Autocrine INSL5 promotes tumor progression and glycolysis via activation of STAT5 signaling

Shi-Bing Li, Yan-Yan Liu, Li Yuan, Ming-Fang Ji, Ao Zhang, Hui-Yu Li, Lin-Quan Tang, Shuo-Gui Fang, Hua Zhang, Shan Xing, Man-Zhi Li, Qian Zhong, Shao-Jun Lin, Wan-Li Liu, Peng Huang, Yi-xin Zeng, Yu-Ming Zheng, Zhi-Qiang Ling, Jian-Hua Sui, and Mu-Sheng Zeng
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Review Timeline:

Editor: Jingyi Hou

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.)

4th Feb 2020

Dear Prof. Zeng,

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now received feedback from the two referees whom we asked to evaluate your manuscript.

As you will see from the reports below, both referees acknowledge the potential interest of the study. However, they also raise a number of concerns about your work, which should be convincingly addressed in a major revision of the present manuscript. In particular, additional experiments are required to strengthen the mechanisms underlying the pro-oncogenic role of INSL5 and to make the study more conclusive. Referee #1 also requested data on the INLS5 knock-out mice, which we would encourage you to provide.

All other issues raised by the referees need to be satisfactorily addressed as well. We would welcome the submission of a revised version within three months. Please note that EMBO Molecular Medicine strongly supports a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

Please also contact us as soon as possible if similar work is published elsewhere. If other work is published, we may not be able to extend the revision period beyond three months.

Please read below for important editorial formatting and consult our author's guidelines for proper formatting of your revised article for EMBO Molecular Medicine.

I look forward to receiving your revised manuscript.

Yours sincerely,
Jingyi Hou

Jingyi Hou
Editor
EMBO Molecular Medicine

*** Instructions to submit your revised manuscript ***

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In the event of acceptance, this file will be published in conjunction with your paper and will include the anonymous 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 at contact@embomolmed.org.

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When submitting your revised manuscript, please include:

1) a .doc formatted version of the manuscript text (including Figure legends and tables). Please make sure that the changes are highlighted to be clearly visible to referees and editors alike.

2) separate figure files*

3) supplemental information as Expanded View and/or Appendix. Please carefully check the authors guidelines for formatting Expanded view and Appendix figures and tables at https://www.embopress.org/page/journal/17574684/authorguide#expandedview

4) a letter INCLUDING the reviewers' reports and your detailed responses to their comments (as Word file)

Also, and to save some time should your paper be accepted, please read below for additional information regarding some features of our research articles:

5) The paper explained: EMBO Molecular Medicine articles are accompanied by a summary of the articles to emphasize the major findings in the paper and their medical implications for the non-specialist reader. Please provide a draft summary of your article highlighting
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6) For more information: There is space at the end of each article to list relevant web links for further consultation by our readers. Could you identify some relevant ones and provide such information as well? Some examples are patient associations, relevant databases,

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8) EMBO Molecular Medicine now requires a complete author checklist (https://www.embopress.org/page/journal/17574684/authorguide) to be submitted with all revised manuscripts. Please use the checklist as a guideline for the sort of information we need WITHIN the manuscript as well as in the checklist. This is particularly important for animal reporting, antibody dilutions (missing) and exact p-values and n that should be indicated instead of a range.

9) Every published paper now includes a 'Synopsis' to further enhance discoverability. Synopses are displayed on the journal webpage and are freely accessible to all readers. They include a short stand first (maximum of 300 characters, including space) as well as 2-5 one sentence bullet points that summarise the paper. Please write the bullet points to summarise the key NEW findings. They should be designed to be complementary to the abstract - i.e. not repeat the same text. We encourage inclusion of key acronyms and quantitative information (maximum of 30 words / bullet point). Please use the passive voice. Please attach these in a separate file or send them by email, we will incorporate them accordingly.

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Currently, our records indicate that there is no ORCID associated with your account.

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*Additional important information regarding Figures

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Figures are not edited by the production team. All lettering should be the same size and style; figure

panels should be indicated by capital letters (A, B, C etc). Gridlines are not allowed except for log plots. Figures should be numbered in the order of their appearance in the text with Arabic numerals. Each Figure must have a separate legend and a caption is needed for each panel.

*Additional important information regarding figures and illustrations can be found at http://bit.ly/EMBOPressFigurePreparationGuideline

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

Needs additional experiments as detailed in the report

Referee #1 (Remarks for Author):

The manuscript by Li and colleagues is an interesting piece of work convincingly showing the potential of the insulin-like peptide 5 (INSL5) as biomarker of diagnosis and of bad prognosis of patients bearing nasophayngeal carcinomas (NPC). Less developed are the aspects related with the mechanisms that underlie the pro-oncogenic role of INSL5. In this regard, they overexpressed INSL5 or silenced its high-affinity receptor, the G-protein coupled receptor RXFP4 (GPCR142), in different cellular lines to demonstrate that INSL5, or its downstream signaling, favor in vitro proliferation and invasion of the cells and in vivo tumor growth in xenograft mouse models. In the search for a mechanism of action of INSL5, they report that both mRNAs, enzymes and metabolites of the glycolytic pathway are significantly increased upon the action or signaling through INSL5, whereas oxidative phosphorylation and metabolites of the TCA cycle negatively correlate with the activity of INSL5. These and some other additional results led the authors to suggest that INSL5 mechanism of action is through transcriptional upregulation of glycolysis. Mechanistically, they report that INSL5 triggers the phosphorylation and nuclear translocation of transcription factor STAT5, which seems to interact with the promotes of some glycolytic genes and increase their activity upon INSL5 stimulation. Finally, they show that antibodies against INSL5 or its receptor GPCR142 diminish the pro-oncogenic activity of INSL5 in patient-derived tumor xenografts suggesting that they could provide targets for the treatment of NPC. Overall, the paper needs of additional experiments to convey a convincing message.
Major points
1. INSL5- and RXFP4-mediated metabolic reprogramming are poorly characterized and their effects on mitochondrial respiration and oxidative phosphorylation need to be reported. Is the cellular mitochondrial content affected?
2. The two-fold increase in cellular ATP content in response to the overexpression of INSL5 (Fig. 4I) or its sharp decrease in knock-down cells (Fig. 4K) is difficult to reconcile with the marginal changes published in cellular ATP content. What is the effect of 2DG and of oligomycin in cellular ATP content?
3. The phosphorylation and nuclear translocation experiments of STAT5 in response to INSL5 (Fig. 5B-D) are not convincing. They require quantification and the incorporation of the results obtained in histograms in the same figure. STAT5 is known to activate anti-apoptotic genes. Is any of these anti-apoptotic genes activated in response to INSL5? What is the contribution of preventing cell death in tumor accretion mediated by INSL5 action?
4. The INSL5/RXFP4 downstream signaling cascade to STAT5 phosphorylation needs to be delineated. Which kinase (ERK, JAK, Akt...) is involved?
5. Apparently, blocking glycolysis with 2DG has no relevant effect in tumor growth (Fig. 6F), raising

doubts about the relevance of glycolytic reprogramming in the INSL5-mediated pro-oncogenic role. The authors should consider combining the treatment of blocking INSL5 or GPCR142 antibodies with conventional chemotherapy (cisplatin, carboplatin, 5FU or docetaxel) regimens of NPC.

6. Is the INLS5 knock-out mice resistant to the growth of NPC?

Minor points
There are some spelling errors that need amendment in the text (line 131, OASL mRNA....) and in the figures (Fig. 4K, reads Akt when it should be ATP)
Explain why the 3.73 cut-off value has been selected (line 185) in KM analyses
The mouse genes need to be appropriately quoted.

Referee #2 (Comments on Novelty/Model System for Author):

Under my knowledge, INSL5 has not been before involved in the progression of NPC. Moreover, it is shown in this study that INSL5 can be a prognosis and diagnosis marker of this cancer type. This opens the door also to analyze this protein other cancers.

Referee #2 (Remarks for Author):

Li et al. describe in this paper a new role for INSL5 in the progression of nasopharyngeal carcinoma. They start by proving the use of INSL5 quantification as a diagnostic and prognostic marker of NPC in human patients. Next, they use cellular models of NPC to analyze the underlying mechanisms by which INSL5 regulates the proliferation and growth of these cancer cells. They show, using different approaches, that INSL5 through binding to its receptor GPCR142, activates the STAT5 pathway to regulate the expression of genes involved in the control of glycolysis. They claim that through this process, INSL5 facilitates the metabolic switch required for cancer cells to proliferate. The study is very extensive, and covers from studies in human patients of cancer to work in mouse models. Furthermore, through the use of cellular models they unravel the molecular mechanisms underlying the effects of INSL5 on cancer cells. This is a novel and original work that has been well conducted. There is no doubt about the relevance of this study for cancer research and treatment. Some additional work, as is next suggested, would further improve the quality of the manuscript and reinforce the conclusions.

1. The authors claim that the function of INSL5 in the proliferation and growth of NPC cancer cells is to promote the required metabolic switch. While this is well proven in this study, the authors cannot conclude that this is the only, or the most important mechanisms that underlie the effects of INSL5 in cancer progression. Indeed, in the figure 6F, the growth of the tumors is only minimally changed when glycolysis is inhibited by 2-DG.

2. The STAT5 pathway has been involved in tumorigenesis in several types of cancer. Previous studies show that STAT5 can mediate oncogenic signals and regulate cell cycle progression, proliferation and promote cancer cell survival. Are there other STAT5-regulated pathways, in addition to glycolysis, changed in the models used?

3. Are JAK kinases, the upstream activators of the STAT pathway also involved? Are JAK kinase inhibitors or STAT5 knock-down experiments abrogate the effects of INSL5?

4. Several reports suggest that there is an important correlation between the JAK/STAT pathway and cell metabolism, notably the activation of hypoxia-inducible factors and the alteration of mitochondrial activity. In addition to the regulation of glycolysis genes, STAT5 has also been involved in mitochondrial function. A more detailed functional evaluation of mitochondria and glycolytic function of the cells with or without INSL5 (using for instance Seahorse analysis) would further prove the direct involvement of this protein in the control of metabolism in these cancer cells.

5. In the Figure 6 A-B, the authors show the effects of INSL5 inhibition by using a monoclonal antibody. Cell death and apoptosis should be analyzed in these cells.

Point-by-point response

We thank all the reviewers for their constructive comments and suggestions. We have performed additional experiments and revised the manuscript accordingly. We believe that we have addressed all the concerns raised by reviewers in the revised manuscript. All changes in the revised manuscript are highlighted in yellow for your attention. The following is a point-by-point response on how we revised our manuscript.

Referee #1 (Comments on Novelty/Model System for Author):

Needs additional experiments as detailed in the report

Referee #1 (Remarks for Author):

The manuscript by Li and colleagues is an interesting piece of work convincingly showing the potential of the insulin-like peptide 5 (INSL5) as biomarker of diagnosis and of bad prognosis of patients bearing nasophayngeal carcinomas (NPC). Less developed are the aspects related with the mechanisms that underlie the pro-oncogenic role of INSL5. In this regard, they overexpressed INSL5 or silenced its high-affinity receptor, the G-protein coupled receptor RXFP4 (GPCR142), in different cellular lines to demonstrate that INSL5, or its downstream signaling, favor in vitro proliferation and invasion of the cells and in vivo tumor growth in xenograft mouse models. In the search for a mechanism of action of INSL5, they report that both mRNAs, enzymes and metabolites of the glycolytic pathway are significantly increased upon the action or signaling through INSL5, whereas oxidative phosphorylation and metabolites of the TCA cycle negatively correlate with the activity of INSL5. These and some other additional results led the authors to suggest that INSL5 mechanism of action is through transcriptional upregulation of glycolysis. Mechanistically, they report that INSL5 triggers the phosphorylation and nuclear translocation of transcription factor STAT5, which seems to interact with the promotes of some glycolytic genes and increase their activity upon INSL5 stimulation. Finally,

they show that antibodies against INSL5 or its receptor GPCR142 diminish the pro-oncogenic activity of INSL5 in patient-derived tumor xenografts suggesting that they could provide targets for the treatment of NPC. Overall, the paper needs of additional experiments to convey a convincing message.

Major points

1. INSL5- and RXFP4-mediated metabolic reprogramming are poorly characterized and their effects on mitochondrial respiration and oxidative phosphorylation need to be reported. Is the cellular mitochondrial content affected?

Response:

Thank you very much. This is a great point. In the revised manuscript, we have performed Seahorse analysis in three different INSL5 overexpressing cell lines (CNE1, CNE2 and HK1) to detect extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) using a XF96 Extracellular Flux analyzer. We demonstrated that INSL5 overexpression significantly increased glycolysis (Fig 4F-G and Fig S5G) and impaired oxidative phosphorylation (OXPHOS) (Fig S5H), which indicated that INSL5 overexpression could promote metabolism shift from OXPHOS to aerobic glycolysis.

2. The two-fold increase in cellular ATP content in response to the overexpression of INSL5 (Fig. 4I) or its sharp decrease in knock-down cells (Fig. 4K) is difficult to reconcile with the marginal changes published in cellular ATP content. What is the effect of 2DG and of oligomycin in cellular ATP content?

Response:

Thank you very much for your suggestion. We are sorry that we made a mistake before, the ATP concentration should be normalized with protein concentration according to the manufacturer's instructions, but we didn't. In the revised manuscript we detected the ATP level in INSL5 overexpressing cell lines and knock-down cells, we got the similar results after protein concentration normalization. CNE1, CNE2 and

HK1 cell respectively showed about 34%, 22%, and 26% increase of ATP level after INSL5 overexpression. INSL5 knockdown in CNE2-EBV and HNE1-EBV cells respectively showed about 35% and 16% decrease of ATP level. we treated the INSL5 overexpressing cells with oligomycin or 2-DG, we found that oligomycin treatment slightly increased the ATP level, while 2-DG treatment greatly decreased the ATP level, INSL5 overexpressing cells especially displayed a greater extent reduction than control cells, which indicated that INSL5 overexpression indeed promoted glucose metabolism shift from OXPHOS to glycolysis (Fig 4J and Fig S5K).

3. The phosphorylation and nuclear translocation experiments of STAT5 in response to INSL5 (Fig. 5B-D) are not convincing. They require quantification and the incorporation of the results obtained in histograms in the same figure. STAT5 is known to activate anti-apoptotic genes. Is any of these anti-apoptotic genes activated in response to INSL5? What is the contribution of preventing cell death in tumor accretion mediated by INSL5 action?

Response:

Thanks for your helpful suggestion. For Fig.5B-D, we quantified the western blotting results by Image J and labeled the fold change just above the indicated bands. We agreed with you that STAT5 is known to activate anti-apoptotic genes, like c-myc, BCL2 and BCL-xL. We detected those genes in INSL5 overexpressing and control cells, and found that INSL5 overexpression only increased c-myc expression, not BCL2 and BCL-xL (Fig S6D). We also detected cell apoptosis under conventional chemotherapy (5-FU and DDP), the results showed that INSL5 overexpression suppressed the sensitivity of NPC cells to 5-FU or DDP. Furthermore, we detected the apoptosis pathway, and found that INSL5 overexpression could suppress the cleavage of caspase 3, caspase7 and caspase 9. Taken together, all of those data suggested that INSL5 overexpression could promote chemoresistance to 5-FU or DDP via inhibiting cell apoptosis (Fig S6E-G).

4. The INSL5/RXFP4 downstream signaling cascade to STAT5 phosphorylation needs to be delineated. Which kinase (ERK, JAK, Akt...) is involved?

Response:

Thank you, this is an excellent question. As reported INSL5 could activate ERK and Akt, we detected the activation of ERK, Akt and JAK1, found that INSL5 could promote the activation of ERK, Akt and JAK1. Meanwhile, we treated the cell with different kinase inhibitor to detect the phosphorylation of STAT5 and found that JAK1 (Ruxolitinib) and ERK1/2 (U0126) inhibitors, not Akt inhibitor (MK2206), could reverse STAT5 activation. Taken together, those results indicated that JAK and ERK contributed to STAT5 activation induced by INSL5/RXFP4 (Fig 5E).

5. Apparently, blocking glycolysis with 2DG has no relevant effect in tumor growth (Fig. 6F), raising doubts about the relevance of glycolytic reprogramming in the INSL5-mediated pro-oncogenic role. The authors should consider combining the treatment of blocking INSL5 or GPCR142 antibodies with conventional chemotherapy (cisplatin, carboplatin, 5FU or docetaxel) regimens of NPC.

Response:

Thanks for your great suggestion. We agree that glycolysis inhibitor 2-DG only shows minor effect in tumor growth. As suggested, we examine whether 2-DG could reverse INSL5 enhanced chemoresistance. We found that 2-DG could sensitize INSL5 highly expressed NPC to chemotherapy (Fig 6F and Fig S7C). Additionally, we combined the treatment of blocking INSL5 or GPCR142 antibodies with conventional chemotherapy (DDP) in tumor-bearing mice, and we found that INSL5 overexpression displayed chemoresistance to DDP treatment, which can be reversed by INSL5 or GPCR142 antibodies treatment (Fig S7E-G). Taken together, those results indicated that Anti-INSL5/GPCR142 neutralized antibodies could diminish the chemoresistance induced by INSL5 overexpression

6. Is the INLS5 knock-out mice resistant to the growth of NPC?

Response:

We are very grateful for your nice suggestion. Indeed, transgenic INSL5 knock-out or overexpressing mice will make our conclusions stronger. But there is no ideal animal model for NPC and we also can not get the transgenic mice in the limited time, we will follow up this exploration as your suggestion in the future. Anyway, thank you very much for your suggestion.

Minor points

There are some spelling errors that need amendment in the text (line 131, OASL mRNA....) and in the figures (Fig. 4K, reads Akt when it should be ATP)

Response:

We have corrected those mistakes in the revised manuscript as suggested. Thank you very much for the careful correction, which help us avoid these unthoughtful mistakes.

Explain why the 3.73 cut-off value has been selected (line 185) in KM analyses

Response:

Thank you. We mentioned this in the methods (line 557). According to many reported methods, we preformed receiver operating characteristic (ROC) curve analysis to identify the optimum cut-off value of plasma INSL5 level for prognosis. We divided all the NPC patients into two groups, progression group and progression free group, then we drawn the ROC curve to predict tumor progression and calculated the Yuden index. Finally, the cut-off value for INSL5 was defined as the value with the maximization of Yuden index.

The mouse genes need to be appropriately quoted.

Response:

Thank you for your reminding. We have corrected all the mouse genes to italics and lowercase letters to distinguish from human genes.

Referee #2 (Comments on Novelty/Model System for Author):

Under my knowledge, INSL5 has not been before involved in the progression of NPC. Moreover, it is shown in this study that INSL5 can be a prognosis and diagnosis marker of this cancer type. This opens the door also to analyze this protein other cancers.

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Response:

Thank you very much. We agree with you that metabolic switch is not the only mechanism to underlie the effects of INSL5 in cancer progression. As your helpful suggestion, we also found that INSL5 could enhance cell cycle progression and suppress cell apoptosis (Fig S6). 2-DG had minimal effect on tumor growth, but in our revised manuscript we found that 2-DG could reverse the chemoresistance induced by INSL5, which indicated that 2-DG could sensitize INSL5 expressed NPC to chemotherapy (Fig 6F and Fig S7C). Based on those finding, besides to metabolic switch, we corrected our conclusion that INSL5 induced metabolism reprograming at least partly contributed the accelerated proliferation in the discussion

2. The STAT5 pathway has been involved in tumorigenesis in several types of cancer. Previous studies show that STAT5 can mediate oncogenic signals and regulate cell cycle progression, proliferation and promote cancer cell survival. Are there other STAT5-regulated pathways, in addition to glycolysis, changed in the models used?

Response:

Thanks for your great suggestion. Indeed, STAT5 was involved in many oncogenic signals as you mentioned. In the revised manuscript we added the new data about cell cycle, which suggested that INSL5 overexpression could promote cell cycle

progression. We also detected the key cyclins and p27, and found that INSL5 overexpression could enhance cyclin D and cyclin E expression, and decreased p27 level (Fig S6A-B). All of those suggested that INSL5 could also promote cell cycle progression in addition to glycolysis.

3. Are JAK kinases, the upstream activators of the STAT pathway also involved? Are JAK kinase inhibitors or STAT5 knock-down experiments abrogate the effects of INSL5?

Response:

We really appreciate your suggestion. We detected several possible upstream activators (Akt, ERK1/2 and JAK) of STAT pathway and found that INSL5 could increase the phosphorylation of Akt, ERK1/2 as reported, also JAK1 and STAT5. Meanwhile, we treated the cell with different kinase inhibitor to detect the phosphorylation of STAT5 and found that JAK1 (Ruxolitinib) and ERK1/2 (U0126) inhibitors, not Akt inhibitor (MK2206), could reverse STAT5 activation (Fig 5E). JAK1 inhibitor and STAT5 knock-down could decrease INSL5 induced cell proliferation and glucose uptake (Fig 5H-J). Taken together, those data suggested that INSL5 activated STAT5 through the JAK kinases in the upstream and JAK kinase inhibitor or STAT5 knock-down could abrogate the pro-tumor effect of INSL5

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Response:

Thanks for your helpful suggestion. We performed Seahorse analysis in three different INSL5 overexpressing cell lines (CNE1, CNE2 and HK1) to detect extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) using a XF96 Extracellular Flux analyzer. We demonstrated that INSL5 overexpression significantly increased glycolysis (Fig 4F-G and Fig S5G) and impaired oxidative phosphorylation (OXPHOS) (Fig S5H), which indicated that INSL5 overexpression could promote metabolism shift from OXPHOS to aerobic glycolysis.

5. In the Figure 6 A-B, the authors show the effects of INSL5 inhibition by using a monoclonal antibody. Cell death and apoptosis should be analyzed in these cells.

Response:

We performed the same treatment as Figure 6A-B, and then detected the cell death and apoptosis by flow cytometry after annexin V/propidium iodide (PI) staining. The results showed that the monoclonal antibody along had no effects on cell death and apoptosis (Fig S7A).

25th May 2020

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed report from the two referees who were asked to re-assess it. As you will see the referees are now supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following amendments.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

The revised version of the manuscript has satisfactorily addressed my previous concerns about mechanistic aspects of the study.

Referee #1 (Remarks for Author):

The revised version of the manuscript has satisfactorily addressed my previous concerns about mechanistic aspects of the study.

Referee #2 (Remarks for Author):

The authors have properly addressed my concerns and suggestions. The manuscript has improved the quality.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Yu-Ming Zheng,Zhi-Qiang Ling,Jian-Hua Sui,Mu-Sheng Zeng
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2020-12050-V3

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 χ2 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)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample sizes were chosen according to minimum of three independent replicates use, and larger numbers were included in some cases to allow more robust analysis.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample sizes for animal studies were chosen following previously reported studies that have used PDX model or subcutaneous tumor formation experimenr ; at least 6-10 independent mice per experimental set
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	Yes, all animals were handled by standardized housing procedures and kept in exactly the same environmental conditions. Age- and sex-matched animals were grouped with random to plant tumor cells or PDX samples in each experimental set, all the control or experimental mice were treated with the same procedure and manipulation.
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	Yes, when performing histopathological analysis, slides were examined independently by different members of the laboratory.
4.b. For animal studies, include a statement about blinding even if no blinding was done	All animals taken into the study were genotyped at 4 weeks of age and received a randomized number by which they were identified. No blinding was done, but all mice were randomly caged and analyzed to avoid group or genotype-specific effects due to timing of experiments or handling of animals.
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

Is there an estimate of variation within each group of data?	Yes, we showed standard error of the mean (SEM) or SD for each group of data analyzed.
Is the variance similar between the groups that are being statistically compared?	Yes, statistical analysis was done using the software GraphPad Prism which calculated the F values for variances, and data was presented as data points for each independent experiment.

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).	Detailed information of all the antibodies used in this study has been included in materials and methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HK1 and C666 cell lines were great gift from George Tsao, HongKong university . All cell lines have been tested for micoplasma contamination (Genecopoeia).

* 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.	Detailed information has been included in materials and methods section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal use and care were approved by the Sun Yat-Sen University Cancer Center Animal Care Committee in accordance with the institutional animal care and use committee guidelines.
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.	Yes

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Institutional Research Ethics Committee of Sun Yat-Sen University Cancer Center
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

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	NA
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).	NA
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).	NA
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.	NA

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.	NA