EMBO reports

Fusobacterium nucleatum promotes colorectal cancer by inducing Wnt/b-catenin modulator Annexin A1

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Transaction Report:

Please note that the manuscript was previously reviewed at another journal and the reports were taken into account in the decision-making process at EMBO reports. Since the original reviews are outside of the EMBO Press transparent review process policy, the reports and author response cannot be published.

1st Editorial Decision 14 January 2019

Thank you for the submission of your manuscript, the referee reports and your point-by-point response from your previous submission (to another journal) to our editorial offices. I now read your manuscript, went through the referee reports and the related files, and discussed your manuscript with my colleagues. We feel that the submitted revised version adequately addresses the concerns of the referees. As EMBO reports emphasizes novel functional over detailed mechanistic insight, remaining questions regarding mechanism are not a concern that will prevent publication here.

We have also contacted an external expert advisor, who examined your manuscript, the referee reports and the point-by-point response, and felt that the revised paper is technically sound, and should be published in EMBO reports.

However, the advisor indicated two points that we ask you to address in a final revised version of your manuscript:

- The paper is about fusobacteria as an inducer of cancer. In fact, the authors do not show tumor induction, but enhanced cell growth, which is not the same. Thus, the authors should temper their conclusions, and call the effect 'enhanced cell growth of cancerous cells'.

- In addition, I believe that the authors would do themselves and the emerging field a favor to also mentioning other bacteria (e.g. Salmonella) that may also induce colon (and gallbladder) cancer (shown in mice and suggested in epidemiology). If multiple bacteria can use different mechanisms for transformation and cell growth, the acceptance of bacteria as inducers of cancer may be more easily accepted in the cancer society. And that would make the paper more important as well.

Thus, please put your findings in a broader context (in the Introduction and the Discussion) and tone down the cancer promoting conclusions. Please provide the final manuscript with track changes, in order that I can assess the amendments.

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

- Please check the labeling of the panels in Figs. 3 and 5. Both show two panels B.

- Please add scale bars to ALL microscopic images (without any writing on them), and define them in the respective figure legends.

- Could you provide the source data for the Western blots shown in the manuscript (including the EV figures)? The source data will be published in separate source data files online along with the accepted manuscript and will be linked to the relevant figures. Please submit scans of entire blots together with the revised manuscript. Please include size markers, label the scans with figure and panel number, and send one PDF file per figure.

- Please remove the phrase 'uncategorized references' below the heading 'References'.

- Why do the panels in Figs. 4D, 5C (which should be 5E) and EV4 show the borders of neighbouring images? Please remove these, or explain/mention in the figure legends why these are shown.

- Please clearly indicate the number of replicates for the data shown in figure 3B. If the experiments have been done only twice, statistical testing does not make sense.

- Please indicate the number of replicates for the data shown in panels 7C-F. How many samples or patients are shown?

- Could statistical testing and the number of replicates for the data shown in panels EV2A/B be provided?

- There is a Table S1 mentioned in the text. However, you uploaded the primer data as Table EV1. Thus, please re-name these call-outs in the text, or provide the information as Appendix, including the table as Appendix Table S1.

- The movie file needs a proper call out. Please use Movie EV1, upload it with this name and amend the call-outs accordingly. Please also provide a legend/description for this movie as text file. Please ZIP the movie file and the legend file together, and upload it as single file.

When submitting your revised manuscript, we will require:
- a Microsoft Word file (.doc) of the final revised manuscript text with track changes
- editable TIFF or EPS-formatted figure files (main figures and EV figures) in high resolution (of those with adjusted panels or labels and added scale bars).

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.

1st Revision - authors' response 23 January 2019

1. “The paper is about fusobacteria as an inducer of cancer. In fact, the authors do not show tumor induction, but enhanced cell growth, which is not the same. Thus, the authors should temper their conclusions, and call the effect 'enhanced cell growth of cancerous cells'.

   In addition, I believe that the authors would do themselves and the emerging field a favor to also mentioning other bacteria (Salmonella, ...) that may also induce colon (and gallbladder) cancer (shown in mice and suggested in epidemiology). If multiple bacteria can use different mechanisms for transformation and cell growth, the acceptance of bacteria as inducers of

cancer may be more easily accepted in the cancer society. And that would make the paper more important as well.

Thus, please put your findings in a broader context (in the Introduction and the Discussion) and tone down the cancer promoting conclusions. Please provide the final manuscript with track changes, in order that I can assess the amendments."

For the comment about *Fusobacteria* as "inducer", we looked carefully all over the ms but could not find such a statement. As a matter of fact, we propose bacteria as the "second hit", meaning they can only facilitate carcinogenesis once the host becomes predisposed. Nevertheless, we have "softened" the choice of words, as you would find in the track-changed text.

We appreciate your suggestion of putting our findings in a broader context and have added a new paragraph in the Discussion. We feel it helps strengthen the "two-hit" model.

2. Please check the labelling of the panels in Figs. 3 and 5. Both show two panels B.
We apologize for the oversight. The labeling has been corrected.

3. Could you provide the source data for the Western blots shown in the manuscript (including the EV figures)? The source data will be published in separate source data files online along with the accepted manuscript and will be linked to the relevant figures. Please submit scans of entire blots together with the revised manuscript. Please include size markers, label the scans with figure and panel number, and send one PDF file per figure.
Source data provided.

4. Please remove the phrase 'uncategorized references' below the heading 'References'.
Removed.

5. Why do the panels in Figs. 4D, 5C (which should be 5E) and EV4 show the borders of neighbouring images? Please remove these, or explain/mention in the figure legends why these are shown.
Sorry for the confusion. They are side-views. We have removed them in Fig 4D because they don't add much, but kept those in Fig 5D & 5E, and added explanation in the figure legend.

6. Please indicate the number of replicates for the data shown in panels 7C-F. How many samples or patients are shown?
This information has been added in the figure legend.

7. Could statistical testing and the number of replicates for the data shown in panels EV2A/B be provided?
Yes, statistics has been added.

8. There is a Table S1 mentioned in the text. However, you uploaded the primer data as Table EV1. Thus, please re-name these call-outs in the text, or provide the information as Appendix, including the table as Appendix Table S1.
Sorry about the confusion. We changed the text to "Table EV1".

9. The movie file needs a proper call out. Please use Movie EV1, upload it with this name and amend the call-outs accordingly. Please also provide a legend/description for this movie as text file. Please ZIP the movie file and the legend file together, and upload it as single file.
Movie EV1 is included and called out in the text.

10. A Microsoft Word file (.doc) of the final revised manuscript text with track changes.
Yes, included.

11. Editable TIFF or EPS-formatted figure files (main figures and EV figures) in high resolution (of those with adjusted panels or labels and added scale bars).
TIFF files included.

12. A short, two-sentence summary of the manuscript:
"*F. nucleatum* stimulates the growth of colorectal carcinoma cells, but not the pre-cancerous adenoma cells, through induction of Annexin A1, a previously unrecognized modulator of Wnt/b-catenin signaling. We propose a "two-hit" model of carcinogenesis in which the host driver mutations serve as the first "hit", and the microbes, e.g. *F. nucleatum*, as the second "hit", to exacerbate cancer progression."

13. Two to three bullet points highlighting the key findings of your study
- *F. nucleatum* stimulates the growth of colorectal cancer cells without affecting the pre-cancerous adenoma cells.
- Annexin A1, a previously unrecognized modulator of Wnt/b-catenin signaling, is specifically expressed in proliferating colorectal cancer cells and is a novel predictor of poor prognosis independent of cancer stage, grade, age and sex.
- The FadA adhesin from *F. nucleatum* up-regulates Annexin A1 expression through E-cadherin. A positive feedback loop between FadA and Annexin A1 is identified in the cancerous cells, absent in the non-cancerous cells.

14. 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.
Included. Due to limitation of the pixels, we use the second half of Fig 9, the 'two-hit" model.

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Corresponding Author Name: Yiping W. Han
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

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

USEFUL LINKS FOR COMPLETING THIS FORM

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

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-tun

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/

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?	N/A
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For the Apc min/+ mice, we first collected the tissues for pathological analysis and later for DNA/RNA extraction. Fig 7 includes the total number of Apc min/+ animals used.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
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.	For in vitro studies, cells were seed at the same time for each treatment. In the animal studie we use multiple litters, animals from same litter were distribute across different treatment groups.
For animal studies, include a statement about randomization even if no randomization was used.	In the animal studie we use multiple litters, animals from same litter were distributed across different treatment 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.	For the xenographs experiment with DLD1 (Fig EV2) the investigator measuring the tumors was blinded.
4.b. For animal studies, include a statement about blinding even if no blinding was done	For Fig 7, the same investigator gavaged and sacrificed the animals. No blinding was possible.
5. For every figure, are statistical tests justified as appropriate?	Yes. To compare difference among 2 groups t-tests were perfomed. To compare difference among 3 or more groups one-way ANOVA was performed and when two different factors were tested (ie siRNA treatment and bacteria infection), two-way ANOVA were performed. When the interaction was significative, simple effect analyses was carried out.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	For every test normal distribution and homoscedasticity was tested. If the samples didn't pass the tests, a transformation was carried out and re tested for normal distribution and homoscedasticity.
Is there an estimate of variation within each group of data?	No
Is the variance similar between the groups that are being statistically compared?	Yes, please see above.

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).	Antibodies used: Annexin A1: Thermo Fisher Scientific, 71-3400 Cyclin D1 Santa Cruz, sc-8396 β-catenin Thermo Fisher Scientific, 71-2700 β-actin Abcam, ab6276 β-actin Cell Signaling Technology, 3700S anti-FadA 5G11 (Xu et al, 2007), E-cadherin Cell Signaling Technology, 3195S E-cadherin polyclonal antibodies R&D Systems, AF648
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The sources of the cell lines are stated in Acknowledgement.

* 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.	For Apc min/+ mice, both males and females were tested. For nude mice, only females were used. This is described in Materials & Methods. The source and husbandry are also described in Materials & Methods.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The protocal was approved by the IACUC committee of Columbia University, as stated inMaterials & Methods.
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.	N/A.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The protocal was approved by the Internal Review Board at Columbia University, as stated in Materials & Methods.
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 tissues from the biobank had consent forms. The specimens were provided to us de-identified.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A.
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).	N/A.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A.