Reviewer's report

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

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Reviewer: Pedro Borralho

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.

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 ressuspended and added to the cells.

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

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.

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

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…)

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.

5) In the NF-kB 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-kB in the phenotype switching should be further investigated by using specific inhibitors or siRNA, and evaluate is this signaling interferes with phenotype alteration.

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.

**Level of interest:** An article of limited interest

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

I declare that I have no competing interests