EMBO reports

pH regulates hematopoietic stem cell potential via polyamines

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1st Editorial Decision **7th Jun 2022**

Dear Prof. Geiger,

Thank you for the transfer of your research manuscript to EMBO reports. I have now received the 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, the referees think that these findings are of interest. However, all referees have several comments, concerns, and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all their points need to be addressed, I will not detail them here.

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 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. Please contact me to discuss the revision (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

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. 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 the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

3) 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

4) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this in a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

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. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

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

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10) For microscopic images, please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

11) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

12) Please add up to five keywords to the title page.

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.

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Achim Breiling
Senior Editor

EMBO Reports

Referee #1:

In this manuscript, Sachin et al. reported that pH regulates the maintenance of murine hematopoietic stem cells (HSCs) via polyamine pathway. Mouse HSC stemness was maintained ex vivo (40 hours) in medium at a pH of 6.9 but not 7.4. When HSCs were cultured at pH 6.9, cells were smaller, less metabolically active and more quiescent. Mechanistically, pH6.9 attenuates the polyamine pathway in HSCs. Inhibition of polyamine pathway by DFMO mimicked the effect of pH6.9 on the maintenance of mouse HSCs.
This study is of potential interest. However, to well explore the role of pH in HSC maintenance and improve the present manuscript, many questions need to be addressed.

Major:
1. The authors claimed that pH in BM cavity is 7.2, and then they found that pH6.9 is the best for the maintenance of murine HSCs. Is it possible to determine the pH in different BM niches, endosteal niches and perivascular niches? As is known, in different BM niches, the oxygen levels re quite different. The H+ concentration may vary in different sites in vivo.
2. In order to study the role of pH on HSC maintenance, the authors ex-vivo cultured the murine HSCs for 40 hours. 40 hours is too short for ex vivo culturing. In such a short time period, the stemness of HSCs are maintained usually well. The authors should do these experiments for at least 4-7 days.
3. In this study, the experiments are performed using FBS containing IMDM medium. For the maintenance or expansion of HSCs, serum free mediums such as SFEM or Stemline medium are used. The authors need do at least some important experiments using commercially available serum free medium.
4. Generally, mitochondrial metabolism is negatively correlated to the stemness of HSCs. Why pH6.9 stimulate OCR activity?
5. How extracellular pH or Proton regulates polyamine pathway? Does it activate a potential cell surface receptor and transduce extracellular signaling into intracellular matrix?
6. The authors show that pH6.9 causes change of intracellular pH. If intracellular environment becomes acidic, the function of those proteins or molecules is still normal ? The HSCs are still healthy in this state?
7. In order to prove the effect of pH6.9 on HSC maintenance, the authors need to check if it is conserved in human HSC maintenance or ex vivo expansion using serum free medium, including polyamine pathway inhibitors.

Minor:
1. Fig2A, LT-HSCs expand 4 times in 40 hours?
2. Fig2B,F, would be better to include untreated cell group.
3. Fig3B, would be better to include untreated cell group.
4. How is polyamine pathway in untreated HSCs ?
5. FigS1F, CD45.2/CD45.1 F1 mice are not used, why CD45.2 and CD45.1 double positive cells are shown in the FACS plot?

Referee #2:

Kumar et al. demonstrated that small changes in culture medium pH could support ex vivo hematopoietic stem cell maintenance. They used competitive transplantation analysis and discovered that HSCs cultured in pH 6.9 condition conferred a higher rate of chimerism, comparable to uncultured HSCs. They also found that lower extracellular pH could influence growth and metabolic processes, potentially increasing stemness. Although the idea of adjusting the pH of the culture medium to prolong HSC maintenance ex vivo is intriguing, the main concern is regarding cellular responses to the adjustment, particularly cell viability.

Major concerns:
1. The authors observed a pH as lowest as 7.1 in bone marrow (Fig.1A). However, the pH in the culture condition used in the experiments is 6.9, which has a proton concentration approximately 1.5-fold higher than the measured physiological condition in the bone marrow. I doubt that HSCs would normally respond to this condition. It would be great if they could provide more information based on their RNAseq analysis to confirm that HSCs respond to the pH 6.9 culture condition similar to the pH 7.4 culture condition.
2. Interestingly, the pH 6.9 culture condition improved HSC function in mice, which could be useful for future research into human HSC transplantation. HSCs cultured in pH 6.9 appear to have a lower expansion rate (Fig. 2A), which can be interpreted as they mentioned that the cells are maintaining their stemness. However, cell death also can cause less expansion. Therefore, I believe the authors should include experiments to demonstrate that decreasing pH has no effect on cell viability.
The gene ontology analysis of gene sets at pH 6.9 versus pH 7.4 (Fig. 3E) revealed an up-regulated apoptotic process. Could the pH 6.9 culture condition cause apoptosis?
3. The percentages of donor-derived cells in Fig. 4I-J have seemed to have heavily deviated. Secondary recipients' bone marrow in the DMFO+SPN group, for example, may contain 0 to 100 % donor derived cells. Indeed, we can see a trend of increasing percentages of donor derived cells in the DMFO treated group and statistical significance. I am still concerned about the data quality because we cannot see such a large deviation in Fig 1.

4. Although the author mentioned deceased ROS production (Fig.2D) and autophagy (Fig.2E-H), as well as changes in metabolic profile (Fig. 4A-B) in HSCs cultured in pH 6.9 condition, the gene ontology analysis did not enrich any of these pathways from the RNAseq dataset (Fig. 3E-F). What is the reason for these discrepancies?
5. Fig. 4D showed that DFMO treatment resulted in a decrease in intracellular pH. However, in HSCs cultured at pH 6.9, lower pH causes a decrease in spermidine (Fig.4B). These findings contradict each other in terms of cause and effect. If the authors believe it could be the other way around, they should provide a more rational explanation.
In addition, the DFMO concentration used in the experiment for Fig. 4D is in the 2-5mM range, but only one bar is shown for the DFMO-treated group. The authors must repeat the experiment with a single concentration or separate each concentration into its own bar.
6. There is a variation in sample size (n) within each experiment. The number of biological repeats in some experiments was not stated. In Fig. 1B, for example, the author stated "n = at least 14 mice per group and 4-5 experiments". The authors must explain why they included different sample sizes and specify the number for each experimental group.

Minor concerns:
1. Flow cytometry plots need to be revised for consistency and clarity.
- They should state the marker and fluorochrome used in the axis label (e.g. CD4-FITC) and include numbers along the axis scale of flow cytometry plots.
2. I do not think the flow cytometry plots and the bar graphs in Fig. 1I-J correspond well. For example, in pH 6.9 and 7.4 culture conditions, the LT-HSC population was around 10.2% and 5.8%, respectively, but the bar graph showed more than 10% of the LT-HSC population in both conditions.
3. The length of each scale bar in ultra-structure TEM images of uncultured fresh HSC is missing. Furthermore, cell size appears to be inconsistent between 3D construction (Fig. 2B) and ultra-structure TEM images (Fig. 2H). In 3D construction images, we can see a significant difference in cell size between pH 6.9 and pH 7.4 culture conditions, but not in ultra-structure TEM images.
4. The authors should double-check the consistency of graph labeling. For example, in Fig. 1, you used donor derived BM chimera, whereas, in Fig. 4, you used donor derived cells.

Referee #3:

Kumar et al present a cohesive manuscript that evaluates the effect of pH on HSC potential. They establish that reducing the pH to 6.9 for ex vivo culturing helps to maintain more stem-cell potential. This reduced pH lowers metabolic stress and was associated with many features suggestive of maintained functional HSC potential and the authors further establish the metabolite changes that may contribute to the maintained potential and showed drug targeting of the polyamine pathways was able to recapitulate the ex vivo culture benefits in lower pH. The manuscript is well written (but requires minor editing) and presents a novel mechanism that could lead to maintained HSC potential during ex vivo culturing / expansion.

Minor points:
1) While these data are interesting regardless of species, it would be relevant to include "mouse" or "murine" in the title. There is a major difference in HSC competence ex vivo between the species and it is important to state to avoid misleading others.

2) The authors chose an ex vivo incubation time of 40 hrs. This time frame is small and does not really allow for a robust expansion of HSCs: Why whas this time point chosen? Would longer culture periods lead to larger expansion of the more potent HSCs, or is this the optimal improvement / expansion of these HSCs?

3) The PCA analysis would be strengthened by including the freshly isolated HSCs to the plot and demonstrating the 6.9 pH cultured HSCs had more similar profiles to the freshly isolated ones (similar to their improved functional potential).

4) Clarification of secondary transplant protocols is needed for the interpretations.

5) There needs to be clarification also of the statement on Page 6, first paragraph, last sentence: "These data indicate that HPC respond distinctly, in comparison to the response of HSCs". There were no HSCs in the experiment, so how is there an indication that this is distinct? Further, as this is mentioned that they respond distinctly, it is odd that LSK cells are used to examine the metabolic changes (though it is appreciated the numbers of cells required for Seahorse Assays is difficult to acquire for HSCs).

6) The EM images presented to display more intact mitochondria are difficult to evaluate.

7) A seemingly small semantics question, but and important disctiction needs to be clarified in the evaluation of the DEGs between the 6.9 and 7.4 pH culture, the authors state that correlations showed..."ph 6.9 compared to 7.4 was highly correlated with multiple gene signatures." Do the authors mean enriched for expression of these pathways or was a correlation analysis performed? It is an important distinction.

8) In the materials and methods section, culture conditions: Please expand this section to include all details needed to repeat the

experiment. What are the exact cytokines used (company and cat#)?. What types of plates were used how many wells, round or flat bottom, TC treated or not)? This paper expands our understanding of HSC ex vivo cultures and these kind of details are very important to others in the field.

Point-to-point response to the comments of the Referees

Referee #1:

In this manuscript, Sachin et al. reported that pH regulates the maintenance of murine hematopoietic stem cells (HSCs) via polyamine pathway. Mouse HSC stemness was maintained ex vivo (40 hours) in medium at a pH of 6.9 but not 7.4. When HSCs were cultured at pH 6.9, cells were smaller, less metabolically active and more quiescent. Mechanistically, pH6.9 attenuates the polyamine pathway in HSCs. Inhibition of polyamine pathway by DFMO mimicked the effect of pH6.9 on the maintenance of mouse HSCs.
This study is of potential interest. However, to well explore the role of pH in HSC maintenance and improve the present manuscript, many questions need to be addressed.

We thank the reviewer for her/his encouraging comments on the novelty of our findings, and the comment on the interest of the data to the community.

Major:

1. The authors claimed that pH in BM cavity is 7.2, and then they found that pH6.9 is the best for the maintenance of murine HSCs. Is it possible to determine the pH in different BM niches, endosteal niches and perivascular niches? As is known, in different BM niches, the oxygen levels re quite different. The H+ concentration may vary in different sites in vivo.

We provide additional novel data to further substantiate our claim of different levels of pH within the bone marrow. Multi-photon live microscopy was employed using an established cell impermeable pH sensitive ratiometric probe, 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) to further determine whether pH distribution within BM is uniform or heterogeneous (**MS page 4** and **Figure EV1C-G**) (Ray et al., 2012). Using selective landmarks as regions of interest (ROI) at the bone surface and within BM including cells with likely osteoblastic and osteoclastic nature or known blood vessels, the conclusion from this type of data is that the pH within bone marrow is not uniform and rather heterogeneous. pH values within the BM of young mice might range from pH 6.4 to a pH of 7.8. It is to the best of our knowledge currently technically not feasible to combine HSC identification and thus localization and pH measurements directly in BM *in vivo*. Such experiments will need to await development of novel technologies in the near future.

2. In order to study the role of pH on HSC maintenance, the authors ex-vivo cultured the murine HSCs for 40 hours. 40 hours is too short for ex vivo culturing. In such a short time period, the stemness of HSCs are maintained usually well. The authors should do these experiments for at least 4-7 days.

A short-term, 40-hour culture period is primarily used for transduction protocols for HSCs, both in basic science as well as in clinical settings. Our data support that a short-term cultivation for 40 hours at a pH of 7.4 results in a significantly reduced stem cell potential (**Figure 1E,H**). Cultivation for 40 hours though will not be able to address the question to what extent changes in pH might result in a change/expansion of HSC potential ex vivo upon long-term culture. To address this question, we added a large set of novel data to the manuscript. In these experiments, HSCs were cultured for a total of 6 days before transplantation. Cultivation at both pH 6.9 or 7.4 for 6 days resulted in a reduction in stem cell potential compared uncultured cells. (**MS page 6 and 14 and Figure EV2E-G**). These data demonstrate the limitations on the effect of pH ex vivo on the potential of HSCs: pH 6.9 positively supports short-term culture of HSCs (murine as well as human, see **point 7 of this referee**), while there is no or even a slightly negative effect upon 6 days of culture. Additional screens will be necessary to define the optimal level of pH for long-term expansion protocols.

3. In this study, the experiments are performed using FBS containing IMDM medium. For the maintenance or expansion of HSCs, serum free mediums such as SFEM or Stemline medium are used. The authors need do at least some important experiments using commercially available serum free medium.

We included novel data from novel experiments in which we used StemSpanSFEM medium in our cultures systems (**MS page 11-12 and Figure 4L, Figure EV5C-F**) and present data on reconstitution of primary recipients as the central experiment to test for HSC potential. The data consistent with experiments in which we used fetal bovine serum (FBS) containing IMDM medium. We observe better reconstitution with HSC cultured at pH 6.9 or pH 7.4 and DFMO treatment in comparison to pH 7.4 condition in STEM medium for 2 days. Cultivation for 6 days at pH 6.9, pH 7.4 or pH 7.4 with DFMO treatment resulted also in serum free medium, similar to serum containing medium (**Figure EV2E-G)** in a reduced reconstitution potential (data not shown). These results support that pH 6.9 or DFMO treatment at pH 7.4 maintain potential of HSC upon short-term ex vivo culture also in serum-free medium used in the clinic.

4. Generally, mitochondrial metabolism is negatively correlated to the stemness of HSCs. Why pH6.9 stimulate OCR activity?

Indeed, HSCs at pH 6.9 show increased metabolic activity compared to HSCs at pH 7.4 (**Figure EV4B-E**). This is though similar to a previously reported effect of pH on the mitochondrial function in yeast (Hughes & Gottschling, 2012). Moreover, TEM analysis suggested stressed mitochondria and autophagy markers at a pH of 7.4 compared to 6.9, this further suggested a reduced frequency of autophagy vacuoles and more intact mitochondria. In aggregation, these data show “healthier” mitochondria at a pH 6.9, though with higher metabolism. Elevated levels of metabolism have indeed been associated with less HSC potential, but when determined in cells all exposed to pH 7.4 only. Our data might imply that the connection between stemness and mitochondria potential could be linked to the context the cell is in, and one additional variable in the context might be pH level (aka proton concentration), which is indeed linked to mitochondria function.

5. How extracellular pH or Proton regulates polyamine pathway? Does it activate a potential cell surface receptor and transduce extracellular signaling into intracellular matrix?

We provide data on likely mechanisms of the action of pH on the potential of HSCs, including the polyamine pathway, a change in metabolism and changes in the epigenetic make-up of HSCs. We are also aware that additional studies on how pH mechanistically affects the polyamine pathways will need to be addressed with a large set of novel approaches to unequivocally test the likely multiple causative molecular targets for the pH dependent functional rejuvenation of HSCs, but see this to be beyond the current scope of this manuscript.

6. The authors show that pH6.9 causes change of intracellular pH. If intracellular environment becomes acidic, the function of those proteins or molecules is still normal？The HSCs are still healthy in this state?

We indeed assume that a change in intracellular pH will affect protein function (structure, folding, pK values, see also **MS page 12, first paragraph of discussion**). We assume though that these changes are positive for HSCs function, as pH 6.9 results in a positive maintenance of HSCs ex vivo.

7. In order to prove the effect of pH6.9 on HSC maintenance, the authors need to check if it is

conserved in human HSC maintenance or ex vivo expansion using serum free medium, including polyamine pathway inhibitors.

Based on our positive experience with serum free medium in mouse, and based on the requirement of serum-free medium for any clinical translation of our findings, we evaluated the effect of pH 6.9 or DFMO at pH 7.4 on human HSC maintenance in StemSpanSFEM medium. We focused in these novel experiments on the gold standard to determine human HSCs potential, the xeno-transplant assay. Consistent with our data on murine HSCs, human HSC cultivated for 2 days at pH 6.9 or in the presence of DFMO supported a higher level of chimerism compared to HSCs cultivated at pH 7.4 (**MS page 11-12 and Figure 4L, Figure EV5D,F).**

Minor:
1. Fig2A, LT-HSCs expand 4 times in 40 hours?

A 4-fold expansion corresponds to only 2 cell-division. The data is based on multiple repeats of this assay. We have already published that indeed already individually cultivated LT-HSCs in single wells can undergo 2 divisions in 40 hours (PMID: 30235201), while we in general observe even faster kinetics among LT-HSCs cultured in bulk.

2. Fig2B,F, would be better to include untreated cell group.

We now include this novel data in **Figure 4K** and discuss it on **page 11 of the MS: "**DFMO treated pH 7.4 HSCs also resembled to a large extent pH 6.9 HSCs with respect to the low level of LAMP2 seen in pH 6.9 HSCs as well as in untreated HSCs **(Figure 4K, Figure 2F)"**

3. Fig3B, would be better to include untreated cell group.

While we agree with the reviewer that this seems initially the most obvious comparison, we did not generate this control data. Such a control will contain all non-activated HSCs that are not actively cycling. In a comparative analysis of activated/cycling vs. non-cycling/quiescent state, this difference will simply dominate any type of comparison (similarity in stem cell genes). It remains very difficult to almost impossible to adequately correct for that in comparative analyses. The non-activated/non-cycling expression profile might thus not really serve as a reference for "the standard HSC potential expression signature" also for activated, cycling HSCs (Hinge et al., 2020).

4. How is polyamine pathway in untreated HSCs?
We now include that data in **Figure 4C and Figure EV5B** and discuss it in the **MS on page 10**: "Interestingly, uncultivated HSCs showed only very low levels of Spermidine and Spermine. (**Figure 4C, Figure EV5B**). Upon cultivation of HSCs, the levels of both Spermidine and Spermine increased, but to a much lower level in pH 6.9 HSC compared to pH 7.4 HSCs (**Figure 4C**)".

5. FigS1F, CD45.2/CD45.1 F1 mice are not used, why CD45.2 and CD45.1 double positive cells are shown in the FACS plot?

This cell population actually appears frequently, but with a small number/frequency present in experiments in which chimerism is determined especially in blood. This is also reported in a large number of other laboratories, see for example also https://lists.purdue.edu/pipermail/cytometry/2007-October/033575.html or (Mercier et al., 2016), Figure 1 in that publication).
The frequency increases with the density of cells in the probe, or whether we stain directly in blood for speed and economic reasons. This population will not be further analyzed, and it is corrected for in chimerism calculations.

Referee #2:

Kumar et al. demonstrated that small changes in culture medium pH could support ex vivo hematopoietic stem cell maintenance. They used competitive transplantation analysis and discovered that HSCs cultured in pH 6.9 condition conferred a higher rate of chimerism, comparable to uncultured HSCs. They also found that lower extracellular pH could influence growth and metabolic processes, potentially increasing stemness. Although the idea of adjusting the pH of the culture medium to prolong HSC maintenance ex vivo is intriguing, the main concern is regarding cellular responses to the adjustment, particularly cell viability.

We thank the reviewer for his encouraging comments on the novelty of our data and approach.

Major concerns:
1. The authors observed a pH as lowest as 7.1 in bone marrow (Fig.1A). However, the pH in the culture condition used in the experiments is 6.9, which has a proton concentration approximately 1.5-fold higher than the measured physiological condition in the bone marrow. I doubt that HSCs would normally respond to this condition. It would be great if they could provide more information based on their RNAseq analysis to confirm that HSCs respond to the pH 6.9 culture condition similar to the pH 7.4 culture condition.

The glass combination probe used for the detection of pH in the bone marrow measures only the overall/average pH in a damaged BM environment. We provide additional novel data to further substantiate our claim of different levels of pH within the bone marrow. Multi-photon live microscopy was employed using an established cell impermeable pH sensitive ratiometric probe, 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) to further determine whether pH distribution within BM is uniform or heterogeneous (**Figure EV1C-G and MS page 4**) (Ray et al., 2012). Using selective landmarks as regions of interest (ROI) at the bone surface and within BM including cells with likely osteoblastic and osteoclastic nature or known blood vessels, the conclusion from this type of data is that the pH within bone marrow is not uniform and rather heterogeneous. pH values within the BM of young mice might range from pH 6.4 to a pH of 7.8. It is to the best of our knowledge currently technically not feasible to combine HSC identification and thus localization and pH measurements directly in BM *in vivo*. Such experiments will need to await development of novel technologies in the near future. Furthermore, experiments were performed at pH 6.4, 6.6, 6.9 to initially evaluate the reconstitution potential of HSC cultured within this lower pH range (**Figure EV1G**). In aggregation, a pH of 6.9 was determined to be best for maintenance of HSC function ex vivo. HSC.

2. Interestingly, the pH 6.9 culture condition improved HSC function in mice, which could be useful for future research into human HSC transplantation. HSCs cultured in pH 6.9 appear to have a lower expansion rate (Fig. 2A), which can be interpreted as they mentioned that the cells are maintaining their stemness. However, cell death also can cause less expansion. Therefore, I believe the authors should include experiments to demonstrate that decreasing pH has no effect on cell viability. The gene ontology analysis of gene sets at pH 6.9 versus pH 7.4 (Fig. 3E) revealed an up-regulated apoptotic process. Could the pH 6.9 culture condition cause apoptosis?

We performed additional experiments to determine the level of apoptosis in HSC cultivated at pH 6.9, pH 7.4 with DFMO or pH 7.4 in IMDM or STEMSpan SFEM medium (**Figure EV5E,F and MS page 11-12).** As the reviewer suspected, there is indeed an increase in the level of apoptosis upon cultivation at pH 6.9 and at 7.4 with DFMO compared to pH 7.4, which is consistent with the gene ontology analyses.

This elevated level of apoptosis at least for murine HSCs might indeed contribute to the reduced number of cells upon expansion of HSCs at pH 6.9/7.4 and DFMO, while at the same time there is elevated maintenance of HSCs potential at pH 6.9 or pH 7.4 and DFMO.

We summarized the data in the MS the following: “Murine pH 6.9 and especially 7.4+ DMSO HSCs, whether cultivated for 2 days with or without serum, showed elevated levels of apoptosis when compared to pH 7.4 HSCs, which might, besides the reduced number of cells in S-phase (Figure 2C) contribute to the lower number of cells seen under the pH 6.9/pH7.4+DFMO conditions (**Figure EV5E**), while there was no difference in the level of apoptosis among the different cultivation conditions for human HSPCs (**Figure EV5F**), they also show a clear difference in cell number upon pH 6.9 or pH 7.4+DFMO (**Figure EV5D**). The role of apoptosis for the reduced expansion of HSCs or human HSPCs at pH 6.9 or pH7.4+DFMO might thus likely be limited”.

3. The percentages of donor-derived cells in Fig. 4I-J have seemed to have heavily deviated. Secondary recipients' bone marrow in the DMFO+SPN group, for example, may contain 0 to 100 % donor derived cells. Indeed, we can see a trend of increasing percentages of donor derived cells in the DMFO treated group and statistical significance. I am still concerned about the data quality because we cannot see such a large deviation in Fig 1.

The differences are significantly different in both PB and BM of both primary and secondary recipients and thus come with a very high level of confidence. Data in **Figure 1** is simply based on quite a large set of experiments and animals transplanted (n = at least 18 mice per group, and for some data it is actually more than 20 mice). Within **Figure 4**, there is about half the number of recipients per group. Similar to Figure 1, data on the pH 6.9 and pH 7.4 with DFMO group in the primary recipients for example are still quite homogeneous, so the main level of heterogeneity, also to our surprise, has been actually seen within the untreated group. This is though not a general issue, as for example the deviation of the untreated group within the data presented in **Figure 1** is similar to the one of the treated groups.

4. Although the author mentioned deceased ROS production (Fig.2D) and autophagy (Fig.2E-H), as well as changes in metabolic profile (Fig. 4A-B) in HSCs cultured in pH 6.9 condition, the gene ontology analysis did not enrich any of these pathways from the RNAseq dataset (Fig. 3E-F). What is the reason for these discrepancies?

The GO analyses did enrich for metabolic processes, so the GO analysis does, in retrospect, contain at least in part pathways that are altered in HSCs cultivated pH 6.9 and pH 7.4. We also find genes directly linked to the polyamine pathway differentially expressed in 7.4 vs. 6.9 HSCs (**Figure EV5A**). GO terms test for changes in expression of genes listed with distinct groups, while ROS production and autophagy primarily are changes in function of an entity that might not always be directly linked to differences in expression of whole GO sets of genes. Our data might be a good example for the observation that distinct experimental approaches (gene expression, function, metabolomics) do have their limitations, and in our course differences in function of pathways seem to be more important/visible than changes in the expression of whole GO families. At the end, the GO classification and the gene expression analyses, while informative on the overall gene expression being more stem cell like, could not really provide enough in-depth information on functional changes in pathways that allow for further in depth mechanistic analyses.

5. Fig. 4D showed that DFMO treatment resulted in a decrease in intracellular pH. However, in HSCs cultured at pH 6.9, lower pH causes a decrease in spermidine (Fig.4B). These findings contradict each other in terms of cause and effect. If the authors believe it could be the other way around, they should

provide a more rational explanation. In addition, the DFMO concentration used in the experiment for Fig. 4D is in the 2-5mM range, but only one bar is shown for the DFMO-treated group. The authors must repeat the experiment with a single concentration or separate each concentration into its own bar.

We apologize for this confusion. We initially used a range of 1-10 mM of DMFO to investigate the effect on HSCs. Both 2mM and 5 mM showed a similar response, so the first round of in depth experiments were conducted with both concentrations. As similarity of the data remained, we only focused on 5 mM for additional repeats of the DFMO treated group and all other subsequent analyses of DFMO on HSCs, like on human HSCs. The quantitative data shown in the MS though has been always only the data generated with 5mM of DFMO. We now adjust this within the MS.

The reviewer is right that our data supports a reciprocal interaction of the polyamine pathway and level of pH. The expression of genes involved in polyamine biosynthesis (PAOX, SRM) were decreased, while the level of expression of genes involved in polyamine catabolic processes (SAT1, SMOX) was increased in pH 6.9 compared to pH 7.4 HSCs **(Figure EV5A).** Moreover, pH 6.9 HSC show low levels of Spermidine and Spermine compared to pH7.4 HSCs (**Figure 4B-C, FigureEV5B**) which in aggregation **implies that the level of pH regulates the polyamine pathway**. In addition, literature supports a role of the polyamine pathway in regulating intracellular pH, which is consistent with our finding of lower pH in DFMO treated cells. The reviewer is therefore also right that our data support a view in which lower pH is the cause of lower levels of Spermidine. We now added additional information to the MS to emphasize a likely reciprocal interaction of pH and the polyamine pathway (**end of page 14 of the MS**: “The detailed mechanisms on how a low intracellular pH and low polyamines affect HSC growth and metabolic conditions and their causal, maybe reciprocal interrelationship needs further investigations”.

6. There is a variation in sample size (n) within each experiment. The number of biological repeats in some experiments was not stated. In Fig. 1B, for example, the author stated "n = at least 14 mice per group and 4-5 experiments". The authors must explain why they included different sample sizes and specify the number for each experimental group.

The difference in sample size stems from variations in the number of HSCs that could be obtained from each sort, and the subsequent allocation of these HSCs to experimental groups. For example, the group untreated was performed with a lower sample size. We provide now in the revised version also more detailed information on the number of independent repeats and the statistical analyses in the manuscript (see all **Figure Legends and Material and Methods**)

Minor concerns:
1. Flow cytometry plots need to be revised for consistency and clarity.
- They should state the marker and fluorochrome used in the axis label (e.g. CD4-FITC) and include numbers along the axis scale of flow cytometry plots.

We revised the cytometry plots for consistency and clarity.

2. I do not think the flow cytometry plots and the bar graphs in Fig. 1I-J correspond well. For example, in pH 6.9 and 7.4 culture conditions, the LT-HSC population was around 10.2% and 5.8%, respectively, but the bar graph showed more than 10% of the LT-HSC population in both conditions.

We thank the referee for this recommendation. We now use other primary flow data just from different samples from the same experiment that better reflect the average numbers depicted in the bar diagrams (final data) in **Figure 1I,J**.

3. The length of each scale bar in ultra-structure TEM images of uncultured fresh HSC is missing. Furthermore, cell size appears to be inconsistent between 3D construction (Fig. 2B) and ultra-structure TEM images (Fig. 2H). In 3D construction images, we can see a significant difference in cell size between pH 6.9 and pH 7.4 culture conditions, but not in ultra-structure TEM images.

We have now included scale bar in ultra-structure TEM images of uncultured fresh HSC as well. In TEM, we have a 70nm thick 2D section without further 3 D information, and it is not known whether that is the plane with the highest diameter etc., while data in **Figure 2B** is indeed real 3D information on the size. Differences in size of pH 6.9 and pH 7.4 HSCs could be confirmed in the laboratory now by multiple approaches that are based on 3D information of the cell (see also **Figure 4K**).

4. The authors should double-check the consistency of graph labeling. For example, in Fig. 1, you used donor derived BM chimera, whereas, in Fig. 4, you used donor derived cells.

We checked the consistency of graph labeling and corrected them where appropriate.

Referee #3:

Kumar et al present a cohesive manuscript that evaluates the effect of pH on HSC potential. They establish that reducing the pH to 6.9 for ex vivo culturing helps to maintain more stem-cell potential. This reduced pH lowers metabolic stress and was associated with many features suggestive of maintained functional HSC potential and the authors further establish the metabolite changes that may contribute to the maintained potential and showed drug targeting of the polyamine pathways was able to recapitulate the ex vivo culture benefits in lower pH. The manuscript is well written (but requires minor editing) and presents a novel mechanism that could lead to maintained HSC potential during ex vivo culturing / expansion.

We thank the reviewer for these encouraging statements on novelty of and mechanism within our data.

Minor points:
1) While these data are interesting regardless of species, it would be relevant to include "mouse" or "murine" in the title. There is a major difference in HSC competence ex vivo between the species and it is important to state to avoid misleading others.

Based on our positive experience with serum free medium in mouse, and based on the requirement of serum-free medium for any clinical translation of our findings, we evaluated the effect of pH 6.9 or DFMO at pH 7.4 on human HSC maintenance in StemSpanSFEM medium. We focused in these novel experiments on the gold standard to determine human HSCs potential, the xeno-transplant assay. Consistent with our data on murine HSCs, human HSC cultivated for 2 days at pH 6.9 or in the presence of DFMO supported a higher level of chimerism compared to HSCs cultivated at pH 7.4 (**MS page 11-12 and Figure 4L, Figure EV5D,F).**

2) The authors chose an ex vivo incubation time of 40 hrs. This time frame is small and does not really allow for a robust expansion of HSCs: Why whas this time point chosen? Would longer culture periods lead to larger expansion of the more potent HSCs, or is this the optimal improvement / expansion of these HSCs?

A short-term, 40-hour culture period is primarily used for transduction protocols for HSCs, both in basic

science as well as in clinical settings. Our data support that a short-term cultivation for 40 hours at a pH of 7.4 results in a significantly reduced stem cell potential (**Figure 1E,H**). Cultivation for 40 hours though will not be able to address the question to what extent changes in pH might result in a change/expansion of HSC potential ex vivo upon long-term culture. To address this question, we added a large set of novel data to the manuscript. In these experiments, HSCs were cultured for a total of 6 days before transplantation. Cultivation at both pH 6.9 or 7.4 for 6 days resulted in a reduction in stem cell potential compared uncultured cells. (**MS page 6 and 14 and Figure EV2E-G**). These data demonstrate the limitations on the effect of pH ex vivo on the potential of HSCs: pH 6.9 positively supports short-term culture of HSCs (murine as well as human, see **point 7 of this referee**), while there is no or even a slightly negative effect upon 6 days of culture. Additional screens will be necessary to define the optimal level of pH for long-term expansion protocols.

3) The PCA analysis would be strengthened by including the freshly isolated HSCs to the plot and demonstrating the 6.9 pH cultured HSCs had more similar profiles to the freshly isolated ones (similar to their improved functional potential).

While we agree with the reviewer that this seems initially the most obvious comparison, we did not generate this control data. Such a control will contain all non-activated HSCs that are not actively cycling. In a comparative analysis of activated/cycling vs. non-cycling/quiescent state, this difference will simply dominate any type of comparison (similarity in stem cell genes). It remains very difficult to almost impossible to adequately correct for that in comparative analyses. The non-activated/non-cycling expression profile might thus not really serve as a reference for “the standard HSC potential expression signature” also for activated, cycling HSCs (for example (Hinge et al., 2020)).

4) Clarification of secondary transplant protocols is needed for the interpretations.

We provide more information on the secondary transplant protocol (**Material and Methods, page 18**): “ Primary transplanted mice were sacrificed after 8 to 20 weeks and BM chimerism was determined by FACS analysis. For secondary transplants, $3x10^6$ BM cells from an individual primary recipient mouse were injected into an individual secondary, lethally irradiated recipient BoyJ mouse”.

5) There needs to be clarification also of the statement on Page 6, first paragraph, last sentence: "These data indicate that HPC respond distinctly, in comparison to the response of HSCs". There were no HSCs in the experiment, so how is there an indication that this is distinct? Further, as this is mentioned that they respond distinctly, it is odd that LSK cells are used to examine the metabolic changes (though it is appreciated the numbers of cells required for Seahorse Assays is difficult to acquire for HSCs).

The reviewer is correct that it is not valid to imply a difference of the behavior of HPCs in comparison to HSCs, as we did actually not analyze all of these phenotypes also directly in HSCs. We now state in the **MS page 6, paragraph 2**: “The data support that changes in extracellular pH also affect the function of HPCs. The full extent to which changes in pH will affect HPCs function will need to be further investigated.”

6) The EM images presented to display more intact mitochondria are difficult to evaluate.

We now provide enlarged EM images in **Figure EV4F** to better inform the reader on the intact mitochondria in our EM image.

7) A seemingly small semantics question, but and important disctiction needs to be clarified in the

evaluation of the DEGs between the 6.9 and 7.4 pH culture, the authors state that correlations showed..."ph 6.9 compared to 7.4 was highly correlated with multiple gene signatures." Do the authors mean enriched for expression of these pathways or was a correlation analysis performed? It is an important distinction.

We agree with the reviewer on the importance of this distinction. We meant “enriched for expression of these pathways”, and now state this in the manuscript **(MS page 9)**.

8) In the materials and methods section, culture conditions: Please expand this section to include all details needed to repeat the experiment. What are the exact cytokines used (company and cat#)?. What types of plates were used how many wells, round or flat bottom, TC treated or not)? This paper expands our understanding of HSC ex vivo cultures and these kind of details are very important to others in the field.

We now provide all details needed to repeat the experiment in Material and Methods **(MS page 16-17)** as well as more detailed reagent information in the reagent list provided as **Appendix.**

References:

Hinge A, He J, Bartram J, Javier J, Xu J, Fjellman E, Sesaki H, Li T, Yu J, Wunderlich M, Mulloy J, Kofron M, Salomonis N, Grimes HL, Filippi MD (2020) Asymmetrically Segregated Mitochondria Provide Cellular Memory of Hematopoietic Stem Cell Replicative History and Drive HSC Attrition. Cell Stem Cell 26: 420-430 e6

Hughes AL, Gottschling DE (2012) An early age increase in vacuolar pH limits mitochondrial function and lifespan in yeast. Nature 492: 261-5

Mercier FE, Sykes DB, Scadden DT (2016) Single Targeted Exon Mutation Creates a True Congenic Mouse for Competitive Hematopoietic Stem Cell Transplantation: The C57BL/6-CD45.1(STEM) Mouse. Stem Cell Reports 6: 985-992

Ray A, Lee YE, Kim G, Kopelman R (2012) Two-photon fluorescence imaging super-enhanced by multishell nanophotonic particles, with application to subcellular pH. Small 8: 2213-21

Dear Prof. Geiger,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from two of the three referees that I asked to re-evaluate your study, you will find below. As you will see, the referees now fully support the publication of your study in EMBO reports. Referee #2 was completely unresponsive to the invitation to re-assess the study. However, going through your point-by-point-response, I consider her/his points as adequately addressed.

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The datasets produced in this study are available in the following databases:

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- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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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.

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Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The authors have addressed my concerns appropriately in the revised version.

Referee #3:

The authors have added significant experiments that further support the pH of ex-vivo culture media affects both murine and human HSCs and adequately addressed the initial concerns raised.

Point-to-point response to the editorial suggestions/comments.

Before I will proceed with formal acceptance, I have these editorial requests I also ask you to address:

We thank the reviewer for her/his encouraging comments that helped us to improve the manuscript substantially. We are very glad with the outcome that this finding will be part of EMBO reports. Here we have considered editorial request in point wise manner, see below.

- Please provide the abstract with not more than 175 words and written in present tense throughout.

Abstract: less than 176 words in present tense.

- Please remove the summary and the information on word count and figures from the title page.

Removed.

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- We plan to publish your manuscript in the Report format, as there are not more than 5 main figures. For a Scientific Report we require that results and discussion sections are combined in a single chapter called "Results & Discussion". Please do this for your manuscript. For more details please refer to our guide to authors: http://www.embopress.org/page/journal/14693178/authorguide#researcharticleguide

Results & Discussion is merged to match the Report format.

- Please complete the 'Data Availability' section (DAS) of the manuscript. Please note that the Data Availability Section is restricted to new primary data that are part of this study. Thus, please remove the statement 'All data reported in this paper will be shared by the lead contact upon request' from this section, but provide the accession numbers and database that follows the model below:

Removed.

Data availability

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Gene Expression Omnibus GSE225267 is updated in the MS file.

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We removed the author contribution section from the MS. CRediT terminology is now used to specify the contributions of each author in the journal submission system.

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- Please provide one 'material and methods' section including what is presently listed separately as 'Additional material and methods for Expanded view figures'.

Additional material and methods for Expanded View figures is now merged with the 'material and methods' section.

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Movie names are updated and legends are removed from MS text file.

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- a schematic summary figure that provides a sketch of the major findings (not a data image) 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.

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Prof. Hartmut Geiger
University of Ulm
Institute of Molecular Medicine
Meyerhofstrasse
Ulm 89081
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EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January 2022)
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Material and Methods and Reagents and Tools Table

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Material and Methods and Reagents and Tools Table

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Material and Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Material and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Material and Methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgements

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Yes	Material and Methods

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Material and Methds, Figure Legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	There was no full blinding of samples to the person performing the experiments.
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	see Material and Methods and Figure Legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure Legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure Legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Material and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Submission is in progress, Data Availability Section.
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Manuscript