Author's response to reviews

Title: Extracellular vesicle-mediated phenotype switching in malignant and non-malignant colon cells

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Author's response to reviews: see over
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Antonio Mazzocca, M.D., Ph.D.,
Associate Editor
BMC Cancer

Dear Dr. Mazzocca,

We are resubmitting MS: 1801427843141495,
Extracellular vesicle-mediated phenotype switching in malignant and non-malignant colon cells. We have addressed all of the reviewers’ comments. This included performing more experiments, revising the text and figures and adding additional references. We feel that our extensive revisions will make our manuscript suitable for publication in BMC Cancer. Our response to the reviewers is copied below.

Sincerely,

Devasis Chatterjee, Ph.D.

Reviewer 1

Reviewer's report: The authors have investigated into the effect of extracellular vesicles (EVs) derived from malignant and normal colon cells. They have focused on the importance of 14-3-3 zeta/delta in transfer of malignant phenotype by using siRNA and evaluated NF-κB signaling pathway in terms of luciferase reporter assay. There are several points the authors should revise.

Major Essential Points
1) In addition to luciferase reporter assay of Figure-4, the authors should evaluate the activity of NF-κB signaling pathway. For instance, the localization of p65 in the nucleus and cytoplasm should be investigated by Western blotting analysis.

Response: We examined the localization of p65 and there was no correlation with NF-κB activity. This is probably due to the fact that EVs contain a cargo of proteins that can enhance NF-κB transcription independent of p65.

2) The authors should perform the Western blot analysis of 14-3-3 protein isoforms other than 14-3-3 zeta/delta. 14-3-3 protein maintains Raf-1 in an inactive state in the absence of activation signals but promotes Raf1 activation and stabilizes its active conformation when such signals are received, so that they should rule out the possibility that other isoforms affect the expression of STAT-1 and Raf-1.

Response: We have now included Western blot data of 2 other 14-3-3 isoforms (sigma/epsilon).
3) The following 10 literatures should be included as references and add further explanation and discussion. EVs and exosomes of wide range of cancer tissues should be introduced or discussed in this manuscript, and the signal transduction related to IQGAP1, RAF1, RKIP, NF-kB, and 14-3-3 family should be further mentioned in details.

- Variable NF-κB pathway responses in colon cancer cells treated with chemotherapeutic drugs (BMC Cancer 2014, 14:599)
- Molecular characterization of exosome-like vesicles from breast cancer cells (BMC Cancer 2014, 14:44)
- IQGAP1 selectively interacts with K-Ras but not with H-Ras and modulates K-Ras function. (doi: 10.1016/j.bbrc.2014.01.041.)
- MicroRNA-224 targets RKIP to control cell invasion and expression of metastasis genes in human breast cancer cells (DOI: 10.1016/j.bbrc.2012.07.025)
- PI3K is required for the physical interaction and functional inhibition of NF-κB by β-catenin in colorectal cancer cells (DOI: 10.1016/j.bbrc.2013.03.135)
- P53 suppresses expression of the 14-3-3gamma oncogene (BMC Cancer. 2011; 11: 378.)
- The role of 14-3-3 in transcriptional activation of estrogen receptor and its involvement in proliferation of breast cancer cells. (doi: 10.1016/j.bbrc.2011.09.056.)
- 14-3-3 induces heat shock protein 70 expression in hepatocellular carcinoma (BMC Cancer 2014, 14:425)

Response: We have added many of the references. These are highlighted in yellow. However, we feel that some of them do not relate to our studies. These include:

- Very specific about a kinase that we don’t even mention and not in the scope of the paper.
- This involves another aspect of NF-κB, unrelated to what we are discussing.
- We don't examine the gamma form, and although interesting, it does not directly apply to the scope of our study.
The role of 14-3-3# in transcriptional activation of estrogen receptor # and its involvement in proliferation of breast cancer cells. (doi: 10.1016/j.bbrc.2011.09.056.) This follows with our reasoning for the above point.

Minor Essential Points
1) The pictures of soft agar assay in Figure-1 and 2 should be replaced with more clear images stained with crystal biolet. Furthermore, the authors should present the size of colonies as well as the number of colonies.

Response: We believe that Figure 2 is of publication quality, based on our previous publication (Mol. Cancer. 2013 12(1):118. PMID: 2410342). We have replaced the soft agar pictures from Figure 1. We can not perform crystal violet staining because Colo and 004 CT are cells derived from primary biopsies and have a finite lifespan in culture. Therefore, these cell lines are no longer available. Soft agar colony sizes have been included.

2) The authors should add the schematic conclusion to help readers to better understand the molecular mechanism how 14-3-3 zeta/delta enhances the malignant potential as a putative drug target for the treatment of colorectal cancer.

Response: A schematic has been added.

Reviewer no. 2
Reviewer's report: The study is very interesting and related to a very hot topic. However the data provided by the authors raises several concerns, highlighted below.

The authors isolated Extracellular vesicles (EVs) from HCT116 colon cancer cell line and also from fresh primary colon tumors, by centrifuging supernatants from 7-day cultures of HCT116 or from macrodissected fresh colon tumors. The authors incubated 1459 cells with these EVs for 7 days and next performed soft agar assays, indicating that 1459 acquire a malignant phenotype, since the number of colonies formed in soft agar assay is increased upon exposure to EVs from HCT116 or primary tumors, and that EVs isolated from 1459 cells are able to reduce the number of colonies formed by HCT116, reverting their malignant phenotype.

Next the authors evaluate the protein content by mass spectrometry in 1459 cultured with the various EVS, and using HCT116 cells as control, identifying 14-3-3 proteins as potentially relevant in this phenotype switch. Next the authors show that 1459 cells co-cultured with malignant EVs showed a significant increase in NF-kB transcriptional activity, and that silencing 14-3-3 zeta with siRNA in HCT116 reduces extracellular-vesicle mediated induction of malignant colon cancer phenotype in 1459 cells, leading to reduced colony formation in soft agar assay.

Although the study aim is very interesting, the present manuscript raises several concerns
(major compulsory revisions):

1) Regarding the 1459 cells used, it is not clear if these are indeed epithelial cells or CRL-1459 (ATCC) fibroblasts. This aspect warrants clarification.

Response: These cells are fibroblasts, as stated by ATCC and now included in the text.

2) Regarding the method for isolations of EVs, it includes a pre-clearing at 300g, followed by a centrifugation at 28000g, and this pellet containing the EVs from 30-1000nM is resuspended and added to the cells.

2.1) No characterization of these EVS was provided (size distribution, cargo, etc.), which is relevant information.

Response: The size range of the vesicles was provided in the methods section. Does the reviewer want a complete analysis of lipids, RNA and microRNA that are in the vesicles? This is not the purpose of the study. We are only focusing on proteins transferred, and this was included.

2.2) Can these isolated EVs contain apoptotic bodies, as they have also been shown to have an impact on other cells? The long time in culture may stress the cells and induce increased cell death. Apoptosis should be evaluated under the author's experimental settings, and also the presence of apoptotic bodies should be checked for. This is particularly relevant for EVs isolated from primary tumor samples, which lead to massive cell death and debris in the cell culture. This could be avoided or minimized by isolating epithelial cells by FACS sorting to eliminate debris and increase cell and culture media purity.

Response: Cells were growing continuously. Therefore debris was not of concern. We perform a 2000 x g spin before the 100,000 x g spin to remove apoptotic bodies.

2.3) The authors could physically degrade the EVs and incubate cells with lysed EVs to evaluate reversal of their effects on phenotype switch.

Response: Lysing or degrading EVs damages the protein components in the EVs and will abolish the reversal effects of EVs.

3) The authors evaluate malignant phenotype increase or decrease only by performing soft agar colony formations assays. Additional methods should be used to demonstrate this phenotype switch (cell cycle, proliferation, migration, others).

Response: We have included new data on cell migration.

4) Regarding the evaluation of protein content by mass spectrometry in 1459 cells cultured with the various EVS, and using HCT116 cells as control, it would be interesting the perform this evaluation also in proteins isolated from EVs and not only from cells.
incubated with EVs, to understand if EVs contribute to these changes in protein expression by altering cell signaling or by delivering high amounts of a particular protein.

Response: We have fractionated 3 compartments of EVs from HCT116 cells: The microvesicle, exosome and total (extracellular vesicle) (microvesicle+exosome). We have detected 14-3-3 zeta/delta in the total fraction by Western blot analysis (revised Figure 5). This is suggests that the increase in 14-3-3 zeta/delta we detect in Figure 3 is a function of the content of the EVs that is transferred/delivered to recipient 1459 cells.

5) In the NF-κB transcriptional activity assay, it is unclear which construct was used, nor is there indication of the use of and internal firefly luciferase control, nor of a Renilla Luciferase control for normalization of transfection efficiency. In addition the relevance of NF-κB in the phenotype switching should be further investigated by using specific inhibitors or siRNA, and evaluate is this signaling interferes with phenotype alteration.

Response: The construct that was used has been used in previously published work by our group (Mol. Cell. Biol., 21: 7207-7217, 2001. PMID:11585904) and our collaborators (Crit Rev Oncog. 2011;16(3-4):211-26; Int J Oncol. 2011 Jun;38(6):1683-94; Genes Cancer. 2010 May;1(5):409-420). We have followed the same experimental protocol stated in theses publications and they have been referenced in our manuscript.

We have performed reporter and migration assays with the NF-κB inhibitor, BAY117082 to measure phenotype alterations.

6) Regarding siRNA experiments with 14-3-3 silencing in HCT116 prior to EV isolation, it would be interesting to see the experiment performed also in cell isolated from primary tumors to test if it will behave similarly or not.

Response: We agree and these are studies currently being conducted in the lab as biopsied samples become available.