Journal of Clinical and Translational Research
Peer review process file 08.202206.015

Bone marrow mesenchymal stem cell-derived exosomal LINC00847 inhibits the proliferation, migration, and invasion of Ewing sarcoma

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Review timeline:

Received: 22 September, 2022
Editorial decision: 13 October, 2022
Revision received: 7 November, 2022
Editorial decision: 8 November, 2022
Published online: 24 November, 2022

1st Editorial decision
13-Oct-2022

Ref.: Ms. No. JCTRes-D-22-00142
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Dear Prof. Cao,

Reviewers have now commented on your paper. Reviewer 1 has suggested a major revision, whereas reviewer 3 recommended a reject based on poor legibility of figures and the fact that the data were extrapolated from in vitro analyses without in vivo corroboration. The latter reviewer therefore concluded that the extent of novelty and validation in a higher-order biological system does not warrant publication. We have discussed these critiques within the editorial board and wish to give the authors the opportunity to rebut and modify the manuscript accordingly. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

For your guidance, reviewers' comments are appended below.

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you submit the revised manuscript. Also, please ensure that the track changes function is switched on when implementing the revisions. This enables the reviewers to rapidly verify all changes made.

Your revision is due by Nov 12, 2022.

To submit a revision, go to https://www.editorialmanager.com/jctres/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Reviewers' comments:

Reviewer #1: In this communication, the authors report on the biological impact of bone marrow mesenchymal stem cell-derived exosomal long non-coding RNA LINC00847 on Ewing sarcoma cells in vitro. They provide evidence for exosomal LINC00847 to modulate Ewing sarcoma cell growth and potentially also tumor cell invasion and migration. Using an in silico approach they identified seed sequences for 5 microRNAs potentially sponged by the lncRNA, and narrowed the list of respective miRNA target genes down to 4 candidates, which they found positively correlated with LINC00847 across many normal tissues and cancers supporting their hypothesis of a competing endogenous RNA network as the source of the observed growth suppressive phenotype.
Even though experimental proof for the suggested mechanism of action is still missing, these in silico findings are intriguing as they may identify a novel Ewing sarcoma growth-regulatory component in MSC-derived exosomal cargo as a putative therapeutic agent. However, important informations for several presented data are missing as detailed below. The work was performed exclusively using commercial bone marrow derived mesenchymal stem cells and established Ewing sarcoma cell lines, as well as publicly accessible gene expression databases. Thus, it does not contain any ex vivo, in vivo, or clinical component, as is required by the journal. In addition, the manuscript needs extensive proofreading by a native English speaker.

Detailed comments:
Results, lines 184-186: "Based on the GEO dataset (GSE48022, GSE90970, GSE17618), we found that LINC00847 was down regulated in ES tissues (Figure 1B) and cells (Figure 1C)". Compared to what? Legend says BMSC. Please indicate also in the text. Please, also indicate age of MSC donor, as transcriptomes may be different in an age-dependent manner (Riggi et al., DOI: gad.1899710 [pii]10.1101/gad.1899710).

Lines 190-194 (Figures 1E, F): Please, indicate and describe the data set that has been used in survival analyses and describe cut-off values for high and low expression.

Figure 2: Text and figure legend lack information if the data represent the consequences of transient (for how long) or stable LINC00847 overexpression. Additionally, the Materials and

Methods section lacks details on the expression vector, LINC expression-driving promoter, and selection of transfected cells. Also, the level of overexpression compared to control transfected Ewing sarcoma cell lines and to MSC should be presented. I presume, it was much higher than physiological levels in MSC?
Please, also provide technical details on the migration/invasion experiment in Figure 2D/E (transient or stable LINC expression, number of cells, duration, chemoattractant, quantification method). How can the authors exclude that the reduced number of cells migrating through the transwell membrane are not the consequence of reduced proliferation?

Figure 4: Neither the Materials and Methods section, nor the text or the figure legend mention the number of extracellular vesicles added to the culture medium, and if there was any pre-incubation with them before start of the proliferation assay, or any medium change/renewal of EVs during the 4 days of the proliferation assay. Same is true for the migration/invasion assays. Also, as stated above for the LINC expressing MSC, how can the authors exclude that the reduced migration/invasion are independent from the anti-proliferative effect of the EVs on Ewing cell lines?

Line 239: The data set GSE18546 used to identify upregulated miRNAs is from synovial sarcoma but not Ewing sarcoma.

Line 242: Which data set was used to identify under-expressed mRNAs in Ewing sarcoma (compared to what?)? I guess the authors meant logFC<-1 rather than >1 as indicated in line 243?

Reviewer #3: In this work, the author aims at demonstrating the consequences of LINC00847 in the context of Ewing sarcoma. Unfortunately, the quality of the provided figures makes it unsuitable to review.
For instance,
Figure 1A entities is not readable
Figure 1E-F numbers are not readable.
Figure 3G is not interpretable (blur blue dots…),
Figure 5C and F and 6A, D, E text is not readable, 6A

Overall, this study seems very correlative and do not provide any new insights into LINC00847 biology. Effects of LINC00847 overexpression (direct or indirect through EVs) on Ewing cells proliferation appear very moderate.
Many potentially interesting observations are discussed but remain purely theorical and extrapolate form other studies (very long discussion section).

Authors' response

Reviewers' comments:

Reviewer #1:
In this communication, the authors report on the biological impact of bone marrow mesenchymal stem cell-derived exosomal long non-coding RNA LINC00847 on Ewing sarcoma cells in vitro. They provide evidence for exosomal LINC00847 to modulate Ewing sarcoma cell growth and potentially also tumor cell invasion and migration. Using an in silico

approach they identified seed sequences for 5 microRNAs potentially sponged by the lncRNA, and narrowed the list of respective miRNA target genes down to 4 candidates, which they found positively correlated with LINC00847 across many normal tissues and cancers supporting their hypothesis of a competing endogenous RNA network as the source of the observed growth suppressive phenotype.
Even though experimental proof for the suggested mechanism of action is still missing, these in silico findings are intriguing as they may identify a novel Ewing sarcoma growth-regulatory component in MSC-derived exosomal cargo as a putative therapeutic agent. However, important informations for several presented data are missing as detailed below. The work was performed exclusively using commercial bone marrow derived mesenchymal stem cells and established Ewing sarcoma cell lines, as well as publicly accessible gene expression databases. Thus, it does not contain any ex vivo, in vivo, or clinical component, as is required by the journal. In addition, the manuscript needs extensive proofreading by a native English speaker.

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RE: Thank you for your detailed and very constructive critique. In the resubmitted manuscript, we have made revisions accordingly.

In addition, we checked the age of 7 BM-MSCs donors in the GSE48022 and GSE90970 datasets separately. We found that the age of 3 BM-MSCs donors (from GSE90970) was 20. Nevertheless, the age of other BM-MSCs donors (from GSE48022) could not be found. It is the shortcoming of this paper. We also hope to obtain more clinical samples to explore further the expression and function of LINC00847 in MSCs and ES.

Q 2: Lines 190-194 (Figures 1E, F): Please, indicate and describe the data set that has been used in survival analyses and describe cut-off values for high and low expression.

RE: Thank you for reading the manuscript so carefully. The details of survival analysis were added in the Materials and Methods section of the manuscript, as described in "2.2. Survival prognostic analysis".

The survival prognosis analysis was performed on 44 ES patients in the GEO dataset (GSE17618). Median values were used as the cutoff values for high and low expression levels of LINC00847.

Q 3. Figure 2: Text and figure legend lack information if the data represent the consequences of transient (for how long) or stable LINC00847 overexpression. Additionally, the Materials and Methods section lacks details on the expression vector, LINC expression-driving promoter, and selection of transfected cells. Also, the level of overexpression compared to control transfected Ewing sarcoma cell lines and to MSC should be presented. I presume, it was much higher than physiological levels in MSC?

Please, also provide technical details on the migration/invasion experiment in Figure 2D/E (transient or stable LINC expression, number of cells, duration, chemoattractant, quantification

method). How can the authors exclude that the reduced number of cells migrating through the transwell membrane are not the consequence of reduced proliferation?

RE: We are so appreciative and grateful for your critical evaluation of our manuscript. We have revised the manuscript accordingly. The LINC00847 overexpression plasmid was transiently transfected into ES cells. The details of the LINC00847 overexpression vector and transfected cells have been added in the "Materials and Methods" section (2.7. Cell migration and invasion assays). Also, we did not simultaneously examine the expression levels of LINC00847 in LINC00847 overexpressed Ewing sarcoma cells, transfected control Ewing sarcoma cells, and BMSCs. It is also a deficiency of this article. We hope to verify the expression levels in further research experimentally.

The technical details on the migration/invasion experiment in Figure 2D/E have been added in the "Materials and Methods" section (2.7. Cell migration and invasion assays). In the migration/invasion experiment, cells were suspended using a serum-free medium. Serum starvation induces G0/G1 cell cycler arrest and inhibits cell proliferation(Cooper S. Reappraisal of serum starvation, the restriction point, G0, and G1 phase arrest points. FASEB J. 2003 Mar;17(3):333-40. doi: 10.1096/fj.02-0352rev. PMID: 12631573.). Under this experiment condition for 48 h (the length for migration and invasion transwell assays), LINC00847 does not have an obvious effect on cell proliferation as well as cell viability. Furthermore, cell proliferation assays showed insignificant differences in Ewing sarcoma cell proliferation within 48 hours of transient transfection of LINC00847 plasmid compared to the control group. In summary, the effects of LINC00847 in migration/invasion assays are independent of the anti-proliferative effect of LINC00847 on Ewing cell lines.

Q 4. Figure 4: Neither the Materials and Methods section, nor the text or the figure legend mention the number of extracellular vesicles added to the culture medium, and if there was any pre-incubation with them before start of the proliferation assay, or any medium change/renewal of EVs during the 4 days of the proliferation assay. Same is true for the migration/invasion assays. Also, as stated above for the LINC expressing MSC, how can the authors exclude that the reduced migration/invasion are independent from the anti-proliferative effect of the EVs on Ewing cell lines?

RE: Thanks for raising this important question. In the "Materials and Methods" section (2.12. Coculture assay of extracellular vesicles and ES cells), we added the coculture assay details. After 48 hours of coculture with extracellular vesicle (20 μg/ml), the cells were collected and subjected to cell proliferation, migration, and invasion assays as described previously.

Cells were suspended in a serum-free medium to inhibit cell proliferation in the migration/invasion experiment, excluding the EVs' anti-proliferative effect on Ewing cell lines.

Q 5. Line 239: The data set GSE18546 used to identify upregulated miRNAs is from synovial sarcoma but not Ewing sarcoma.

RE: Thank you for your comment. We have added details of all GEO samples in Table S1. In the GSE18546 dataset, up-regulated miRNAs were obtained from 5 Ewing sarcoma samples compared to 5 normal skeletal muscles.

Q 6. Line 242: Which data set was used to identify under-expressed mRNAs in Ewing sarcoma

(compared to what?)? I guess the authors meant logFC<-1 rather than >1 as indicated in line 243?

RE：Thank you for your detailed and very constructive critique. According to your advice, we revised the manuscript. Downregulated mRNAs were obtained from Ewing sarcoma cell lines (11 Ewing sarcoma cell lines from GSE17618) compared to BM-MSCs (4 BM-MSCs from GSE48022, 3 BM-MSCs from GSE90970). LogFC < -1 and adjust.p.value < 0.05 were set as downregulated mRNA screened criteria.

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Overall, this study seems very correlative and do not provide any new insights into LINC00847 biology. Effects of LINC00847 overexpression (direct or indirect through EVs) on Ewing cells proliferation appear very moderate.
Many potentially interesting observations are discussed but remain purely theorical and extrapolate form other studies (very long discussion section).

RE: We are so appreciative and grateful for your critical evaluation of our manuscript. According to your suggestions, we have improved the quality of the pictures. You can click the link above to view the original image if the images are still blurry.

In our study, overexpression of LINC00847 had a mild effect on the proliferation of Ewing sarcoma cells but a substantial effect on the migration and invasion of Ewing sarcoma cells. The malignant phenotype of tumors is not limited to proliferation, migration, and invasion, and it is unknown whether LINC00847 can also affect other malignant phenotypes of Ewing sarcoma. We hope to verify the other functions of LINC00847 in future research experimentally.

2nd Editorial decision

Ref.: Ms. No. JCTRes-D-22-00142R1
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Dear authors,

I am pleased to inform you that your manuscript has been accepted for publication in the Journal of Clinical and Translational Research.

You will receive the proofs of your article shortly, which we kindly ask you to thoroughly

review for any errors.
Please notify our assistant editor/production editor when you receive the proofs if your article should belong a speical issue specifying the issue's title.

Thank you for submitting your work to JCTR.

Kindest regards,

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Comments from the editors and reviewers: