis suppressor gene in breast cancer together with ZNF395. The findings by Goodarzi et al. seem to be controversial to the results by this group, as they described that “advanced breast cancers with knockdown of APP are more prone to enter into apoptosis” in the present study (lines 16-17, page 18). Please explain the difference of APP function between the 2 systems.

- It is interesting to see the significant role of APP in the regulation of breast cancer. The study demonstrated by Goodarzi et al examined the role of APP in the context of TARBP2 knockdown whereas our study examined direct role of APP in the parental MDA-MB-231 cells without any other combinatorial genetic modifications. These results strongly suggest that APP has a different pathological role depending on the cell context. This point is discussed in the revised manuscript and this reference is now included.

Reviewer: Amit Tuli
Minor points:
1. In the materials & methods sections, the authors should provide the complete information on all the antibodies used. This will help the readers.

- The detailed information of the antibodies has been included in the revised manuscript.

2. In some of the western blots (like Fig 1C, Fig 2B, Fig 3A and 3B and Fig 5), the protein size marker is missing, and that should be included.

- As suggested, the protein size markers have been incorporated.

3. In Figure 1C (top panel) blot, the author should comment why there are two bands immunoreactivity for anti-APP Ab.

- It is due to the post-translational modification (i.e. glycosylation) where the upper molecule represents matured form whereas the lower band correspond to immature form of APP. This has been added in the revised manuscript.
4. I’m not convinced with the blot shown in Fig 5A. I’m not so sure whether EGF stimulation has worked in this case.

- Indeed, we included the Fig. 5A data to show that pERK1/2 levels in MDA-MB-231 cells did not change by knockdown of APP, which were constantly activated due to the oncogenic mutants of KRAS/BRAF, or even in the condition of EGF treatment. So we can rule out the ERK-dependent pathway from the candidates of APP-related major pathways.

Reviewer: Diego Ruano
As a minor point authors must describe RT-PCR in the methods section. Page 7 line 4.

- We are sorry for this oversight. In the revised manuscript, we have added the detailed RT-PCR method.

Additional Editorial requests for revisions
1. Please clarify whether the experiments involving animals have been specifically approved by your local ethics committee. Authors are encouraged to conform to the Animal Research:

- The animal experiment has been approved by the IACUC at Case Western Reserve University and the revised manuscript now includes such information.

3) Please describe in the Methods section the source of the tissue microarray used in the study.

- The tissue array used in the manuscript was purchased from US Biomax (Caht# BRC961). According to the company, “all tissue is collected under the highest ethical standards with the donor being informed completely and with their consent. We make sure we follow standard medical care and protect the donors' privacy. All human tissues are collected under HIPPA approved protocols”. Since we purchased the tissue array from the commercial source, this study does not require our school’s IRB review. The Methods section has been revised accordingly in the revised manuscript.

I would like to thank you for the opportunity to clarify the manuscript according to the reviewers’ comments.

Sincerely,

Hyoung-gon Lee