EMBO reports

Cancer-secreted miRNAs regulate amino-acid-induced mTORC1 signaling and fibroblast protein synthesis

Miranda Fong, Wei Yan, Majid Ghassemian, Xiwei Wu, Xin Zhou, Minghui Cao, Li Jiang, Jessica Wang, Xuxiang Liu, Jin Zhang, and Shizhen Emily Wang
DOI: 10.15252/embr.202051239

Corresponding author(s): Shizhen Emily Wang (emilywang@ucsd.edu)

Review Timeline:

Submission Date:	3rd Jul 20
Editorial Decision:	19th Aug 20
Revision Received:	3rd Nov 20
Editorial Decision:	23rd Nov 20
Revision Received:	24th Nov 20
Accepted:	27th Nov 20

Editor: Achim Breiling

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 19th Aug 2020

Dear Dr. Wang,

Thank you for the submission of your manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think that the findings are of interest, but they also have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication in EMBO reports. As the reports are below, and I think all points need to be addressed, I will not detail them here. In particular however, it will be important to strengthen the physiological/clinical relevance of the findings, e.g. by using patient derived fibroblasts, EVs from biospecimens, a larger panel of cultured cancer cell lines and/or breast cancer tissues.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure

Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

For more details please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#manuscriptpreparation

See also our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please also note our new reference format:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. Please find instructions on how to link the ORCID ID to the account in our manuscript tracking system in our Author guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

In this manuscript, cancer cell secreted vesicles were shown to suppress mTOR signaling and reduced protein translation in normal fibroblasts. The concept is interesting. However, the approach

used is premature in the manuscript. The authors should have used human patient derived and normal fibroblasts. This would have allowed them to compare specific changes induced in normal fibroblasts. 3T3 mouse fibroblasts are not a good model to understand the crosstalk between cancer-stromal cells.

Additionally, the differential changes induced in normal vs cancer fibroblasts would shed light into reprogramming of pathways.

Further, it is surprising that they observed reduced protein synthesis. It has been known that desmoplasia occurs in fibroblasts in contact with cancer cells, meaning that they have upregulated protein secretion. Hence, the functional relevance of their results is unclear.

They should collect EVS from at least three to four cancer cells and use patient derived CAFs and normal fibroblasts.

13C tracing of glucose and glutamine at min should be done in fibroblasts with and without EVs to see metabolic reprogramming.

Authors should characterize activated and quiescent markers in fibroblast with and without cancer cell EVs.

Referee #2:

This manuscript by Fong et al provides mechanistic insights in to how cancer cells could reprogramme stromal cells within the tumour microenvironment to promote tumour growth. Evidence that extracellular vesicles (EVs) from breast cancer cells suppress amino acid-stimulated mTORC1 signalling in WI-38 and NIH3T3 fibroblast cell lines is presented. This in vitro study compares EVs from the triple negative MDA-MB-231 breast cancer cell line to those from MCF10A, which is widely used as a normal breast cell line. Only the EVs from the breast cancer cell line were shown to suppress mTORC1 signalling, mRNA translation and protein synthesis in recipient fibroblast cells. The authors highlight a role for RagC in this mTORC1-regulated effect and implicate miRNA-105 and miRNA-204 as causative EV factors. in having effects. The authors report differential effects on translation that are not reflected at the transcriptional level and therefore conclude that these effects are under translational control. The manuscript has the standard issues of knowing how generic the mechanism identified is, since only one cancer cell line is used and the study lacks in vivo data, but this work will nevertheless attract the attention of those interested in both EV and cancer biology.

The Wang lab has been studying the effect of extracellular vesicle-associated miRNAs on breast cancer development for a number of years and published extensively in this area. This study is logically and concisely constructed and for the most part clearly explained.

I do, however, have some points that I think would be helpful for the authors to consider prior to publication:

1. The preparations used in the study are small EVs produced by ultracentrifugation. It would be helpful to give them this name, because the breast cells will produce other vesicles, which are not analysed in this study.

2. Small EV preparations contain mixtures of microvesicles and exosomes (EVs), and non-vesicular particles (NVPs) - see Jeppesen et al. (2019) Cell 177, 428-445, and the denser NVPs have high levels of RNA-binding proteins. This makes the gradient data (Figure S2) important in confirming association of miRNAs with EVs. However, there is not an exact correlation between EV protein and miRNA fractions in all cases, eg miR-204 spreads into the putative NP fractions. I therefore think it is critical that the fractions are also probed with a marker for NVPs (Ago1, histones?) and perhaps a microvesicle marker (AnxA1) to show how much overlap there is with these markers. Does ND mean not detected or not done in S2?

3. Are miRNA-105 and miRNA-204 located on the inside or outside of small EVs? Could the authors address this by combining RNase treatment with and without detergent?

4. The details of the anti-miRNA treatment appear to be missing. Are the anti-miRNAs added to the secreting cells before collection of small EVs? Has it been confirmed that this does not affect small EV secretion? If added to the target cells, can we be sure that the miRNAs affected were derived from the secreting cells? It is important for the reader to see how these experiments were done.

5. It is notable that miRNA-105 and miRNA-204 are present on EVS from both MDA-MB-231 and MCF10A cells (there only seems to be two-fold increase miR-105 in MDA-MB-231 cells, Fig. S2), but only the MDA-MB-231 cells are shown to have an effect on mTORC1 signalling etc in fibroblast cells. What explanation do the authors have for this? Are there other factors which only allow the miRNAs to work when present in cancer cell small EVs?

6. The authors have indicated that they have used an n of at least three for experiments in which no quantification is shown. I think it would make sense to quantify the data, eg Rag levels, puromycin labelling, etc, if the data are available.

Minor points:

1. Introduce WI-38 and NIH3T3 as fibroblast cell lines
2. Abstract, line 5 - 'mRNA translation, rather than 'protein translation'
3. Page 3, line 4 - 'cancer's exploitation' rather than 'cancer's exploit'
4. Page 4, line 10 - this sentence needs rewording

Referee #3:

The manuscript of Miranda et al. provided evidence to show that breast cancer cells secrete EVs containing miR-105 and miR-204 and that these EVs are taken up by fibroblast and suppress protein synthesis through down regulation of RagC. This is a very interesting study which sheds new light into the mechanism of interaction between cancer cell and tumor microenvironment. However, there are several major and minor concerns and this reviewer has suggestions to further strengthen the manuscript as listed below.

Major questions:

1. This study needs more data to show clinical relevance.

a. The findings were based on a single cell line system, 231 vs MCF10A. It's not clear whether the observation is more general. It's also not clear if this finding is specific to triple negative breast cancer cells. I would suggest to verify their findings in other cancer cell lines such as MCF7 and SKBR3, as well as some breast cancer PDX cells.

b. This study needs more clinical evidence to back up their findings. Such evidence includes, but not limited to verifying miRNAs in EVs from biospecimens (blood or breast cancer tissue), verifying RagC expression in surrounding fibroblast of breast cancer tissue and doing patient survival analysis in relation to the miRNAs.

2. The functional consequence of the findings is not clear. The authors presented the evidence to show that cancer derived EVs inhibited protein synthesis in fibroblast. However, how the "educated" fibroblasts in return affect cancer growth is not clear. The authors can add an animal experiment to address this question. For example, knockdown of the miRNAs in cancer cells and inject them into mice.

3. The authors stated that "Fibroblasts exhibited high and comparable uptake efficiencies for EVs from MDA-MB-231 breast cancer cells and MCF10A non-cancer cells (Fig. S1)". However, Figure S1A is not conclusive. It is not surprising to see comparable uptake in in vitro setting, but this may not be the case in vivo. The authors should test this in mouse by directly injecting labeled EVs. Also, because it may not be easy to compare the uptake by the florescent imaging, FACS analysis may be a better choice for quantification and comparison of the uptake.

4. The authors stated that "Upon re-feeding with a three-AA mixture of leucine, arginine, and lysine or a cocktail of essential AAs, fibroblasts pretreated with MDA-MB-231-derived EVs exhibited a significant suppression of p70S6K phosphorylation compared to fibroblasts treated with EVs from MCF10A cells or with PBS (Fig. 1A)". While the purpose of Figure 1A is to compare the mTORC1 activation among the groups of control, MCF710A EV and 231EV, the western blot data were separated into three panels, probably because they were performed separately. If this is the case, it is difficult to compare.

5. The authors state that "Treatment with MDA-MB-231 EVs, but not MCF10A EVs, significantly downregulated the wild-type RRAGC 3'UTR reporter; both the miR-105 and miR-204 binding sites were required for this effect, as demonstrated by the loss of response in mutant reporters (Fig. 5D)". Can authors add some explanation why both of the binding sites are required? According to Figure 5B, the two miRNAs have separate binding sites on the 3'UTR of RRAGC. Figure 5C indicates that miR-105 or miR-204 alone was able to suppress the RRAGC, which means one binding site is enough. This seems contradicting the observation of Figure 5D, where miR-105 and miR-204 function in a synergistic manner.

Minor points:

1. The authors stated that "To investigate the mechanism through which cancer EVs suppress AA-induced protein synthesis, components of mTORC1/p70S6K signaling were analyzed after EV treatment, with only RAGC consistently showing reduction by MDA-MB-231 EVs in both WI-38 and NIH3T3 fibroblast models (Fig. 4A)". However, the reduction of RAGC seems to be marginal in the WI-38 panel in Figure 4A. I suggest to repeat the western blot or add quantified data.

2. The authors stated that "Ectopic expression of a RagC cDNA construct lacking 3'UTR abolished the effect of cancer EVs on puromycin incorporation (Fig. 4D)". In the RAGC-HA ectopic expression group, why is there a decrease in puro incorporation in the PBS treatment group compared to the MCF610A EV treatment group? The increase of RAGC should be shown by western blot. It is important to know how much of the increase in RAGC could rescue the protein translation from the 231EV treatment.

3. The authors stated that "By using anti-miRNAs to block the selected EV miRNAs, we found that miR-105 and miR-204, but not miR-122, were required for cancer EVs to downregulate RagC (Fig. 5A)". In addition to the level of RagC, the authors also need to directly check if these anti-miRNAs rescue the protein synthesis by doing the Puro incorporation assay.

4. There is no descriptions about most supplemental figures in the main text.

Response to Reviewers

We thank the editor and all reviewers for the constructive comments. Substantial revisions have been made to the manuscript and a considerable amount of new data has been added. We have addressed all points raised, including strengthening the physiological/clinical relevance of the findings by using patient-derived fibroblasts and EVs from a larger panel of cancer cells and by adding staining of breast tumor tissues. Below please find our point-by-point responses. Changes in the text are highlighted to facilitate tracking.

Referee #1:

In this manuscript, cancer cell secreted vesicles were shown to suppress mTOR signaling and reduced protein translation in normal fibroblasts. The concept is interesting. However, the approach used is premature in the manuscript. The authors should have used human patient derived and normal fibroblasts. This would have allowed them to compare specific changes induced in normal fibroblasts. 3T3 mouse fibroblasts are not a good model to understand the crosstalk between cancer-stromal cells. Additionally, the differential changes induced in normal vs cancer fibroblasts would shed light into reprogramming of pathways.

Response: Following the reviewer's suggestion, we have performed new experiments using patient-derived cancer-associated fibroblasts (CAF) and normal human mammary fibroblasts. We also included data with WI-38 cells, which have been widely used as a model of normal fibroblasts. In both CAF and normal fibroblasts (mammary fibroblasts and WI-38), we observed suppression of AA-stimulated protein synthesis by MDA-MB-231-derived EVs (measured by puromycin incorporation into newly synthesized proteins), suggesting this effect of EVs similarly influences CAF and normal fibroblasts. The new data have been added to Fig. 1A-C.

Further, it is surprising that they observed reduced protein synthesis. It has been known that desmoplasia occurs in fibroblasts in contact with cancer cells, meaning that they have upregulated protein secretion. Hence, the functional relevance of their results is unclear.

Response: Increased protein secretion is indeed a hallmark of fibroblast activation by cancer-secreted factors such as TGF-beta. In the current study our focus was on fibroblasts' early response to AA stimulation in terms of the transient mTORC1 activation and protein synthesis, an experimental setting to mimic the periodic nutrient fluctuations in the tumor microenvironment. Under this experimental setting, cancer EVs did not significantly affect global protein synthesis in fibroblasts that were continuously cultured under AA repletion (Fig. 1C), but only suppressed the transient protein synthesis in fibroblasts following AA starvation and re-feeding (Fig. 1B). This indicates that the EVs target the translatomic response of recipient cells to the dynamic changes of AA levels in the environment, rather than the basic components of translational machinery.

They should collect EVS from at least three to four cancer cells and use patient derived CAFs and normal fibroblasts.

Response: We have performed new experiments using patient-derived CAF and EVs from several BC cell lines (MDA-MB-231, SK-BR-3, MCF7, and patient-derived xenograft tumor cells/PDX), and showed suppressed new protein synthesis (puromycin labeling) and RagC expression following treatment with EVs from all BC cell lines but not with EVs from MCF10A non-cancer cells. The new data have been added to Fig. 4E.

13C tracing of glucose and glutamine at min should be done in fibroblasts with and without EVs to see metabolic reprogramming.

Response: We have previously performed ^{13}C tracing of glucose and glutamine in fibroblasts with and without EVs to study metabolic reprogramming, and the results are reported in Yan et al. Nat Cell Biol 20, 597-609. In the current study we instead focused on fibroblasts' response to AA stimulation in terms of mTORC1 activation and protein synthesis.

Authors should characterize activated and quiescent markers in fibroblast with and without cancer cell EVs.

Response: We have performed new experiments to examine the levels of α-smooth muscle actin (SMA) and vimentin. We did not observe significant changes in these proteins in CAF treated with cancer-derived or non-cancer EVs for 48 h (Fig. EV1C).

Referee #2:

This manuscript by Fong et al provides mechanistic insights in to how cancer cells could reprogramme stromal cells within the tumour microenvironment to promote tumour growth. Evidence that extracellular vesicles (EVs) from breast cancer cells suppress amino acid-stimulated mTORC1 signalling in WI-38 and NIH3T3 fibroblast cell lines is presented. This in vitro study compares EVs from the triple negative MDA-MB-231 breast cancer cell line to those from MCF10A, which is widely used as a normal breast cell line. Only the EVs from the breast cancer cell line were shown to suppress mTORC1 signalling, mRNA translation and protein synthesis in recipient fibroblast cells. The authors highlight a role for RagC in this mTORC1-regulated effect and implicate miRNA-105 and miRNA-204 as causative EV factors in having effects. The authors report differential effects on translation that are not reflected at the transcriptional level and therefore conclude that these effects are under translational control. The manuscript has the standard issues of knowing how generic the mechanism identified is, since only one cancer cell line is used and the study lacks in vivo data, but this work will nevertheless attract the attention of those interested in both EV and cancer biology. The Wang lab has been studying the effect of extracellular vesicle-associated miRNAs on breast cancer development for a number of years and published extensively in this area. This study is logically and concisely constructed and for the most part clearly explained. I do, however, have some points that I think would be helpful for the authors to consider prior to publication:

Response: We appreciate the reviewer's kind comments. In addition to our point-by-point response below, we have also assessed levels of miR-105, miR-204, and RagC in human breast tumors and xenograft tumors to strengthen the clinical relevance (new Fig. 7).

1. The preparations used in the study are small EVs produced by ultracentrifugation. It would be helpful to give them this name, because the breast cells will produce other vesicles, which are not analysed in this study.

Response: Following the reviewer's suggestion, we have added the statement that EVs used in this study are similar to "small EVs" defined by others (page 7).

2. Small EV preparations contain mixtures of microvesicles and exosomes (EVs), and non-vesicular particles (NVPs) - see Jeppesen et al. (2019) Cell 177, 428-445, and the denser NVPs have high levels of RNA-binding proteins. This makes the gradient data (Figure S2) important in confirming association of miRNAs with EVs. However, there is not an exact correlation between

EV protein and miRNA fractions in all cases, eg miR-204 spreads into the putative NP fractions. I therefore think it is critical that the fractions are also probed with a marker for NVPs (Ago1, histones?) and perhaps a microvesicle marker (AnxA1) to show how much overlap there is with these markers. Does ND mean not detected or not done in S2?

Response: We have performed new experiments detecting AnxA1 in EV fractions, and added the data to Fig. EV2A,B. In addition to AnxA1, CD9 has also been reported as a marker for microvesicles and was detected in EV fractions. In the EVs prepared in this study, we did not detect Ago proteins, and have included this result in Fig. EV1E. ND means not detected, which has been clarified in the figure legend.

3. Are miRNA-105 and miRNA-204 located on the inside or outside of small EVs? Could the authors address this by combining RNase treatment with and without detergent?

Response: We have performed new experiments using Protease+RNase treatment in the presence or absence of detergent to show that both miR-105 and miR-204 are mainly located inside of EVs. The new data have been added to Fig. EV1F.

4. The details of the anti-miRNA treatment appear to be missing. Are the anti-miRNAs added to the secreting cells before collection of small EVs? Has it been confirmed that this does not affect small EV secretion? If added to the target cells, can we be sure that the miRNAs affected were derived from the secreting cells? It is important for the reader to see how these experiments were done.

Response: The anti-miRNA treatment was added to the target cells via transfection to make these cells resistant to the effect of corresponding EV-transferred miRNA. The experimental procedure has been clarified in the figure legend. We indeed cannot distinguish the effect of endogenous and EV-transferred miRNAs in target cells, but this experiment would still support the regulatory effect of miR-105 and miR-204 on RagC expression and protein synthesis.

5. It is notable that miRNA-105 and miRNA-204 are present on EVS from both MDA-MB-231 and MCF10A cells (there only seems to be two-fold increase miR-105 in MDA-MB-231 cells, Fig. S2), but only the MDA-MB-231 cells are shown to have an effect on mTORC1 signalling etc in fibroblast cells. What explanation do the authors have for this? Are there other factors which only allow the miRNAs to work when present in cancer cell small EVs?

Response: Data in the old figure were not normalized to an equal amount of unfractionated EVs. We have corrected this figure by applying the proper normalization (Fig. EV2D). As shown in our previous publication (Yan et al. Nat Cell Biol 20, 597-609) and in new Fig. EV1F, both miR-105 and miR-204 levels are significantly higher by >10 folds in EVs from MDA-MB-231 cells compared to MCF10A EVs.

6. The authors have indicated that they have used an n of at least three for experiments in which no quantification is shown. I think it would make sense to quantify the data, eg Rag levels, puromycin labelling, etc, if the data are available.

Response: We have included quantification of RagC levels and puromycin labeling in all figures.

Minor points:

1. Introduce WI-38 and NIH3T3 as fibroblast cell lines

Response: We have added this in pages 4 and 5.

2. Abstract, line 5 - 'mRNA translation, rather than 'protein translation'

Response: Corrected.

3. Page 3, line 4 - 'cancer's exploitation' rather than 'cancer's exploit'

Response: Corrected.

4. Page 4, line 10 - this sentence needs rewording

Response: Corrected.

Referee #3:

The manuscript of Miranda et al. provided evidence to show that breast cancer cells secrete EVs containing miR-105 and miR-204 and that these EVs are taken up by fibroblast and suppress protein synthesis through down regulation of RagC. This is a very interesting study which sheds new light into the mechanism of interaction between cancer cell and tumor microenvironment. However, there are several major and minor concerns and this reviewer has suggestions to further strengthen the manuscript as listed below.

Major questions:

1. This study needs more data to show clinical relevance.

a. The findings were based on a single cell line system, 231 vs MCF10A. It's not clear whether the observation is more general. It's also not clear if this finding is specific to triple negative breast cancer cells. I would suggest to verify their findings in other cancer cell lines such as MCF7 and SKBR3, as well as some breast cancer PDX cells.

Response: We have performed new experiments using patient-derived CAF and EVs from several BC cell lines (MDA-MB-231, SK-BR-3, MCF7, and patient-derived xenograft tumor cells/PDX), and showed suppressed new protein synthesis (puromycin labeling) and RagC expression following treatment with EVs from all BC cell lines but not with EVs from MCF10A non-cancer cells. The new data have been added to Fig. 4E.

b. This study needs more clinical evidence to back up their findings. Such evidence includes, but not limited to verifying miRNAs in EVs from biospecimens (blood or breast cancer tissue), verifying RagC expression in surrounding fibroblast of breast cancer tissue and doing patient survival analysis in relation to the miRNAs.

Response: We have performed new experiments to assess the levels of miR-105, miR-204, and RagC in human breast tumors and xenograft tumors to strengthen the clinical relevance. The new data showing a negative correlation between stroma RagC and miR-105 have been added to Fig. 7A,B. Unfortunately, the patient survival data are not available to us.

2. The functional consequence of the findings is not clear. The authors presented the evidence to show that cancer derived EVs inhibited protein synthesis in fibroblast. However, how the

"educated" fibroblasts in return affect cancer growth is not clear. The authors can add an animal experiment to address this question. For example, knockdown of the miRNAs in cancer cells and inject them into mice.

Response: We added new data showing that miR-105, but not miR-204, was negatively associated with stroma RagC in human breast tumors. We further showed that stroma RagC was downregulated in MCFDCIS xenograft tumors when miR-105 was overexpressed in cancer cells (Fig. 7). Our previous and current studies thus together indicate a multifaceted function of cancer-secreted miR-105 in modifying the tumor microenvironment to promote tumor growth and metastasis, including metabolic reprogramming of CAF (Yan et al. Nat Cell Biol 20, 597-609), an altered protein synthesis response of CAF to the intermittent AA replenishments in the tumor microenvironment (current study), and modulation of the tumor vasculature (Zhou et al. Cancer Cell 25, 501-515). All these mechanisms may contribute to the miR-105-induced tumor growth and metastasis as well as the tumor-suppressive effect of anti-miR-105 treatment that are reported in our previous studies (Yan et al. Nat Cell Biol 20, 597-609; Zhou et al. Cancer Cell 25, 501-515). We have added this discussion to the manuscript (page 11).

3. The authors stated that "Fibroblasts exhibited high and comparable uptake efficiencies for EVs from MDA-MB-231 breast cancer cells and MCF10A non-cancer cells (Fig. S1)". However, Figure S1A is not conclusive. It is not surprising to see comparable uptake in in vitro setting, but this may not be the case in vivo. The authors should test this in mouse by directly injecting labeled EVs. Also, because it may not be easy to compare the uptake by the florescent imaging, FACS analysis may be a better choice for quantification and comparison of the uptake.

Response: We have performed new experiments to confirm fibroblasts' *in vivo* uptake of EVs by injecting DiI-labeled EVs into xenograft tumors. The new data have been added to Fig. EV1B.

4. The authors stated that "Upon re-feeding with a three-AA mixture of leucine, arginine, and lysine or a cocktail of essential AAs, fibroblasts pretreated with MDA-MB-231-derived EVs exhibited a significant suppression of p70S6K phosphorylation compared to fibroblasts treated with EVs from MCF10A cells or with PBS (Fig. 1A)". While the purpose of Figure 1A is to compare the mTORC1 activation among the groups of control, MCF710A EV and 231EV, the western blot data were separated into three panels, probably because they were performed separately. If this is the case, it is difficult to compare.

Response: The experiment was performed together. The cell lysates were separated into three panels because the Western blot gels we used only had 10 lanes. For Fig. 1A it is critical to determine the time course following AA re-feeding, and to compare such time course under PBS, MCF10A EV, and MDA-MB-231 EV treatments. The development was given equal time and the exposure to film was performed together. In all other experiments in this study we have included PBS, MCF10A EV, and MDA-MB-231 EV treatments in the same gels.

5. The authors state that "Treatment with MDA-MB-231 EVs, but not MCF10A EVs, significantly downregulated the wild-type RRAGC 3'UTR reporter; both the miR-105 and miR-204 binding sites were required for this effect, as demonstrated by the loss of response in mutant reporters (Fig. 5D)". Can authors add some explanation why both of the binding sites are required? According to Figure 5B, the two miRNAs have separate binding sites on the 3'UTR of RRAGC. Figure 5C indicates that miR-105 or miR-204 alone was able to suppress the RRAGC, which means one binding site is enough. This seems contradicting the observation of Figure 5D, where miR-105 and miR-204 function in a synergistic manner.

Response: We added interpretation of this result to page 7. Although individual transfection of synthetic mimics of miR-105 and miR-204 showed that both miRNAs act on the 3'UTR reporter (Fig. 5C), Fig. 5D showed that loss of either miRNA binding sites abolished the effect of MDA-MB-231 EVs. This could be due to the lower abundances of both miRNAs in the EVs compared to miRNA mimics used in transfection assay. Previous studies have revealed synergistic action of miRNAs targeting the same genes, which we discussed in the manuscript as a possible mechanism.

Minor points:

1. The authors stated that "To investigate the mechanism through which cancer EVs suppress AA-induced protein synthesis, components of mTORC1/p70S6K signaling were analyzed after EV treatment, with only RAGC consistently showing reduction by MDA-MB-231 EVs in both WI-38 and NIH3T3 fibroblast models (Fig. 4A)". However, the reduction of RAGC seems to be marginal in the WI-38 panel in Figure 4A. I suggest to repeat the western blot or add quantified data.

Response: We have repeated the Western blot, and have included quantification of RagC levels and puromycin labeling in all figures.

2. The authors stated that "Ectopic expression of a RagC cDNA construct lacking 3'UTR abolished the effect of cancer EVs on puromycin incorporation (Fig. 4D)". In the RAGC-HA ectopic expression group, why is there a decrease in puro incorporation in the PBS treatment group compared to the MCF10A EV treatment group? The increase of RAGC should be shown by western blot. It is important to know how much of the increase in RAGC could rescue the protein translation from the 231EV treatment.

Response: We have repeated the Western blot and added RagC blots to Fig. 4D.

3. The authors stated that "By using anti-miRNAs to block the selected EV miRNAs, we found that miR-105 and miR-204, but not miR-122, were required for cancer EVs to downregulate RagC (Fig. 5A)". In addition to the level of RagC, the authors also need to directly check if these anti-miRNAs rescue the protein synthesis by doing the Puro incorporation assay.

Response: We have added puromycin incorporation assay to Fig. 5A to show that the anti-miRNAs also rescue protein synthesis.

4. There is no descriptions about most supplemental figures in the main text.

Response: We have added the descriptions in page 7.

1st Revision - Editorial Decision 23rd Nov 2020

Dear Dr. Wang,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your study in EMBO reports.

Before we can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised manuscript:

- Table EV1 is too large to be displayed in full in the published manuscript. This is a dataset. Please name this file Dataset EV1 and put its legend on the first TAB of the excel file. Then change the callouts in the manuscript text accordingly. Finally, please remove the legend from the manuscript text file.

- There is a callout to Table S1 in the manuscript, but there is no such table. Please check.

- Please remove the referee access information from the data availability section, and make sure the data get public upon publication of the paper.

- As most of the Western blots shown in the study are significantly cropped, could you please provide the source data for the blots. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data (scans of entire blots) together with the final revised manuscript. Please include size markers for the scans of entire blots, label the scans with figure and panel number, and send one PDF file per figure (main and EV figures).

- Could statistical testing be performed for the diagram shown in Fig. EV1F?

- Please make sure that the funding information added in the online submission system is complete and similar to the one in the manuscript. Please also add the funding for the core facility into the online form.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Kind regards,

Achim

Achim Breiling
Editor

EMBO Reports

Referee #1:

I am satisfied with the new experiments and comments from the authors. Hence, the manuscript is now suitable for publication.

Referee #2:

The authors have satisfactorily addressed my comments and I am happy to recommend publication in its current form.

Referee #3:

All comments were addressed and it is now suitable for publication.

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 27th Nov 2020

Dr. Shizhen Emily Wang
University of California San Diego
Pathology
9500 Gilman Drive
MC 0612
La Jolla, CA 92093
United States

Dear Dr. Wang,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

****

THINGS TO DO NOW:

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2020-51239V3 and be addressed to emboreports@wiley.com.

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Shizhen Emily Wang
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2020-51239

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was generally chosen based on preliminary data indicating the variance within each group and the differences between groups.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size for animal study was chosen based on previous data indicating the variance within each group and the differences between groups. We did not use a statisical method to determine sample size for animal study in this research.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All samples were included for the analyses.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Mice were randomized before tumor cell injection.
For animal studies, include a statement about randomization even if no randomization was used.	Mice were randomized before tumor cell injection.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The IHC slides were analyzed by a researcher blinded to the tumor group.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The IHC slides were analyzed by a researcher blinded to the tumor group.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, as determined by GraphPad Prism 7.01 and SPSS 22.
Is there an estimate of variation within each group of data?	Yes. We presented data as mean +/- SEM or mean +/- SD. Error bars were shown to indicate variation within each group of data.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The antibodies were described in the Materials and Methods section in detail.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The cell line information was included in the Materials and Methods section in detail.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Female NOD/SCID/IL2Rγ-null (NSG) mice of 8 week old were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed at a facility that maintains a 12:12h light/dark cycle with Zeitgeber time 12=lights off.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal experiments were approved by the institutional animal care and use committees at the University of California San Diego and the Beckman Research Institute of the City of Hope. The study is compliant with all relevant ethical regulations regarding animal research.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	All aspects of animal studies meet the compliance criteria of the ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The datasets produced in this study have been deposited in public g databases, as described under "Data availability" in the Materials and Methods section.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Please see above.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No