Deletion of F4L (ribonucleotide reductase) in vaccinia virus produces a selective oncolytic virus and promotes anti-tumor immunity with superior safety in bladder cancer models

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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 Molecular Medicine. Since the original reviews are not subject to EMBO's transparent review process policy, the reports and author response cannot be published.)

Editor: Roberto Buccione

1st Editorial Decision 24 November 2016

We have now heard from the expert external advisor whom we asked to help us on making a decision on your manuscript.

The advisor was provided with the full manuscript, the previous Reviewers' comments and your Point-by-Point rebuttal.

As you will see, s/he agrees that the manuscript would make an interesting and worthy contribution, pending an experimental concern and a number of issues that need to be addressed. After internal discussion we agreed that, provided you successfully address these concerns, we would be happy to proceed with your manuscript without further revision other than going back to the advisor for a final check, if necessary.

I look forward to seeing a revised form of your manuscript as soon as possible.

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

Referee #1 (Remarks):

I have now had chance to look at the paper in more detail. When the key finding of the paper is

clear, I think it does carry significance for the field. Hence my overall recommendation is to accept it with a few amendments - including sharpening of the title and abstract to convey the key findings more clearly.

In my view the most important finding of this manuscript is that deletion of the RRM2 gene homologue from vaccinia virus gives it cancer selectivity under low serum conditions (used to mimic conditions within a tumour environment). The immune effects are consequential to this, but are not the key aspect. Hence I think the title should be modified to something such as: " Deletion of F4L (ribonucleotide reductase) in vaccinia virus produces a selective oncolytic phenotype and promotes anti-tumor immunity with superior safety in bladder cancer models". I would suggest the Abstract is also sharpened to make the key finding obvious.

1. It is not very clear to me why N60 fibroblasts were used as a 'normal' cell control, when the bladder cancer cell lines are epithelial - why not use an immortalised normal epithelial cell such as Human Airway Epithelial Cells etc?

2. They imply that low serum acts at least partly through effects on the cell cycle; is there data somewhere showing that the low serum conditions does stall the cell cycle in those cells? Is it feasible that the only difference between the cancer cells and the normal fibroblasts is that the former were not able to quiesce, and this explains the effects on the F4L-deleted viruses? That does not really undermine the interpretation, although it is important and should definitely be discussed.

3. Also the Discussion should contain a paragraph discussing what metabolic deregulation may have occurred in the cancer cell lines that allows complementation of the RRM2 deletion even under low serum conditions.

It is notable that the majority of comments from the previous referees have focused on the immunostimulatory properties of the viruses, which presumably just reflects the different patterns of virus activity in vivo. Personally I think the animal data are sufficiently convincing, but the authors need to pay more attention to the cellular mechanisms that govern the oncolytic activity observed - as outlined above.

1st Revision - authors' response 26 January 2017

Referee #1 (Remarks):

I have now had chance to look at the paper in more detail. When the key finding of the paper is clear, I think it does carry significance for the field. Hence my overall recommendation is to accept it with a few amendments - including sharpening of the title and abstract to convey the key findings more clearly.

In my view the most important finding of this manuscript is that deletion of the RRM2 gene homologue from vaccinia virus gives it cancer selectivity under low serum conditions (used to mimic conditions within a tumour environment). The immune effects are consequential to this, but are not the key aspect. Hence I think the title should be modified to something such as: "Deletion of F4L (ribonucleotide reductase) in vaccinia virus produces a selective oncolytic phenotype and promotes anti-tumor immunity with superior safety in bladder cancer models". I would suggest the Abstract is also sharpened to make the key finding obvious.

The title has been modified based on the reviewer’s suggestions. We have also made some revisions to the abstract.

1. It is not very clear to me why N60 fibroblasts were used as a 'normal' cell control, when the bladder cancer cell lines are epithelial - why not use an immortalised normal epithelial cell such as Human Airway Epithelial Cells etc?

We have added a normal epithelial kidney (NKC) cell line to our growth curve and cytotoxicity panels (Figures EV2 and EV3). The results are similar to what we observed with N60 fibroblasts.

2. They imply that low serum acts at least partly through effects on the cell cycle; is there data somewhere showing that the low serum conditions does stall the cell cycle in those cells? Is it feasible that the only difference between the cancer cells and the normal fibroblasts is that the former were not able to quiesce, and this explains the effects on the F4L-deleted viruses? That does not really undermine the interpretation, although it is important and should definitely be discussed.

We have analyzed distribution of human RT112-luc bladder cancer cellsunder both regular and low serum conditions. We have also carried out cell cycle analysis on the N60 cell line and the NKC cell line under the same conditions (Figure EV4). This is discussed in the text (Page 6).

3. Also the Discussion should contain a paragraph discussing what metabolic deregulation may have occurred in the cancer cell lines that allows complementation of the RRM2 deletion even under low serum conditions.

A paragraph has been added to the discussion to address the metabolic deregulation that may occur leading to complementation of ΔF4L VACV (Page 15).

It is notable that the majority of comments from the previous referees have focused on the immunostimulatory properties of the viruses, which presumably just reflects the different patterns of virus activity in vivo. Personally I think the animal data are sufficiently convincing, but the authors need to pay more attention to the cellular mechanisms that govern the oncolytic activity observed - as outlined above.

2nd Editorial Decision 09 February 2017

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed report from the reviewer who was asked to re-assess it. As you will see the s/he is now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final minor amendments:

1) Please provide a running title in the title page

2) We noticed the figure callout for Appendix Figure S5A-D on page 10, which is most likely a mistake. Did you mean perhaps Appendix Figure S6A-D? Also, and probably connected to this, you mention Appendix Figure S6A and B but not C and D.

3) Please remove the Appendix methods from the main text

4) The scale bars for Fig. 7A are very difficult to see, please improve

5) We noted that in Fig. 7A, the mCherry heat Map mock panel appears to be empty. Could you please verify and eventually clarify?

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible so that we can proceed with formal acceptance.

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

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

The paper has been revised to address my previous concerns and now contains a clearer focus on the dependence of the virus on tumour metabolism alongside their observations about immunogenicity and creation of an anticancer immune response. The work now fits well into the emerging field of oncolytic viruses and should be well cited. All my concerns have been adequately addressed.

2nd Revision - authors' response 10 February 2017

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed report from the reviewer who was asked to re-assess it. As you will see the s/he is now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final minor amendments:

1) Please provide a running title in the title page

We have added the following running title "ΔF4L oncolytic VACV in bladder cancer"

2) We noticed the figure callout for Appendix Figure S5A-D on page 10, which is most likely a mistake. Did you mean perhaps Appendix Figure S6A-D? Also, and probably connected to this, you mention Appendix Figure S6A and B but not C and D.

Errors have been corrected.

3) Please remove the Appendix methods from the main text

Appendix text has been removed from the end of the manuscript.

4) The scale bars for Fig. 7A are very difficult to see, please improve

We have increased the size and clarity of the scale bars.

5) We noted that in Fig. 7A, the mCherry heat Map mock panel appears to be empty. Could you please verify and eventually clarify?

mCherry signal in the mock panel is correct. There is no visible signal generated from the mock infected tissue.

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible so that we can proceed with formal acceptance.

We also made a formatting correction to the Appendix document as well.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: DAVID EVANS
Journal Submitted to: EMBO MOLECULAR MEDICINE
Manuscript Number: EMM-2016-07296

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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 χ^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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample sizes were chosen based on experience from previous experiments along with the power calculation described in 1.b.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	With ten animals per group, 80% power, and a 5% significance level, we are able to distinguish differences in survival of presented data. For orthotopic xenograft model and IV treated subcutaneous xenograft models groups of five were used unless otherwise noted. These experiments were proof of principle experiments and detailed survival was not determined.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Two animals suffered seizures and were censored from Figure 5G. All other animals that developed tumors were used in the studies.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Yes, animals were allocated into treatment groups following initial tumor volume measurements to ensure an even distribution of tumor sizes between groups.
For animal studies, include a statement about randomization even if no randomization was used.	Animals were not randomized, however they were sorted into groups based on tumor measurements in order to equalize average tumor size and distribution of sizes between 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.	Where possible methods of unbiased quantification were used. Quantification of ex vivo infected tissues was performed blinded.
4.b. For animal studies, include a statement about blinding even if no blinding was done	None of the animal experiments were blinded.
5. For every figure, are statistical tests justified as appropriate?	Yes, to the best of our knowledge we used the appropriate statistical method for each experimetal analysis.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	GraphPad Prism was used for statistical analyses.
Is there an estimate of variation within each group of data?	Variations are shown for each group as SEM.
Is the variance similar between the groups that are being statistically compared?	The groups have similar numbers resulting in comparible variances between groups.

C- Reagents

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://jij.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	Antibody catalog numbers are provided in the Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The identities of the cell lines were confirmed using a 16-marker AmpFLSTR® Identifiler® system and tests performed by the TCAG facility at the University of Toronto.

* 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.	Details on animals are provided in the manuscript. Housing conditions included standard pellet food and water, as well as regular health monitoring.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All the studies reported in this communication were conducted with the approval of the University of Alberta Health Sciences Animal Care and Use Committee in accordance with guidelines from the Canadian Council for Animal Care.
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.	The ARRIVE guidelines were consulted.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	University of Alberta Health Research Ethics Board.
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.	Primary cancer and adjacent normal tissues were obtained from consenting patients undergoing surgery.
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.	Bladder cancer patient microarray expression data were retrieved from Sanchez-Carbayo M., et al. Patient-derived cell lines are regulated by availability and institutional MTAs.
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 accession codes for deposited data. See author guidelines, under '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. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	N/A
22. 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

23. 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