EMBO Molecular Medicine

Lentiviral Vectors Escape Innate Sensing but Trigger p53 In Human Hematopoietic Stem and Progenitor Cells

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Transaction Report:

(Note: 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 17 May 2017

Thank you for the submission of your manuscript and previous review correspondence to EMBO Molecular Medicine.

We have now heard back for the expert advisor who was asked to evaluate it. As you will see, s/he is globally positive but points to a few issues that need further clarification. You will also note that, not surprisingly in my opinion, a few reservations are expressed concerning the degree of clinical impact, that call for some deemphasizing.

I am prepared to make an editorial decision on the next, final version of your manuscript, provided you carefully and fully address the advisor's concerns. Please highlight the changes in the manuscript text.

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

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

Referee #1 (Remarks):

• Well performed study
• This is novel since reporting consequences (innate immune-DDR responses) of LV (γRV, AAV)

transduction of human hematopoietic progenitor cells had not yet been done. It is a significant advance in describing the biology underlining gene therapy based on viral vectors
• Potential to increase early stages of hematopoietic reconstitution following LV mediated GT is a little but overemphasized since :
a) neutropenia related death as mentioned p19, following SCTs is no longer as high as reported in the cited 2009 paper
b) it is not excluded that p53 inhibition or ATM inhibition might exert unwanted effects not seen in the experimental setting. I therefore suggest to temper the presentation of the medical interest of this approach
• P3, DAI (NLR family) could also be cited as a cytosolic receptor of DNA
• All over the manuscript, the term "HSC" is used to define cord blood or bone marrow CD34+ cells. This is not fully accurate since only small fractions are bonafide HSC. The term of hematopoietic progenitor cells would thus be more appropriate.

1st Revision - authors' response 30 May 2017

We thank the reviewers for retaining our work technically well performed, novel and of significant advance in understanding the biology underlying gene therapy based on viral vectors. We have modified the manuscript based on the reviewers' insightful criticisms and suggestions and believe to have properly addressed most of his/her concerns and to have significantly improved the overall quality of this work.

Briefly, we have now modified the manuscript to address the concerns specifically raised by the Reviewer as follows:

Referee #1 (Remarks):

• Potential to increase early stages of hematopoietic reconstitution following LV mediated GT is a little but overemphasized since :
a) neutropenia related death as mentioned p19, following SCTs is no longer as high as reported in the cited 2009 paper

Regarding this specific point, we thank the Reviewer for this observation and have now modified the discussion on neutropenia-related mortality in hematopoietic stem cell transplantation (HSCT) accordingly and have included more recent references, page 18 of the revised manuscript, as suggested by the Reviewer.

b) it is not excluded that p53 inhibition or ATM inhibition might exert unwanted effects not seen in the experimental setting. I therefore suggest to temper the presentation of the medical interest of this approach

As the Reviewer correctly suggests, there are certainly some safety concerns associated with the potential application of transient ATM inhibition during ex vivo HSPC gene therapy to be taken into account and carefully addressed before any clinical implementation can be foreseen. Based on this useful comment, we have now tempered the discussion regarding the applicability of ATM inhibition, page 19 of the revised manuscript, acknowledging these relevant safety concerns, as rightly pointed out by the Reviewer.

• P3, DAI (NLR family) could also be cited as a cytosolic receptor of DNA

As the Reviewer rightly points out, also other cytosolic nucleic acid sensor may be involved in vector sensing in HSPC. In particular, we have now cited also the DNA-dependent activator of interferon-regulatory factors (DAI) as a potential sensor of vector nucleic acids in the introduction, page 3 of the revised manuscript, as suggested by the Reviewer

• All over the manuscript, the term "HSC" is used to define cord blood or bone marrow CD34+ cells. This is not fully accurate since only small fractions are bonafide HSC. The term of hematopoietic progenitor cells would thus be more appropriate.

We fully agree with the Reviewer that including the progenitor compartment in our definition of CD34+ population is more appropriate. Accordingly, we have modified the term "HSC" to "HSPC" throughout the revised version of manuscript in order to refer more correctly to the heterogeneous population of $CD34^+$ hematopoietic stem and progenitor cells, as kindly suggested by the Reviewer.

Finally, we have also modified our manuscript to comply to the EMBO Molecular Medicine editorial requirements as follows:

- We have included five keywords, page 1 of the revised manuscript;
- We have moved the methods and related references of Viral Vectors Colony-forming unit (CFU) assay and Transplantation of human HSPC in NSG mice, Statistical analysis, Limiting Dilution Assay and Homing Assay from the supplementary materials to the main manuscript as requested, pages 21-24;
- We have updated the references to comply the EMBO Molecular Medicine guidelines;
- We have updated all figure legends to comply to the Author Guidelines regarding statistical testing, pages 32-36;
- We have filled-in the complete Author Checklist;
- We have included source data for all the Western blots shown in the main and supplementary figures of the manuscript;
- We have included a statement in the Materials and Methods section of the revised manuscript identifying the institutional and/or licensing committee approving the experiments, page 22;
- We have included a Synopsis of our findings and prepared a visual abstract accompanying it;
- We have included the ORCID ID for the corresponding author.

2nd Editorial Decision 02 June 2017

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine.

I am pleased to inform you that we will be able to accept your manuscript pending a few final amendments concerning items that were not fully dealt with:

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

2nd Revision - authors' response 06 June 2017

Authors made requested editorial changes.

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: Anna Kajaste-Rudnitski
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2017-07922

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).

USEFUL LINKS FOR COMPLETING THIS FORM

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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?	No prior studies were used to estimate the statistical power of the study
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The animal sample size was chosen in agreement with available published work in which similar experiments were performed (Zonari et al., Stem Cell reports, 2017; Nucera et al, Cancer Cell, 2016, Giustacchini et al. , Cell stem cell, 2012)
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All the mice used in this study were female NSG, transplanted between 8-10 weeks of age. In the homing and LDA assay both pre-established criteria and stringent internal controls (not transplanted mice) were used to establish exlusion/inclusion of the mice in the analysis. For example, in the LDA experiment a mouse was considered as engrafted if the percentages of human CD45+ within the BM was above 0.10 (basal level of noise signal at flow cytometer observed in the negative controls), and if both human myeloid and lymphoid cells were observed in the mouse (pre-established criteria based on the expected stem cell repopulation capacity). In the primary transplant experiment (Fig 3A-B) we excluded all the mice of one experiment due to technical issues during the transplant procedure that lead to unaccettably low and random levels of engraftment among the groups (also in untreated controls).
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, the in vitro experiments were mostly performed with pools of CD34+ donors to decrease the impact of interindividual biological variability.
For animal studies, include a statement about randomization even if no randomization was used.	The animal study was randomized.
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.	No blinding
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding
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.	Data were analyzed with Graph Pad Prism version 5.0a and expressed as the mean ± standard error mean (SEM) if not otherwise stated. Non-parametric Wilcoxon Signed Rank test was used to assess the different level of expression of specific genes respect to internal control set as 1. Non-parametric (Kruskall-Wallis) was used for unpaired dataset, while the non-parametric (Friedman
Is there an estimate of variation within each group of data?	No estimation of variation was performed as all tests were non-parametric. For further details, please refer to Material and Methods of the manuscript
Is the variance similar between the groups that are being statistically compared?	NA

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 for FACS: Protein, Fluorophore, Dilution, Clone, Company and cat. hCD235a, APC, 1:25, GA-R2, BD Biosciences 551336 hCD33, BV421, 1:25, WM53, BD Biosciences 562854 Anti human FCR Blocking, 1:50, Miltenyi Biotec 120-000-442 Anti murine FCR Blocking, 1:100, 2.4G2, BD Pharmigen 553142 hCD45, APC-Cy7, 1:33, HI30, eBiosciences 47-0459-42 hCD19, PE, 1:25, SJ25C1, BD Biosciences 345789 hCD33, PeCy7, 1:25, P67.6, BD Biosciences 333952 hCD3, APC, 1:25, UCHT1, BD Biosciences 555335 hCD13, BV 1:25, WM15, BD Biosciences 562596 hCD34, PeCy7, 1:25, 8G12, BD Biosciences 348811 hCD38, V450, 1:25, HB7, BD Biosciences 646851 hCD90, APC, 1:25, 5E10, BD Biosciences 559869 hCD45RA, VioBlue, 1:10, T6D11, Miltenyi Biotec 130-095-464 hCD133, PE, 1:15, 293C3, Miltenyi Biotec 130-090-853 hCD38, APC ,1:10, IB6, Miltenyi Biotec 130-092-261 Ki-67, PE, B56, BD Bioesciences 556027 hCD90, Brillant Violet, 1:30, Biolegend 328122 Annexin V, Pacific Blue, 1:20, Biolegend 640918 Antibody for WB: p53Ser15, Rabbit, 1:1000, Polyclonal, Cell signaling Technology 284S p53, Mouse, 1:200, DO-1, Santa Cruz Sc126 p21, Rabbit, 1:1000, 12D1, Cell signaling Technology 2947 γ-H2AX, Rabbit, 1:1000, 20E3, Cell signaling Technology 9718 pSer1981ATM, Rabbit, 1:1000, D25E5 Cell signaling Technology 13050 ATM, Rabbit, 1:1000, D2E2, Cell signaling Technology 2873
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines were tested negative for Mycoplasma.

* 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.	All the mice used in this study werefemale NSG, purchased from Jackson lab, transplanted between 8-10 weeks of age. Animals were housed at the Ospedale San Raffaele animal facility in sterility conditions complying with the immunosuppressed phenotype of the NSG mice. Transplantions, bleeding and sacrifice were performed within thwe animal facility BSL2 room, according to National and European regulatory standards.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were performed according to National and European regulation in the context of a protocol approved by the Ospedale San Raffaele Ethical Committee and the Italian Ministry of Health (IACUC 611).
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.	All animal experiments comply.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	All experiments involving primary human cord blood-derived CD34+ cells collected at the the Ospedale San Raffaele refer to the study protocol approved by the Ospedale San Raffaele Ethical Committee (TIGET01).
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.	The TIGET01 study protocol is conform and informed consent was obtained from all subjects.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide 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	The complete RNA-Seq dataset is available at NCBI, accession number GSE92652.
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. 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	NA
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.	NA

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.	NA