EMBO reports

Lysine demethylase LSD1 delivered via small extracellular vesicles promotes gastric cancer cell stemness

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(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 22nd Jun 2020

Dear Prof. Zheng,

Thank you for the submission of your research 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. Most importantly, all the missing information on the experimental setup need to be provided (as detailed by referee #2). Moreover, it will be important to demonstrate clinical relevance, and to define the molecular characteristics and differences of donor GC cells and recipient GC cells (see report of referee #3).

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.

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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').

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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:
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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:

The study by Zhao et al. "Small extracellular vesicles-delivered LSD1 promotes gastric cancer cell stemness" examines the impact of LSD1 on cancer stem cell proliferation. Unexpectedly, the demethylase is delivered to tumor cells via small extracellular vesicles. The level of secretion seems to correlate with the amount of LSD1 in gastric cells lines and interestingly, is also elevated in gastric cancer and plasma from these patients in comparison to healthy donors. The authors

correlate LSD1 and SOX2 transcription, well known to be a critical gene for stem cell maintenance. The underlying molecular mechanism is based on the established stabilizing effect of LSD1 on SOX2, which is, if methylated at K42 and K117, degraded by endosomes. Furthermore, the authors could show that small extracellular vesicle-derived LSD1 promotes chemotherapy resistance in gastric cancer cell lines and in xenografts. Importantly, Zhao et al. demonstrate that the enzymatic activity is essential for the impact of LSD1 revealing the possibility to therapeutically interfere with gastric cancer stem cell maintenance.

In summary, the study is extensive and mostly convincing. The findings are novel and of general interest for the understanding of tumor biology as well for its clinical implications for tumor resistance, diagnosis, and a potential new strategy for tumor therapy targeting cancer stem cells. However, as described in detail below, some data are premature and not adequately controlled.

- The analysis of Sox2 methylation levels is premature, not convincing, and in need of further clarification.

- Figure 3I: Quantification of SOX2 mRNA is required to show that LSD1 increases the stability of the SOX2 protein rather than influencing transcription.

- Figure 3K: The description of the experiment is not sufficient. Which antibody was used for immunoprecipitation? In addition, a description of the Kme1/2 antibody is missing. The authors MUST in detail verify the specificity of the antibody. Are other K-methylated proteins recognized? The authors must verify the methylation level of SOX2 K42me and K117me in dependence of LSD1 by mass spectrometry.

Minor point:
Please include size standards for all Western blots.

Referee #2:

LSD1 has been described as an oncogene in gastric cancer. In this study Zhao et al. aim to study whether LSD1 is loaded in small EVs and whether LSD1 loaded small EVs are delivered to recipient cells and exert functions in stemness and oxaliplatin resistance. Given the strong focus of the manuscript on the identification of LSD1 in small EVs and the function of those small EVs there are multiple major comments related to the experimental set-up of the study.

1)In the material and methods section the authors indicate that EVs are separated by differential ultracentrifugation or total exosome isolation. It is important to recognize that both methods do not separate EVs with high specificity. It is unclear which method has been used to produce the different results/figures. This is however important to be able to evaluate whether the results and the conclusions presented in this manuscript are adequate. To demonstrate that LSD1 is part of the EV cargo the authors do include control experiments, however this is less so for the functional experiments. So from the functional experiments it is unclear whether the reported effects are due to small EVs or any other component that can be part of the small EV preparation obtained by differential ultracentrifugation or total exosome isolation.

2)For the functional experiments the authors implement small EVs from LSD1 control versus KO cells. The authors should provide an in depth analysis on EVs obtained from LSD1 control and KO cells including impact on number of small EVs released but also profile their full protein content by

mass spectrometry to interpret the overall impact of LSD1 KO on the small EV secretion and composition. How have those small EVs been prepared, which separation method? This is crucial information to understand the conclusions and results. Also here dose reponse curves should be provided.

3)The authors compare the small EVs from several cell lines. They demonstrate that only small EVs from LSD1 positive cells promote sphere formation. But how were those experiments performed? Specify the treatment conditions and include dose reponse curves.

4)Cells are treated with GW4869, but how was the treatment performed? What was the dose used? Using this inhibitor the authors show functional effects. But what are the effects of this inhibitor on the small EVs that are studied? Can the authors confirm effects on the release of small EVs containing LSD1?

5)The authors implement protease and tritonx100 treatment but how was the treatment performed? Which concentrations of the enzyme and detergent have been used? CD9 is still detectable after protease K treatment. So we can assume the antibody used does not recognize the extracellular part of CD9? As a control, include an antibody that does recognize the extracellular part to confirm the results. Finally, the treatment does not seem so efficient since LSD1 and TSG101 are still detected in the presence of both tritonx100 and proteaseK?

6)EM images are not clear. Provide clear wide field and close up images for EV obtained by differential ultracentrifugation and total exosome isolation. How were EVs obtained from patient blood plasma? By differential ultracentrifugation? This does not allow to conclude that LSD1 is actually part of the EV cargo in patient blood plasma. A density gradient or treatment with protease K and tritonx100 is absolutely required to support this conclusion. Did the authors compare the result between patients diagnosed with LSD1 high and low tumors?

7)The authors use PKH26 labeling to demonstrate that small EVs are transferred between cells. Nevertheless, also here experimental information lacks. What is the dose? How many small EVs have been delivered to how many cells? How were those EVs separated? In addition, density gradient should be included to remove PKH26 micelles from the preparation. It is impossible to distinguish those micelles from small EVs. Did the authors include a PKH26 label only condition?

8)Cells have been cultured serum free for 36 hours? What is the morphology and cell viability of those cells? Taking into account that serum free conditions alter the composition of EVs it is important to explain why the authors have selected these conditions.

9)Submit experimental parameters to the EV-TRACK knowledge. Report the EV-TRACK ID in the material and methods section. This will assist the authors in transparently reporting the material and methods issue as indicated above.

Referee #3:

Zhao L.J. et al reported EV-mediated transfer of LSD1 and its role in stemness of gastric cancer cells. The used experimental techniques were adequate and the results were reasonably drawn from them one by one.

However, before discussing about all these phenomena and molecular mechanisms, the main

premise that EV-LSD1 would be transferred from GC cells to GC cells is logically difficult to accept. To elucidate the biological significance and prove the actual existence of this phenomenon in a human body, authors have to at first define the molecular characteristics and difference of donor GC cells and recipient GC cells. Indeed, authors only observed transfer of EV-LSD1 between the identical cell lines (MGC-803) in Figure 2.

Furthermore, clinical relevance of EV-LSD1 was not shown at all, such as relationship between plasma EV-LSD1 level and stemness of GC cells within tissue specimen, or distribution of donor and recipient cells in IHC-stained tissue samples.

Thus, without clear definition of donor and recipient cells they claimed, it is difficult to consider the importance of this study.

Response to referees

Referee #1:

The study by Zhao et al. "Small extracellular vesicles-delivered LSD1 promotes gastric cancer cell stemness" examines the impact of LSD1 on cancer stem cell proliferation. Unexpectedly, the demethylase is delivered to tumor cells via small extracellular vesicles. The level of secretion seems to correlate with the amount of LSD1 in gastric cells lines and interestingly, is also elevated in gastric cancer and plasma from these patients in comparison to healthy donors. The authors correlate LSD1 and SOX2 transcription, well known to be a critical gene for stem cell maintenance. The underlying molecular mechanism is based on the established stabilizing effect of LSD1 on SOX2, which is, if methylated at K42 and K117, degraded by endosomes. Furthermore, the authors could show that small extracellular vesicle-derived LSD1 promotes chemotherapy resistance in gastric cancer cell lines and in xenografts. Importantly, Zhao et al. demonstrate that the enzymatic activity is essential for the impact of LSD1 revealing the possibility to therapeutically interfere with gastric cancer stem cell maintenance.

In summary, the study is extensive and mostly convincing. The findings are novel and of general interest for the understanding of tumor biology as well for its clinical implications for tumor resistance, diagnosis, and a potential new strategy for tumor therapy targeting cancer stem cells. However, as described in detail below, some data are premature and not adequately controlled.

(1) The analysis of Sox2 methylation levels is premature, not convincing, and in need of further clarification. Figure 3I: Quantification of SOX2 mRNA is required to show that LSD1 increases the stability of the SOX2 protein rather than influencing transcription.

Re: Thank you for your suggestion. We have quantified the mRNA level of SOX2 in MGC-803 and MGC-803 LSD1 KO cells. As shown in Figure 3I, LSD1 deletion did not have a significant impact on SOX2 mRNA.

(2) Figure 3K: The description of the experiment is not sufficient. Which antibody was used for immunoprecipitation? In addition, a description of the Kme1/2 antibody is missing. The authors MUST in detail verify the specificity of the antibody. Are other K-methylated proteins recognized? The authors must verify the methylation level of SOX2 K42me and K117me in dependence of LSD1 by mass spectrometry.

Re: Thank you for your reminding. Kme1/2 antibody was bought from PTM biolabs. According to the instruction from the manufacture, it specifically recognizes proteins with mono-, dimethyl lysine residues but not the trimethyl lysine, acetyl lysine or unmodified lysine residues. As it has been reported in detail that LSD1 acted on both methylated K42 and K117 of SOX2 to prevent the proteolysis (DOI: 10.1074/jbc.RA117.000342, DOI: 10.1074/jbc.RA118.005336), meanwhile, we have proved that LSD1 KO had no effect on SOX2 mRNA (Figure 3I), but increased SOX2 degradation rate (Figure 3J & K), therefore, we only verified the effect of LSD1 on SOX2 methylation level here. As shown in Figure 3L & M, both SOX2 IP assay and Kme1/2 IP assay results displayed that LSD1 could remove methyl group of SOX2 in gastric cancer cells.

Minor point:
Please include size standards for all Western blots.
Re: Thanks for your reminding. We have marked the size standards for all bands.

Referee #2:

LSD1 has been described as an oncogene in gastric cancer. In this study Zhao et al. aim to study whether LSD1 is loaded in small EVs and whether LSD1 loaded small EVs are delivered to recipient cells and exert functions in stemness and oxaliplatin resistance. Given the strong focus of the manuscript on the identification of LSD1 in small EVs and the function of those small EVs there are multiple major comments related to the experimental set-up of the study.

(1) In the material and methods section the authors indicate that EVs are separated by differential ultracentrifugation or total exosome isolation. It is important to recognize that both methods do not separate EVs with high specificity. It is unclear which method has been used to produce the different results/figures. This is however important to be able to evaluate whether the results and the conclusions presented in this manuscript are adequate. To demonstrate that LSD1 is part of the EV cargo the authors do include control experiments, however this is less so for the functional experiments. So, from the functional experiments it is unclear whether the reported effects are due to small EVs or any other component that can be part of the small EV preparation obtained by differential ultracentrifugation or total exosome isolation.
Re: Thanks for your reminding. In this article, unless otherwise specified, sEVs were obtained by differential ultracentrifugation. And the exosomes extracted by kit was only used in Figure 1I to verify the existence of LSD1 in sEVs by a different method. All the sEVs were filtered through 0.22 μm membrane filters before functional experiments. And we have added these instructions in the the material and methods section.
For the functional experiments, firstly, we isolated sEVs from MGC-803 cells and GW4869 treated MGC-803 cells. As a result, compared with MGC-803 cell, smaller amount sEVs was obtained from GW4869 treated cells than the untreated cells (Figure EV1A), and the ability to promote the sphere formation of recipient cells was also decreased either (Figure EV1B). This suggested that it was sEVs who played an important role in sphere formation of recipient cells. Secondly, to demonstrate LSD1 was the main component of sEVs that promoted the sphere formation of recipient cells, we extracted and compared the biological functions of sEVs from LSD1-overexpression and LSD1-mutation HEK293T cells. Consistent with the hypothesis, we observed that sEVs from LSD1-overexpressed cells could promote sphere formation ability of recipient cells, while sEVs from LSD1-mutation cells could not (Figure 4F).

(2) For the functional experiments the authors implement small EVs from LSD1 control versus KO cells. The authors should provide an in-depth analysis on EVs obtained from LSD1 control and KO cells including impact on number of small EVs released but also profile their full protein content by mass spectrometry to interpret the overall impact of LSD1 KO on the small EV

secretion and composition. How have those small EVs been prepared, which separation method? This is crucial information to understand the conclusions and results. Also, here dose reponse curves should be provided.

Re: Thank you for your suggestion. To analyze sEVs obtained from LSD1 control and KO cells in depth, we profiled their full protein content by mass spectrometry. As shown in Figure EV1E, there was no significant difference in protein distribution with different mass. In addition, whether LSD1 directly regulates sEVs secretion has not been reported. In this article, to avoid the difference in sEVs number from individual cells, same amount of sEVs was used in each assay.

The sEVs were isolated by differential ultracentrifugation. In order to understand the results and conclusions more clearly, we added a detailed description of the extraction process of sEVs. At the same time, we added a dose-dependent experiment. As shown in Figure EV1C, sEVs could promote the sphere formation of recipient cells in a dose-dependent manner. This further proved that sEVs could promote the sphere formation of recipient cells.

(3) The authors compare the small EVs from several cell lines. They demonstrate that only small EVs from LSD1 positive cells promote sphere formation. But how were those experiments performed? Specify the treatment conditions and include dose reponse curves.

Re: First, sEVs from different cells were accumulated individually and filtered through a 0.22 μm filter membrane, then, MGC-803 cells were incubated with 20 μg/mL sEVs from different cell lines. As shown in Figure 1D ＆ E, the sphere formation ability of recipient cells was positively associated with the amount of LSD1 in sEVs and their parent cells. Further does response experiment also confirmed that sEVs treatment can lead to the accumulation of LSD1 in recipient cells in a dose dependent manner (Figure EV1D).

(4) Cells are treated with GW4869, but how was the treatment performed? What was the dose used? Using this inhibitor, the authors show functional effects. But what are the effects of this inhibitor on the small EVs that are studied? Can the authors confirm effects on the release of small EVs containing LSD1?

Re: Thank you for your reminding. In Figure 1B, MGC-803 cell was treated with GW4869 (10 μM) for two days, then the medium was collected and used for 3D cell culture of MGC-803. And we have added this statement in material and methods section. GW4869 has been widely studied and applied as an inhibitor of exosome secretion (doi: 10.1038/s41408-018-0139-7, doi: 10.1021/acsnano.7b01087). To confirm the effect on the release of sEVs, the sEVs marker proteins were detected by western blotting to reflect the amount of sEVs secreted by equal quantity of cells, and results confirmed that GW4869 could effectively inhibit the secretion of sEVs in gastric cancer cells (Figure EV1A).

(5) The authors implement protease and tritonX-100 treatment but how was the treatment performed? Which concentrations of the enzyme and detergent have been used? CD9 is still detectable after protease K treatment. So, we can assume the antibody used does not recognize the extracellular part of CD9? As a control, include an antibody that does recognize the extracellular

part to confirm the results. Finally, the treatment does not seem so efficient since LSD1 and TSG101 are still detected in the presence of both tritonx100 and protease K?

Re: Thank you for your question. For the preparation of sEVs samples to prove LSD1 was harbored in sEVs, same amount (5 μg) of sEVs were resuspended with PBS, then one group was kept without treatment, one group was treated with 300 ng/ml Proteinase K for 6 mins at room temperature, and one group was treated with 0.01% Triton X-100 for 10 mins firstly and then treated with 300 ng/ml Proteinase K for 6 mins at room temperature. After treatment, sEVs were mixed with the loading buffer, denatured and subjected to western blotting. Due to the low concentration of Proteinase K, short treatment time and the enrichment of CD9 in sEVs, CD9 could still be detectable after treatment. So we optimized the experimental method, and the experiment was changed to 1 μg/ml Proteinase K for 20 mins treatment at 37℃, with or without 0.1% Triton X-100 for 20 mins. With the optimized experiment, CD9, CD63 and LSD1 were detectable with Proteinase K treatment only, but no longer detectable with both Triton X-100 and Proteinase K treatment, which fully proved that LSD1 was protected by the sEVs membrane (Figure 1J).

(6) TEM images are not clear. Provide clear wide field and close up images for EV obtained by differential ultracentrifugation and total exosome isolation. How were EVs obtained from patient blood plasma? By differential ultracentrifugation? This does not allow to conclude that LSD1 is actually part of the EV cargo in patient blood plasma. A density gradient or treatment with protease K and tritonx100 is absolutely required to support this conclusion. Did the authors compare the result between patients diagnosed with LSD1 high and low tumors?

Re: Thank you for your reminding. We have provided clear wide field and close up images for sEVs obtained by differential ultracentrifugation and total exosome isolation in revised manuscripts. For the sEVs from patient plasma, to prove that LSD1 is part of the sEVs cargo, we added proteinase K and triton X-100 treatment experiment in Figure EV4C, and the result also showed that LSD1 was packaged in sEVs.

As shown in Figure 3A-C, all these data gave solid evidence that LSD1 was overexpressed in gastric cancer tissues and associated with poor outcome. So LSD1 may contribute to gastric cancer diagnosed in clinic. As to the relationship between sEVs-LSD1 and clinical diagnosis, we plan to collect more patient information for in-depth analysis and research, which is waiting for the ethics approval. Therefore, we will carry out further research on the clinical application of sEVs-LSD1 in future work.

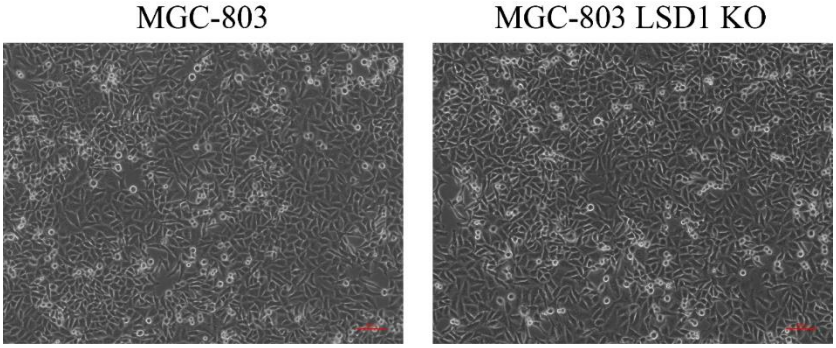
(7) The authors use PKH26 labeling to demonstrate that small EVs are transferred between cells. Nevertheless, also here experimental information lacks. What is the dose? How many small EVs have been delivered to how many cells? How were those EVs separated? In addition, density gradient should be included to remove PKH26 micelles from the preparation. It is impossible to distinguish those micelles from small EVs. Did the authors include a PKH26 label only condition?

Re: Thank you for your reminding. Purified sEVs were labeled with PKH26 red fluorescent labeling kit (MINI26-1KT, Sigma, Germany) as per manufacturer's protocol. In brief, sEVs were mixed with diluted PKH26 in a volume ratio of 1:1 for 5 mins, and size exclusion chromatography was used to remove PKH26 micelles from the labled sEVs. The PKH26 labeled sEVs (20 μg/ml) were incubated with 1.2×10^4 target cells for 12 hours, then target

cells membrane were stained by DiO (C1038, Beyotime, China), and cell nucleus was stained by DAPI (BS130A, Biosharp, China). Finally, PKH26 labeled sEVs were tracked by confocal microscope (Nikon, Japan). We have repeated the experiment and added the PKH26 label only group (Figure 2A). The experiment details were also added in the revised manuscript.

(8) Cells have been cultured serum free for 36 hours? What is the morphology and cell viability of those cells? Taking into account that serum free conditions alter the composition of EVs it is important to explain why the authors have selected these conditions.

Re: Thank you for your question. Before this study, we found many reports indicated that sEVs were isolated after 36 or 48 hours cultured without serum (DOI: 10.1073/pnas.1902513116; DOI: 10.1002/0471143030.cb0322s30; DOI: 10.1007/s40820-019-0285-x). Then, morphology of those cells as below indicated that serum free conditions for 36 hours has little influence on the morphology and cell viability. Hence, cell culture medium was collected after 36 hours cultured without serum and used for the extraction of sEVs for following biological study of sEVs.



The morphology of MGC-803 and MGC-803 LSD1 KO.

(9) Submit experimental parameters to the EV-TRACK knowledge. Report the EV-TRACK ID in the material and methods section. This will assist the authors in transparently reporting the material and methods issue as indicated above.

Re: Thank you for your reminding. We have submitted experimental parameters to the EV-TRACK knowledge, but we have not got the EV-TRACK ID. We used the same protocol with EV200065.

Referee #3:

Zhao L.J. et al reported EV-mediated transfer of LSD1 and its role in stemness of gastric cancer cells. The used experimental techniques were adequate and the results were reasonably drawn from them one by one.

(1) However, before discussing about all these phenomena and molecular mechanisms, the main premise that EV-LSD1 would be transferred from GC cells to GC cells is logically difficult to accept. To elucidate the biological significance and prove the actual existence of this phenomenon in a human body, authors have to at first define the molecular characteristics and difference of

donor GC cells and recipient GC cells. Indeed, authors only observed transfer of EV-LSD1 between the identical cell lines (MGC-803) in Figure 2.

Re: Thank you for your reminding. As we know that gastric cancer is a molecularly and phenotypically highly heterogeneous disease (doi: 10.1016/S0140-6736(20)31288-5). The WHO classification of gastric adenocarcinoma as an intractable cancer for its complexity distinguishing many subtypes (tubular, parietal cell, papillary, micropapillary, mucoepidermoid, mucinous, poorly cohesive, signet ring cell, medullary carcinoma with lymphoid stroma, hepatoid, and Paneth cell type), some of which are very rare. And gastric cancer usually consists different cell subgroups. Therefore, it is particularly important to study the information transmission and biological effects between different types of gastric cancer cells.

To further prove that sEVs can be transferred between different gastric cancer cells, we performed additional experiment in Figure 2B, so that to show that sEVs-LSD1 could be delivered to not only MGC-803 cells but also MKN-45 cells. The sEVs-LSD1 could be transferred between different kinds of gastric cancer cell lines.

(2) Furthermore, clinical relevance of EV-LSD1 was not shown at all, such as relationship between plasma EV-LSD1 level and stemness of GC cells within tissue specimen, or distribution of donor and recipient cells in IHC-stained tissue samples. Thus, without clear definition of donor and recipient cells they claimed, it is difficult to consider the importance of this study.

Re: Thank you for your reminding. In order to explore the relationship between plasma sEVs-LSD1 level and stemness of GC cells within tissue specimens, we detected the SOX2 level in tissues of 10 patients whose plasma were used to isolated sEVs. As shown in Figure EV4D & E, sEVs-LSD1 level had a positive relationship with SOX2 in tissues. This further verified the clinical relevance of sEVs-LSD1 and stemness.

1st Revision - Editorial Decision 13th Nov 2020

Dear Prof. Zheng,

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 #1 and #2 now support the publication of your study in EMBO reports. Nevertheless, both have some remaining concerns and suggestions to improve the manuscript, we ask you to address in a final revised manuscript.

Referee #3 indicates novelty issues. After cross-commenting with the other referees, we ask you to clearly indicate in your manuscript that LSD1 was detected in exosomal vesicles before, and to cite the articles mentioned by the referee and to rephrase the statement that LSD1 "is the first identified histone demethylase in sEVs" in the abstract and also in other parts of your manuscript. Please also underline in the abstract and the manuscript text that the novelty and scientific impact of this manuscript is based on the functional analyses of this exosomal LSD1 in cancer, especially beyond the system of established cell lines.

Moreover, I have these editorial requests I also ask you to address:

- Please provide the abstract written in present tense.

- There are still several typos and grammatical errors in the text. Please have your final manuscript carefully proofread by a native speaker.

- Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also of the EV figures). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable.

- Please check that all microscopic images have scale bars and that they are visible. E.g. the scale bars in Fig. 1C, 4A and 5C are hardly visible (these are just examples - please check all figures). Please provide the scale bars as uniform lines (black or white - depending on the background) without any writing on or near them. Please define their size in the respective figure legend.

- It seems there is no call out for Fig 6I. Please check.

- For Fig. 3J, please separate the blots on the right (MBC-803 LSD1 KO) from those on the left (MBC-803) by a black line. These come from different films (as the SD indicates) and should not be spliced together.

- In Fig. 5I, please use 'Patients' as label on the left.
- Thanks for providing the source data for the Western blots. Please ad size marker to all the blots (molecular weight ladders) shown, as like this it is impossible to evaluate the bands.

- For Fig. 1I it remains unclear where the blot comes from. Are these 2 experiments (it should, as these are different cell lines)? The SD just shows 1 empty film. Please indicate with size markers and a frame where the image shown in the panel comes from. Please also explain in the legend

what UC and Kit means.

- Please provide all the source data for each figure in one pdf file. One file per figure.

- Please call the methods section 'Materials and Methods'.

- Please remove the list of abbreviations. Please define all abbreviations upon their first mention in the text.

- Please fuse the two data availability sections.

- Please name the part 'Competing interests' 'Conflict of interest statement'.

- Please remove the part 'Consent for publication'. This is declared by the corresponding author in the submission system. We do not need that in the manuscript text.

- Please remove the TOC (extra title part) for the EV figure legends. Please just list these after the main figure legends as 'Expanded Vew Figure Legends'.

- Please make sure that the funding information added in the online submission system is complete and similar to the one in the manuscript (in the Acknowledgements). Please call the paragraph 'Acknowledgements'.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

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.

Yours sincerely

Achim Breiling
Editor
EMBO Reports

Referee #1:

The revised manuscript by Zhao et al. "Small extracellular vesicles-delivered LSD1 promotes gastric cancer cell stemness" completely addressed my comment in regards to Figure 3I and size standards. I also appreciate the addition of the immunoprecipitation description in the method section.

In contrast, the addition of a Western blot decorated with Sox2 does not address if, indeed methylated Sox2 was immunoprecipitated by the pan lysine mono and dimethyl antibodies. This requires analysis by mass spectrometry.

Referee #2:

Overall the authors have properly addressed most of the comments. Especially the additional control experiments performed and included in the revised manuscript further strenghten the overall conclusions.

Nevertheless it is important that the authors ensure the clarity of the additional information for example in the material and methods section as well as in the results section and the figure legends.

The authors should improve the readibility of the newly added information, because as it is, multiple parts across the manuscript contain typos and unclarities given that novel information was added while incomplete information was not properly removed. As an example of this I add the following paragraph:

"All sEVs in this paper were obtained by differential ultracentrifugation except the sEVs extracted by kit in figure 1, the kit was only used in Figure 1 for verification the existence of LSD1 in sEVs by a different method. All the sEVs were filtered through 0.22 μm membrane filters before functinnal experiments. We used the same protocol with EV200065 on EV-TRACK knowledge."

Describe for differential ultracentrifugation the specifics of the protocol, for which figures this method was used etc. And then do the same for the kit. It is unclear what the authors mean with "We used the same protocol with EV200065 on EV-TRACK knowledge". This ID refers to an article published by another research group. The authors should submit the experimental parameters from this study to EV-TRACK. They will receive an EV-TRACK ID for their specific study that can be included in the material and methods section.

This is a recommendation for 1 such paragraph but this recommendation should be followed by the authors across the manuscript.

Referee #3:

Thank you very much for your comments and additional experiments. But I still have a major concern about the novelty and scientific impact of this report.

The authors mentioned in the abstract that "LSD1 in sEVs from gastric cancer cells and gastric cancer patient plasma, which is the first identified histone demethylase in sEVs" and "LSD1 as a sEVs protein to promote stemness for the first time ". However, existence of LSD1 in cancer cell-derived EVs were already reported in many articles, such as hepatocellular carcinoma cells (PMID 26054723), ovarian cancer cells (PMID 23333927), and also thymus tissue (PMID 23844026). Moreover, LSD1 is well known for promoting stemness and chemoresistance as reported in the

following reviews.

"LSD1/KDM1A, a Gate-Keeper of Cancer Stemness and a Promising Therapeutic Target." (Cancers, 2019, 11(12):1821. PMID: 31756917)
"The Histone Demethylase LSD1/KDM1A Mediates Chemoresistance in Breast Cancer via Regulation of a Stem Cell Program." (Cancers, 2019, 11(10):1585. PMID: 31627418)

In consideration of these previous reports, I think that overall novelty and scientific impact of this study are insufficient for publication in EMBO Reports.

Referee #1:

The revised manuscript by Zhao et al. "Small extracellular vesicles-delivered LSD1 promotes gastric cancer cell stemness" completely addressed my comment in regards to Figure 3I and size standards. I also appreciate the addition of the immunoprecipitation description in the method section.

In contrast, the addition of a Western blot decorated with Sox2 does not address if, indeed methylated Sox2 was immunoprecipitated by the pan lysine mono and dimethyl antibodies. This requires analysis by mass spectrometry.

Re: Thanks for your reminding very much. To better confirmed that LSD1 enhanced the stability of SOX2 by removing the methylation of SOX2, we have prepared both mass spectrometry and additional biological experiment for verification. However, the mass spectrometry results were not so perfect that we chose the results of biological experiment here. We prepared mutant of SOX2 containing K42R and K117R to prove that LSD1 enhanced the stability of SOX2 by maintaining the methylation of SOX2. After co-transfection of LSD1-WT and SOX2-WT or LSD1-WT and SOX2-Mut in 293T cells, the Kme1/2 IP assay in Figure 3N showed that K42R and K117R mutations lead to a significant reduction of lysine methylated SOX2. Meanwhile, when compared with the SOX2 stability in co-transfected LSD1-Mut and SOX2-WT HEK293T cells, the SOX2 stability in cells that co-transfected with LSD1-WT and SOX2-WT or LSD1-WT and SOX2-Mut were significantly enhanced (Figure 3O＆P). These results indicated that the decrease of SOX2 methylation caused by LSD1 demethylation or the mutation of SOX2 could enhance the stability of SOX2. However, when SOX2 methylation was maintained by LSD1 mutation, the stability of SOX2 was decreased.

Referee #2:

Overall the authors have properly addressed most of the comments. Especially the additional control experiments performed and included in the revised manuscript further strenghten the overall conclusions.

Nevertheless it is important that the authors ensure the clarity of the additional information for example in the material and methods section as well as in the results section and the figure legends. The authors should improve the readibility of the newly added information, because as it is, multiple parts across the manuscript contain typos and unclarities given that novel information was added while incomplete information was not properly removed. As an example of this I add the following paragraph:

"All sEVs in this paper were obtained by differential ultracentrifugation except the sEVs extracted by kit in figure 1, the kit was only used in Figure 1 for verification the existence of LSD1 in sEVs by a different method. All the sEVs were filtered through 0.22 μm membrane filters before functinnal experiments. We used the same protocol with EV200065 on EV-TRACK knowledge."

Describe for differential ultracentrifugation the specifics of the protocol, for which figures this method was used etc. And then do the same for the kit. It is unclear what the authors mean with "We used the same protocol with EV200065 on EV-TRACK knowledge". This ID refers to an article published by another research group. The authors should submit the experimental parameters from this study to EV-TRACK. They will receive an EV-TRACK ID for their specific study that can be included in the material and methods section.

This is a recommendation for 1 such paragraph but this recommendation should be followed by the authors across the manuscript.

RE: Thanks for your reminding very much. We have carefully checked the writing of the article and corrected the errors in the description to ensure the clarity of the additional information and the description.

We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV200198). You may access and check the submission of experimental parameters to the EV-TRACK knowledgebase via the following URL: http://evtrack.org/review.php. Please use the EV-TRACK ID (EV200198) and the last name of the first author (Zhao) to access our submission.

Referee #3:

Thank you very much for your comments and additional experiments. But I still have a major concern about the novelty and scientific impact of this report.

The authors mentioned in the abstract that "LSD1 in sEVs from gastric cancer cells and gastric cancer patient plasma, which is the first identified histone demethylase in sEVs" and "LSD1 as a sEVs protein to promote stemness for the first time ". However, existence of LSD1 in cancer cell-derived EVs were already reported in many articles, such as hepatocellular carcinoma cells (PMID 26054723), ovarian cancer cells (PMID 23333927), and also thymus tissue (PMID 23844026). Moreover, LSD1 is well known for promoting stemness and chemoresistance as reported in the following reviews.

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"The Histone Demethylase LSD1/KDM1A Mediates Chemoresistance in Breast Cancer via Regulation of a Stem Cell Program." (Cancers, 2019, 11(10):1585. PMID: 31627418)

In consideration of these previous reports, I think that overall novelty and scientific impact of this study are insufficient for publication in EMBO Reports.

RE: Thanks for your reminding very much. I am very sorry for our inaccurate description. We have rephrased the statement that “LSD1 is the first identified histone demethylase in sEVs" in the abstract and in other parts of your manuscript, and cited your listed articles. We want to underline in the manuscript that the novelty and scientific impact of this manuscript is based on the functional analyses and the clinical application prospects of this exosomal LSD1 in gastric cancer, which provides a novel insight for LSD1 in gastric cancer cell stemness.

2nd Revision - Editorial Decision 20th Apr 2021

Dear Prof. Zheng,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the report from the referee that was asked to re-evaluate your study (you will find enclosed below). As you know, the referee has a final concern, indicating that the analysis of the immunoprecipitates by mass spectrometry (Figure 3) would be a prerequisite for publication. However, after discussing this internally, and considering your feedback regarding this point, we decided to proceed without mass spectrometry data. However, we require that you phrase the conclusions of the data in Fig.3 more carefully and acknowledge that you have some evidence that methylation occurs at the two lysines mutated but that you have not provided final proof here. An indirect effect would still be possible. Thus, please re-write the results and discussion part accordingly. Please also provide a final p-b-p response to the remaining referee concern.

Moreover, I have this editorial request:

- I suggest a slightly modified title:
Lysine demethylase LSD1 delivered via small extracellular vesicles promotes gastric cancer cell stemness

- Please have the manuscript carefully proofread. There are still too many grammatical errors that render the manuscript partly difficult to comprehend. We cannot proceed with the paper if this is not improved.

- There are presently 4 figure files uploaded: 'Figure 1G Con sEVs', 'Figure 1G KO sEVs', 'Figure EV4A Healthy' and 'Figure EV4A Patient'. It seems the same images are already part of Figs. 1 and EV4. Is this source data? If yes, please upload this as source data, combined with the SD of the two figures.

- Please remove the referee access information form the DAS and make sure that the datasets are public upon publication of the paper.

- Please provide bigger error bars for Fig. EV1E. They are presently hardly visible.

- Could statistical testing be performed for the diagrams in Figs. 1B, 1E, 5J and EV1D. Please further check that statistical testing has been done were applicable.

Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. Please do that for the co-corresponding author Hong-Min Liu. 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 the final revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,

Achim Breiling
Editor
EMBO Reports

Referee #1:

I appreciate the effort to address my only remaining concern if indeed SOX2 protein is stabilzed by LSD1-mediated methylation in MG3-803 cells. The authors state that in transfected 293 cells SOX2 is stablized by LSD1. This conclusion is in line with data in several carcinoma cell lines and ES cells, but does not answer my question.

I consider the analysis of the immunoprecipitates by mass spectrometry (Figure 3) as an absolute prerequisite for publication.

Dear Professor,

Thanks very much for reviewing our revised manuscript. We have revised our manuscript based on your comments. Please see below point-by-point responses and revised manuscript for details. We hope the current version is suitable for publication in the journal.

Best wishes,
Yi-Chao Zheng
Zhengzhou University

EDITOR'S COMMENTS TO AUTHORS:

1. Thank you for the submission of your revised manuscript to our editorial offices. We have now received the report from the referee that was asked to re-evaluate your study (you will find enclosed below). As you know, the referee has a final concern, indicating that the analysis of the immunoprecipitates by mass spectrometry (Figure 3) would be a prerequisite for publication. However, after discussing this internally, and considering your feedback regarding this point, we decided to proceed without mass spectrometry data. However, we require that you phrase the conclusions of the data in Fig.3 more carefully and acknowledge that you have some evidence that methylation occurs at the two lysines mutated but that you have not provided final proof here. An indirect effect would still be possible. Thus, please re-write the results and discussion part accordingly.

Re: Thanks for your suggestion. We have revised our description and conclusions in Figure 3 more carefully, and added a corresponding discussion in the discussion section.

2. I suggest a slightly modified title:

Lysine demethylase LSD1 delivered via small extracellular vesicles promotes gastric cancer cell stemness

Re: Thanks for your suggestion. We agree with your suggestion and have revised the title.

3. Please have the manuscript carefully proofread. There are still too many grammatical errors that render the manuscript partly difficult to comprehend. We cannot proceed with the paper if this is not improved. Our publisher also offers a manuscript editing service:
https://wileyeditingservices.com/en/english-language-editing/

Re: Thanks for your suggestion. We have improved our manuscript by this recommended manuscript editing service.

4. There are presently 4 figure files uploaded: 'Figure 1G Con sEVs', 'Figure 1G KO sEVs', 'Figure EV4A Healthy' and 'Figure EV4A Patient'. It seems the same images are already part of Figs. 1 and EV4. Is this source data? If yes, please upload this as source data, combined with the SD of the two figures.

Re: Thanks for your suggestion. These four images are the same images in Figure 1G and Figure EV4A. We have deleted these 4 figures and uploaded as source data.

5. Please remove the referee access information form the DAS and make sure that the datasets are public upon publication of the paper.

Re: Thanks for your suggestion. We have deleted the referee access information and confirmed that the dataset is public.

6. Please provide bigger error bars for Fig. EV1E. They are presently hardly visible.

Re: Thanks for your suggestion. We have modified Figure EV1E.

7. Could statistical testing be performed for the diagrams in Figs. 1B, 1E, 5J and EV1D. Please further check that statistical testing has been done were applicable.

Re: Thanks for your suggestion. We have performed and added statistical testing results of Figure 1B, 1E, 5J and EV1D.

8. Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. Please do that for the co-corresponding author Hong-Min Liu. 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

Re: Thanks for your suggestion. We have supplied the ORCID ID of co-corresponding author Hong-Min Liu.

3rd Revision - Editorial Decision **11th May 2021**

Prof. Yi-Chao Zheng
Zhengzhou University
Key Laboratory of Advanced Drug Preparation Technologies, Ministry of Education of China;
Collaborative Innovation Center of New Drug Research and Safety Evaluation
100 Kexue Avenue
Zhenzhou, Henan 450001
China

Dear Prof. Zheng,

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

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THINGS TO DO NOW:

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Corresponding Author Name: Yi-Chao Zheng & Hong-Min Liu
Journal Submitted to: EMBO REPORTS
Manuscript Number: EMBOR-2020-50922V2

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 iustified
- 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)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Generally, one blank or control group is included. Besides, at least three test groups were applied in dose dependent experiment. For all experiments, three independent replications were carried out for each in vitro experiment.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For in vivo limiting dilution assay, there are 6 mice in each group, and this was approved by the Ethics Committee of the Zhengzhou University Health Science Center.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All in vivo/ in vitro data were aquired without exclusion.
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.	Animals were grouped by the technician who does not know about this research.
For animal studies, include a statement about randomization even if no randomization was used.	Yes, we randomly divided the animals into groups.
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.	In vitro experiments were performed by technicians who do not have insight about this research.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The animal operator is unaware of the purpose of the experiment.
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.	SPSS 21.0 was used to detect whether data meet the normal distribution.
Is there an estimate of variation within each group of data?	Standard deviation was used to estimate the 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.The variance was similar between the groups that are being statistically compared.

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 we used in study are LSD1 (ab129195, abcam, England), CD9 (134403, CST, USA), CD63 (ab59479, abcam, England), Calnexin (ab22595, abcam, England), OCT4 (ab181557, abcam, England), SOX2 (14962, CST, USA), Nanog (ab21624, abcam, England), and GAPDH (AB-P-R 001, Hangzhou Goodhere Biotechnology, China),Kme1/2 ((PTM602, PTM biolabs, China) .
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Gastric cancer cell lines MGC-803, BGC-823, NCI-N87 and HGC-27 were purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences. MKN-45 was purchased from Shanghai Bogoo Biotechnology Company.All cell lines were authenticated by STR method and ensured to be mycoplasma free by the third party before experiment.

* 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.	Five weeks old female BALB/c-nude mice were purchased from Jingda Laboratory Animal, Hunan, China. All animals were housed in a pathogen-free environment at an environmental temperature of 24°C and a 12/12 h light/dark cycle with water ad libitum.Besides, experimental protocols were approved by the Ethics Committee of the Zhengzhou University Health Science Center.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Yes, all animal concerning experiments were approved by the Ethics Committee of the Zhengzhou University Health Science Center.
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.	Compliance

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Gastric cancer tissues, adjacent tissues and patient's blood were obtained from the First Affiliated Hospital of Zhengzhou University. All human tissues were collected using protocols approved by the Ethics Committee of the Zhengzhou University Health Science 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.	All donors were informed the sample acquisition and application according to WMA Declaration of Helsinki and the department of heal and human services belmont report.
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.	Additional data or samples acquisition should be licenced by the Ethics Committee of the Zhengzhou University Health Science Center.
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	Yes, it is included in the manuscript.
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