

Peer Review Information

Journal: Nature Cell Biology

Manuscript Title: Unchecked oxidative stress in skeletal muscle prevents outgrowth of disseminated tumour cells

Corresponding author name(s): Professor Cyrus M. Ghajar

Reviewer Comments & Decisions:

Decision Letter, initial version:
--

Subject: Decision on Nature Cell Biology submission NCB-G45082

Message:

*Please delete the link to your author homepage if you wish to forward this email to co-authors.

Dear Cyrus,

Please first accept the delay getting back to you with a decision due to difficulties in retrieving reviewer comments.

Your manuscript, "Unchecked oxidative stress is an insurmountable barrier for tumour cells that disseminate to skeletal muscle", has now been seen by 3 referees, who are experts in skeletal muscle biology (referee 1); metabolism in metastasis (referee 2); and metabolism (referee 3). As you will see from their comments (attached below) they find this work of potential interest, but have raised substantial concerns, which in our view would need to be addressed with considerable revisions before we can consider publication in Nature Cell Biology.

Nature Cell Biology editors discuss the referee reports in detail within the editorial team, including the chief editor, to identify key referee points that should be addressed with priority, and requests that are overruled as being beyond the scope of the current study. To guide the scope of the revisions, I have listed these points below. We are committed to providing a fair and constructive peer-review process, so please feel free to contact me if you would like to discuss any of the referee comments further.

In particular, it would be essential to:

A) Address the potential caveat of not using spontaneous models of metastasis as questioned by Reviewer 2:

"A major limitation of the study is that it does not include spontaneous models of metastasis. This is a limitation because as shown previously, the circulation is a highly oxidative environment. Thus, cancer cells that survive the circulation may already be selected to have a high ROS defense and thus may not be limited by the oxidative environment of skeletal muscle but some other particularities of this environment. The authors should exclude this possibility."

B) Strengthen the evidence of spontaneous dissemination of breast cancer to the skeletal muscle as questioned by reviewers:

Reviewer 2

"Similarly, what is the evidence that breast cancer cells spontaneously travel to the skeletal muscle?"

Reviewer 3

"The authors are able to detect DTCs in the skeletal muscle using qPCR-based amplification. Can the authors validate this finding using an alternative approach, such a qPCR of GFP over-expression in the tumor cells? Do the authors observe DTCs or increased oxidative stress in DTCs in the heart?"

C) Address the concern with the catalase overexpression data as asked by Reviewer 3:

"The authors find that catalase overexpression promotes tumor outgrowth and even colonization in a skeletal muscle environment. Does overexpression of catalase in the skeletal muscle versus lung cells have a similar effect?"

D) Provide further mechanistic insight as requested by Reviewer 3:

"A large gap in the study is the lack of mechanistic understanding in the source of ROS generated in DTCs in the skeletal muscle versus lung environment under physiological conditions. Is this due to fundamental differences in mitochondrial generation of ROS? This information is important to understand as it would provide additional nodes of regulation to examine in vivo. Further insight into this area would help to clarify the differences between permissive and non-permissive tissues."

E) All other referee concerns pertaining to strengthening existing data, providing controls, methodological details, clarifications and textual changes as appropriate should also be addressed.

F) Finally please pay close attention to our guidelines on statistical and methodological reporting (listed below) as failure to do so may delay the reconsideration of the revised manuscript. In particular please provide:

- a Supplementary Figure including unprocessed images of all gels/blots in the form of a multi-page pdf file. Please ensure that blots/gels are labeled and the sections presented in the figures are clearly indicated.

- a Supplementary Table including all numerical source data in Excel format, with data for different figures provided as different sheets within a single Excel file. The file should include source data giving rise to graphical representations and statistical descriptions in the paper and for all instances where the figures present representative experiments of multiple independent repeats, the source data of all repeats should be provided.

We would be happy to consider a revised manuscript that would satisfactorily address these points, unless a similar paper is published elsewhere, or is accepted for publication in Nature Cell Biology in the meantime.

When revising the manuscript please:

- ensure that it conforms to our format instructions and publication policies (see below and www.nature.com/nature/authors/).

- provide a point-by-point rebuttal to the full referee reports verbatim, as provided at the end of this letter.

- provide the completed Editorial Policy Checklist (found here <https://www.nature.com/authors/policies/Policy.pdf>), and Reporting Summary (found here <https://www.nature.com/authors/policies/ReportingSummary.pdf>). This is essential for reconsideration of the manuscript and these documents will be available to editors and referees in the event of peer review. For more information see <http://www.nature.com/authors/policies/availability.html> or contact me.

Nature Cell Biology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as ‘corresponding author’ on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from

the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit www.springernature.com/orcid.

Please submit the revised manuscript files and the point-by-point rebuttal to the referee comments using this link:

[REDACTED]

*This url links to your confidential home page and associated information about manuscripts you may have submitted or be reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We would like to receive a revised submission within six months. We would be happy to consider a revision even after this timeframe, however if the resubmission deadline is missed and the paper is eventually published, the submission date will be the date when the revised manuscript was received.

We hope that you will find our referees' comments, and editorial guidance helpful. Please do not hesitate to contact me if there is anything you would like to discuss.

Best wishes,
Zhe Wang

Zhe Wang, PhD
Senior Editor
Nature Cell Biology

Tel: +44 (0) 207 843 4924
email: zhe.wang@nature.com

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

The authors of this manuscript report that skeletal muscle exerts an antimetastatic effect on mammary cancer cells. Through elegant experiments, they identify increased glutathione (GSH) metabolism in skeletal muscle metastasis as being associated with reduced ability of metastatic cells to colonize skeletal muscle. The redox-sensitive biosensor Grx-roGFP2 revealed that the MDA-MB-231 mammary

cancer cells experienced oxidative stress when cultured on human skeletal muscle myotubes (SkMc) compared to when cultured on human lung fibroblasts (LFs). These findings were recapitulated when MDA-MB-231 Grx1-roGFP2 cells were injected into recipient mice. ROS production was further documented by a cell-permeable fluorescent dye. In addition to MDA-MB-231 cells, SkMc themselves exhibited elevated ROS levels. To induce oxidative stress, the authors employed the chemogenic HyPer3-DAAO that, in the presence of exogenous D-alanine, generates hydrogen peroxide (H_2O_2). Growth of MDA231-DAAO cells was suppressed in the LF niche in the presence of alanine. Increasing the antioxidant potential of tumor cells by expressing the peroxisomal enzyme catalase (mitochondrial mCAT or cytosol cCAT) that converts H_2O_2 to H_2O and O_2 favored outgrowth of MDA-MB-231 cells and of another cancer cell line, E0771 on SkMc. MDA231-mCAT and MDA231-cCAT cells, directly injected into the TA muscle of NOD-SCID mice generated tumors. However, E0711-mCAT cells were unable to colonize the lung.

This is a very interesting and well-conducted study revealing that oxidative stress suppresses tumor cell engraftment in skeletal muscle. It further documents the existence of a metabolic vulnerability that could be exploited to reduce dissemination of tumor cells. There are some points that need clarification.

COMMENTS:

1. Tumor implant rather than i.c. injection may better recapitulate the events occurring during the metastatic process.
2. Tumor cells identified within muscle were non proliferating (Ki67-). Were they Ki67+ in other organs?
3. It would be important to evaluate GSH metabolism, including GSH:GSSG ratio, in metastatic (4T1) and non-metastatic cell lines before i.c. injection and after isolating the injected cells from metastatic lesions.
4. Figure 3. It is interesting to note that the GSH metabolite sets behave in a specular manner in metastasis SkM/healthy SkM and metastasis lung/healthy lung. How do metabolites compare in healthy SkM and healthy lung?
5. How did the growth of 4T1 cells compare on SkM and LF cultures?
6. HyPer3-DAAO expression in alanine-treated LFs may decrease MDA231 metastases. Has this been evaluated?
7. Figure 4. How was extracellular H_2O_2 production in SkMc and LF niches without MDA-MB-231 co-culture?

8. How comparable were tumors (in growth and size) generated by MDA-MB-231mCAT/cCAT cells to those generated by 4T1 cells?

Reviewer #2:

Remarks to the Author:

Crist and colleagues addressed the question of whether the tissue environment limits the outgrowth of disseminated breast cancer cells thus contributing to organotropism. They relied on intracardiac injections and measured the number of disseminated breast cancer cells in different tissues. They find in multiple tissues including skeletal muscle disseminated breast cancer cells. Yet, in skeletal muscle the disseminated breast cancer cells were barely growing into metastasis. Based on metabolomics analysis and breast cancer cells evolved to grow metastases in skeletal muscle they find that a highly oxidative environment of the muscle prevented metastasis outgrowth. Overexpression of catalase increased the capacity of breast cancer cells to outgrow in skeletal muscle. In principle the study addressed an important and very interesting question.

The following points should be considered:

- 1) The manuscript contains many generalizing statements such as "...revealing that breast cancer cells can and do traffic to skeletal muscle." This and other generalized statements should be rephrased so that they include the model systems and cell lines for which the conclusions can be drawn.
- 2) A major limitation of the study is that it does not include spontaneous models of metastasis. This is a limitation because as shown previously, the circulation is a highly oxidative environment. Thus, cancer cells that survive the circulation may already be selected to have a high ROS defense and thus may not be limited by the oxidative environment of skeletal muscle but some other particularities of this environment. The authors should exclude this possibility.
- 3) Similarly, what is the evidence that breast cancer cells spontaneously travel to the skeletal muscle?

Reviewer #3:

Remarks to the Author:

The manuscript "Unchecked Oxidative Stress is an insurmountable barrier for tumor cells that disseminate to skeletal muscle investigates the difference in the colonization of tumors to metastatic versus non-metastatic sites, with a focus on skeletal muscle as a non-metastatic tissue. The authors employ an innovative and high throughput approach for detecting low levels of disseminated tumor cells (DTCs) involving qPCR-based amplification of AluYB8 elements, which are only found in primates. Using this approach, the authors find that DTCs exist and survive in skeletal muscle, but do not colonize and proliferate due to an inhospitable oxidative environment. These initial observations of examining

the interaction between tumor and host tissue are innovative and timely, however, several points should be addressed to allow for full interpretation of the data.

1. The authors are able to detect DTCs in the skeletal muscle using qPCR-based amplification. Can the authors validate this finding using an alternative approach, such as a qPCR of GFP over-expression in the tumor cells? Do the authors observe DTCs or increased oxidative stress in DTCs in the heart?
2. In Figure 2, the authors observe that live myotubes suppress tumor cell outgrowth. Does the oxygen level affect this phenotype?
3. By several measures, including GSH:GSSH and the use of a HyPer3-DAAO sensitive chemogenetic reporter, DTCs in the skeletal muscle are highly oxidized due to the H₂O₂ levels in the environment and endogenous ROS. Using a culture set up the authors demonstrate that skeletal muscle is a more oxidizing environment compared with lung. However, these conditions do not accurately reflect the vast difference in oxygen in these organs in vivo. How do these levels compare in physiological oxygen levels, which one would expect the lung to be even more oxygenated compared with the skeletal muscle?
4. The authors find that catalase overexpression promotes tumor outgrowth and even colonization in a skeletal muscle environment. Does overexpression of catalase in the skeletal muscle versus lung cells have a similar effect?
5. A large gap in the study is the lack of mechanistic understanding in the source of ROS generated in DTCs in the skeletal muscle versus lung environment under physiological conditions. Is this due to fundamental differences in mitochondrial generation of ROS? This information is important to understand as it would provide additional nodes of regulation to examine in vivo. Further insight into this area would help to clarify the differences between permissive and non-permissive tissues.
6. How is the difference in the redox environment molecularly transmitted to the tumor?

GUIDELINES FOR MANUSCRIPT SUBMISSION TO NATURE CELL BIOLOGY

READABILITY OF MANUSCRIPTS – Nature Cell Biology is read by cell biologists from diverse backgrounds, many of whom are not native English speakers. Authors should aim to communicate their findings clearly, explaining technical jargon that might be unfamiliar to non-specialists, and avoiding non-standard abbreviations. Titles and abstracts should concisely communicate the main findings of the study, and the background, rationale, results and conclusions should be clearly explained in the

manuscript in a manner accessible to a broad cell biology audience. Nature Cell Biology uses British spelling.

MANUSCRIPT FORMAT – please follow the guidelines listed in our Guide to Authors regarding manuscript formats at Nature Cell Biology.

TITLE – should be no more than 100 characters including spaces, without punctuation and avoiding technical terms, abbreviations, and active verbs..

AUTHOR NAMES – should be given in full.

AUTHOR AFFILIATIONS – should be denoted with numerical superscripts (not symbols) preceding the names. Full addresses should be included, with US states in full and providing zip/post codes. The corresponding author is denoted by: "Correspondence should be addressed to [initials]."

ABSTRACT AND MAIN TEXT – please follow the guidelines that are specific to the format of your manuscript, as listed in our Guide to Authors (http://www.nature.com/ncb/pdf/ncb_gta.pdf) Briefly, Nature Cell Biology Articles, Resources and Technical Reports have 3500 words, including a 150 word abstract, and the main text is subdivided in Introduction, Results, and Discussion sections. Nature Cell Biology Letters have up to 2500 words, including a 180 word introductory paragraph (abstract), and the text is not subdivided in sections.

ACKNOWLEDGEMENTS – should be kept brief. Professional titles and affiliations are unnecessary. Grant numbers can be listed.

AUTHOR CONTRIBUTIONS – must be included after the Acknowledgements, detailing the contributions of each author to the paper (e.g. experimental work, project planning, data analysis etc.). Each author should be listed by his/her initials.

FINANCIAL AND NON-FINANCIAL COMPETING INTERESTS – the authors must include one of three declarations: (1) that they have no financial and non-financial competing interests; (2) that they have financial and non-financial competing interests; or (3) that they decline to respond, after the Author Contributions section. This statement will be published with the article, and in cases where financial and non-financial competing interests are declared, these will be itemized in a web supplement to the article. For further details please see <https://www.nature.com/licenceforms/nrg/competing-interests.pdf>.

REFERENCES – are limited to a total of 70 for Articles, Resources, Technical Reports; and 40 for Letters. This includes references in the main text and Methods combined. References must be numbered sequentially as they appear in the main text, tables and figure legends and Methods and must follow the precise style of Nature Cell Biology references. References only cited in the Methods should be numbered consecutively following the last reference cited in the main text. References only associated with Supplementary Information (e.g. in supplementary legends) do not count toward the total reference limit and do not need to be cited in numerical continuity with references in the main text. Only published papers can be cited, and each publication cited should be included in the numbered reference list, which should include the manuscript titles. Footnotes are not permitted.

METHODS – Nature Cell Biology publishes methods online. The methods section should be provided as a separate Word document, which will be copyedited and appended to the manuscript PDF, and incorporated within the HTML format of the paper.

Methods should be written concisely, but should contain all elements necessary to allow interpretation and replication of the results. As a guideline, Methods sections typically do not exceed 3,000 words. The Methods should be divided into subsections listing reagents and techniques. When citing previous methods, accurate references should be provided and any alterations should be noted. Information must be provided about: antibody dilutions, company names, catalogue numbers and clone numbers for monoclonal antibodies; sequences of RNAi and cDNA probes/primers or company names and catalogue numbers if reagents are commercial; cell line names, sources and information on cell line identity and authentication. Animal studies and experiments involving human subjects must be reported in detail, identifying the committees approving the protocols. For studies involving human subjects/samples, a statement must be included confirming that informed consent was obtained. Statistical analyses and information on the reproducibility of experimental results should be provided in a section titled “Statistics and Reproducibility”.

All Nature Cell Biology manuscripts submitted on or after March 21 2016 must include a Data availability statement at the end of the Methods section. For Springer Nature policies on data availability see <http://www.nature.com/authors/policies/availability.html>; for more information on this particular policy see <http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf>. The Data availability statement should include:

- Accession codes for primary datasets (generated during the study under consideration and designated as "primary accessions") and secondary datasets (published datasets reanalysed during the study under consideration, designated as "referenced accessions"). For primary accessions data should be made public to coincide with publication of the manuscript. A list of data types for which submission to community-endorsed public repositories is mandated (including sequence, structure, microarray, deep sequencing data) can be found here <http://www.nature.com/authors/policies/availability.html#data>.

- Unique identifiers (accession codes, DOIs or other unique persistent identifier) and hyperlinks for datasets deposited in an approved repository, but for which data deposition is not mandated (see here for details <http://www.nature.com/sdata/data-policies/repositories>).
- At a minimum, please include a statement confirming that all relevant data are available from the authors, and/or are included with the manuscript (e.g. as source data or supplementary information), listing which data are included (e.g. by figure panels and data types) and mentioning any restrictions on availability.
- If a dataset has a Digital Object Identifier (DOI) as its unique identifier, we strongly encourage including this in the Reference list and citing the dataset in the Methods.

We recommend that you upload the step-by-step protocols used in this manuscript to the Protocol Exchange. More details can found at www.nature.com/protocolexchange/about.

DISPLAY ITEMS – main display items are limited to 6-8 main figures and/or main tables for Articles, Resources, Technical Reports; and 5 main figures and/or main tables for Letters. For Supplementary Information see below.

FIGURES – Colour figure publication costs \$600 for the first, and \$300 for each subsequent colour figure. All panels of a multi-panel figure must be logically connected and arranged as they would appear in the final version. Unnecessary figures and figure panels should be avoided (e.g. data presented in small tables could be stated briefly in the text instead).

All imaging data should be accompanied by scale bars, which should be defined in the legend. Cropped images of gels/blots are acceptable, but need to be accompanied by size markers, and to retain visible background signal within the linear range (i.e. should not be saturated). The boundaries of panels with low background have to be demarked with black lines. Splicing of panels should only be considered if unavoidable, and must be clearly marked on the figure, and noted in the legend with a statement on whether the samples were obtained and processed simultaneously. Quantitative comparisons between samples on different gels/blots are discouraged; if this is unavoidable, it should only be performed for samples derived from the same experiment with gels/blots were processed in parallel, which needs to be stated in the legend.

Figures should be provided at approximately the size that they are to be printed at (single column is 86 mm, double column is 170 mm) and should not exceed an A4 page (8.5 x 11"). Reduction to the scale that will be used on the page is not necessary, but multi-panel figures should be sized so that the whole

figure can be reduced by the same amount at the smallest size at which essential details in each panel are visible. In the interest of our colour-blind readers we ask that you avoid using red and green for contrast in figures. Replacing red with magenta and green with turquoise are two possible colour-safe alternatives. Lines with widths of less than 1 point should be avoided. Sans serif typefaces, such as Helvetica (preferred) or Arial should be used. All text that forms part of a figure should be rewritable and removable.

We accept files from the following graphics packages in either PC or Macintosh format:

- For line art, graphs, charts and schematics we prefer Adobe Illustrator (.AI), Encapsulated PostScript (.EPS) or Portable Document Format (.PDF). Files should be saved or exported as such directly from the application in which they were made, to allow us to restyle them according to our journal house style.
- We accept PowerPoint (.PPT) files if they are fully editable. However, please refrain from adding PowerPoint graphical effects to objects, as this results in them outputting poor quality raster art. Text used for PowerPoint figures should be Helvetica (preferred) or Arial.
- We do not recommend using Adobe Photoshop for designing figures, but we can accept Photoshop generated (.PSD or .TIFF) files only if each element included in the figure (text, labels, pictures, graphs, arrows and scale bars) are on separate layers. All text should be editable in 'type layers' and line-art such as graphs and other simple schematics should be preserved and embedded within 'vector smart objects' - not flattened raster/bitmap graphics.
- Some programs can generate Postscript by 'printing to file' (found in the Print dialogue). If using an application not listed above, save the file in PostScript format or email our Art Editor, Allen Beattie for advice (a.beattie@nature.com).

Regardless of format, all figures must be vector graphic compatible files, not supplied in a flattened raster/bitmap graphics format, but should be fully editable, allowing us to highlight/copy/paste all text and move individual parts of the figures (i.e. arrows, lines, x and y axes, graphs, tick marks, scale bars etc.). The only parts of the figure that should be in pixel raster/bitmap format are photographic images or 3D rendered graphics/complex technical illustrations.

All placed images (i.e. a photo incorporated into a figure) should be on a separate layer and independent from any superimposed scale bars or text. Individual photographic images must be a minimum of 300+ DPI (at actual size) or kept constant from the original picture acquisition and not decreased in resolution post image acquisition. All colour artwork should be RGB format.

FIGURE LEGENDS – must not exceed 350 words for each figure to allow fit on a single printed NCB page together with the figure. They must include a brief title for the whole figure, and short descriptions of each panel with definitions of the symbols used, but without detailing methodology.

TABLES – main tables should be provided as individual Word files, together with a brief title and legend. For supplementary tables see below.

SUPPLEMENTARY INFORMATION – Supplementary information is material directly relevant to the conclusion of a paper, but which cannot be included in the printed version in order to keep the manuscript concise and accessible to the general reader. Supplementary information is an integral part of a Nature Cell Biology publication and should be prepared and presented with as much care as the main display item, but it must not include non-essential data or text, which may be removed at the editor's discretion. All supplementary material is fully peer-reviewed and published online as part of the HTML version of the manuscript. Supplementary Figures and Supplementary Notes are appended at the end of the main PDF of the published manuscript.

Supplementary items should relate to a main text figure, wherever possible, and should be mentioned sequentially in the main manuscript, designated as Supplementary Figure, Table, Video, or Note, and numbered continuously (e.g. Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2 etc.).

Unprocessed scans of all key data generated through electrophoretic separation techniques need to be presented in a supplementary figure that should be labelled and numbered as the final supplementary figure, and should be mentioned in every relevant figure legend. This figure does not count towards the total number of figures and is the only figure that can be displayed over multiple pages, but should be provided as a single file, in PDF or TIFF format. Data in this figure can be displayed in a relatively informal style, but size markers and the figures panels corresponding to the presented data must be indicated.

The total number of Supplementary Figures (not including the “unprocessed scans” Supplementary Figure) should not exceed the number of main display items (figures and/or tables (see our Guide to Authors and March 2012 editorial <http://www.nature.com/ncb/authors/submit/index.html#suppinfo>; <http://www.nature.com/ncb/journal/v14/n3/index.html#ed>). No restrictions apply to Supplementary Tables or Videos, but we advise authors to be selective in including supplemental data.

Each Supplementary Figure should be provided as a single page and as an individual file in one of our accepted figure formats and should be presented according to our figure guidelines (see above). Supplementary Tables should be provided as individual Excel files. Supplementary Videos should be

provided as .avi or .mov files up to 50 MB in size. Supplementary Figures, Tables and Videos must be accompanied by a separate Word document including titles and legends.

GUIDELINES FOR EXPERIMENTAL AND STATISTICAL REPORTING

REPORTING REQUIREMENTS – To improve the quality of methods and statistics reporting in our papers we have recently revised the reporting checklist we introduced in 2013. We are now asking all life sciences authors to complete two items: an Editorial Policy Checklist (found here <https://www.nature.com/authors/policies/Policy.pdf>) that verifies compliance with all required editorial policies and a reporting summary (found here <https://www.nature.com/authors/policies/ReportingSummary.pdf>) that collects information on experimental design and reagents. These documents are available to referees to aid the evaluation of the manuscript. Please note that these forms are dynamic ‘smart pdfs’ and must therefore be downloaded and completed in Adobe Reader. We will then flatten them for ease of use by the reviewers. If you would like to reference the guidance text as you complete the template, please access these flattened versions at <http://www.nature.com/authors/policies/availability.html>.

STATISTICS – Wherever statistics have been derived the legend needs to provide the n number (i.e. the sample size used to derive statistics) as a precise value (not a range), and define what this value represents. Error bars need to be defined in the legends (e.g. SD, SEM) together with a measure of centre (e.g. mean, median). Box plots need to be defined in terms of minima, maxima, centre, and percentiles. Ranges are more appropriate than standard errors for small data sets. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test used needs to be stated in the legend. Statistics such as error bars must not be derived from $n < 3$. For sample sizes of $n < 5$ please plot the individual data points rather than providing bar graphs. Deriving statistics from technical replicate samples, rather than biological replicates is strongly discouraged. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test stated in the legend.

Information on how many times each experiment was repeated independently with similar results needs to be provided in the legends and/or Methods for all experiments, and in particular wherever representative experiments are shown.

We strongly recommend the presentation of source data for graphical and statistical analyses as a separate Supplementary Table, and request that source data for all independent repeats are provided when representative experiments of multiple independent repeats, or averages of two independent experiments are presented. This supplementary table should be in Excel format, with data for different

figures provided as different sheets within a single Excel file. It should be labelled and numbered as one of the supplementary tables, titled “Statistics Source Data”, and mentioned in all relevant figure legends.

----- Please don't hesitate to contact NCB@nature.com should you have queries about any of the above requirements -----

Author Rebuttal to Initial comments
--

Overall response to our reviewers: Thank you for recognizing that our study addresses a novel and interesting question in a technically rigorous and well-presented fashion. Your careful reviews raised key questions that we have addressed over the past six months through thorough experimentation, resulting in **42 new figure panels**. These questions touched on the following major themes:

(1) *What evidence is there that breast tumor cells disseminate spontaneously to skeletal muscle?* We thought the most thorough way to address this question was in humans and in mice. So we first made an amendment to a rapid autopsy protocol to allow for collection of skeletal muscles from metastatic breast cancer patients who succumb to their disease. Two patients had estrogen receptor (ER) and progesterone receptor (PR) positive disease, allowing us to use these markers in addition to pan-cytokeratin to stain for breast tumor cells in muscle. We first confirmed that this cocktail stained primary breast tumors, and also that marker positive cells were absent in the muscles from individuals with no history of cancer. Then, we analyzed 200 μm of tibialis anterior (TA), quadriceps and gastrocnemius from metastatic breast cancer donors. There we found evidence of intra- and extra-vascular (i.e., disseminated) marker-positive tumor cells. This finding establishes that spontaneous dissemination to the muscle occurs in humans and, based on the limited amount of tissue surveyed, suggests that dissemination to muscle is not be a rare phenomenon.

To fully document how often this kind of dissemination can occur, we analyzed the same muscles in mice following orthotopic implantation of fluc-eGFP MDA-MB-231. We detected primate-specific AluYb8 in the TA, quadriceps and gastrocnemius of multiple mice, and verified these findings in a comprehensive second assay where contralateral tissues from the same animals were cleared and imaged whole for tumoral GFP. These two assays allow us to conclude that tumor cell dissemination and persistence within skeletal muscle is not a rare event. Combined with our previous data generated in experimental metastasis assays, we can now state that metastatic breast tumor cells disseminate to at least one of these muscles (the TA) to the same extent that they do to lung initially, and although tumor cells eventually colonize lung they remain neutralized in muscle. This all provides a strong quantitative and qualitative basis to explore and ultimately reveal a mechanism by which skeletal muscle suppresses breast tumor cell outgrowth.

(2) *How does directing ectopic catalase expression to the host (instead of the tumor) influence metastasis in skeletal muscle and in lung?* Addressing this important question allowed us to show more conclusively that that myocyte-derived ROS are the party responsible for suppressing tumor colonization within the skeletal muscle. Inoculating C57BL/6 transgenic hosts expressing mitochondrial-targeted catalase (mCAT) with wild-type, syngeneic mammary tumor cells (EO771) promotes skeletal muscle colonization, but provides no benefit whatsoever in the lung. Strikingly, MCAT hosts promote skeletal muscle metastasis to the same degree that ectopic expression of catalase in the tumor cell does. These and follow-up experiments in culture implicate mitochondrial ROS generated by skeletal muscle myocytes as a principal suppressor of DTC outgrowth and show that establishing redox balance is critical for colonization of skeletal muscle to occur.

(3) *Clarify whether ROS accumulate in the mitochondria of DTCs residing in skeletal muscle.* We directed ROS measurements to the mitochondria using mitochondrial dyes to test this directly. We now show that ROS are elevated dramatically within the mitochondria of tumor cells seeded on skeletal muscle niches compared to those residing on lung niches. But we went a step beyond – our results from MCAT hosts suggest that skeletal muscle myoblasts are a principal source of ROS, and that neutralizing ROS within this population may be sufficient to reduce oxidative stress on the tumor. To test this we derived skeletal muscle myocytes from MCAT hosts and showed that tumor cells exhibit less mitochondrial ROS in this context, resulting in more colonization potential. Together these new data suggest that induction of ROS – or – transmission of ROS from the host to the tumor cell acts potently within the mitochondria to suppress tissue colonization. This opens the door for exploration of the precise mechanisms by which ROS exerts anti-metastatic effects within this organelle and even further how this can be harnessed to combat metastasis at other sites.

In addition to addressing these major questions, we have also corrected and/or responded to all of the minor points that were raised, enhancing the clarity of our manuscript and its message. We believe this has substantially improved our manuscript, and we are excited to hear your feedback.

Crist et al, NCB-G45082

Reviewer #1:

Remarks to the Author:

The authors of this manuscript report that skeletal muscle exerts an antimetastatic effect on mammary cancer cells. **Through elegant experiments, they identify increased glutathione (GSH) metabolism in skeletal muscle metastasis as being associated with reduced ability of metastatic cells to colonize skeletal muscle.** The redox-sensitive biosensor Grx-roGFP2 revealed that the MDA-MB-231 mammary cancer cells experienced oxidative stress when cultured on human skeletal muscle myotubes (SkMc) compared to when cultured on human lung fibroblasts (LFs). These findings were recapitulated when MDA-MB-231 Grx1-roGFP2 cells were injected into recipient mice. ROS production was further documented by a cell-permeable fluorescent dye. In addition to MDA-MB-231 cells, SkMc themselves exhibited elevated ROS levels. To induce oxidative stress, the authors employed the chemogenic HyPer3-DAAO that, in the presence of exogenous D-alanine, generates hydrogen peroxide (H₂O₂). Growth of MDA231-DAAO cells was suppressed in the LF niche in the presence of alanine. Increasing the antioxidant potential of tumor cells by expressing the peroxisomal enzyme catalase (mitochondrial mCAT or cytosol cCAT) that converts H₂O₂ to H₂O and O₂ favored outgrowth of MDA-MB-231 cells and of another cancer cell line, E0771 on SkMc. MDA231-mCAT and MDA231-cCAT cells, directly injected into the TA muscle of NOD-SCID mice generated tumors. However, E0711-mCAT cells were unable to colonize the lung.

This is a very interesting and well-conducted study revealing that oxidative stress suppresses tumor cell engraftment in skeletal muscle. It further documents the existence of a metabolic vulnerability that could be exploited to reduce dissemination of tumor cells. There are some points that need clarification. Thank you sincerely for these encouraging remarks and for your thoughtful and careful review of our work.

Comments:

1. Tumor implant rather than i.c. injection may better recapitulate the events occurring during the metastatic process.

Great insight. There are indeed multiple ways to model metastasis in mice, each with their own advantages and disadvantages. In our original submission, we employed an experimental model of metastasis to quantitatively assess trafficking and residence within skeletal muscles due to our desire to normalize input and determine whether failure of tumor cells to metastasize to skeletal muscle was due to poor trafficking to the tissue, a failure to survive within the tissue, or an inability to proliferate within the skeletal muscle microenvironment. Therefore we adopted a quantitative genomic assay (AluYb8 qPCR) and examined the quadriceps, tibialis anterior (**TA**), gastrocnemius and several other more common metastatic sites at an early (Day 3) and later (Day 42/Week 7) timepoint. This experiment revealed that MDA-MB-231 human breast cancer cells traffic to and persist within multiple skeletal muscles (**Supplementary Figure 2a-e**). This study also allowed us to make an important point that the failure of tumor cells to colonize the muscle remains true even if they achieve an equal level of dissemination initially, as Alu levels measured within the TA and the lung were equivalent at Day 3. Controlling input at the outset of the experiment was an important aspect of reaching this conclusion.

While even the best controlled spontaneous models do not allow us to normalize input in the same fashion due to differences in primary tumor growth and dissemination from mouse to mouse, the reviewer correctly points out that such models do capture the full spectrum of the metastatic cascade. By exclusively relying on experimental metastasis models, we could not rule out that invasion and intravasation, for example, select for a phenotype (e.g., epithelial-to-mesenchymal transition) that poorly navigates skeletal muscle endothelium and/or fails to survive within skeletal muscles. To effectively rule this out, we now employ models of

spontaneous metastasis, as suggested by the reviewer, in **Figure 1**. We also substantiate our Alu qPCR-based findings by clearing and imaging contralateral tissues for DTC GFP expression.

More specifically, we orthotopically injected MDA-MB-231-ffluc-eGFP into the inguinal mammary gland of female NSG mice. In our hands, MDA-MB-231 exhibit more variable kinetics in NOD-SCID mice. Therefore, we utilized NSG hosts because we desired a model with consistent progression kinetics, allowing us to analyze mice with similar tumor volumes at the same timepoint. We resected primary tumors once they reached a volume of ~200 mm³. Three weeks later, bioluminescence revealed that all mice had substantial metastatic burden in their lungs. We then compared disseminated tumor cell (DTC) burden in lung and skeletal muscle via Alu qPCR and by Ce3D whole tissue clearing (Li *et al.*, Nature Protocols 2019) at this timepoint.

This experiment revealed that MDA-MB-231 cells traffic and persist in skeletal muscle sites after orthotopic injection. When compared to uninoculated controls, we observed a 7.5-fold increase of human AluYb8 DNA in lung, a 3.5-fold increase in the quadriceps, a 2.6-fold increase in the gastrocnemius, and a 2.3-fold increase in the TA (**Figure 1f**). The higher burden in lung measured via AluYb8-qPCR was expected based on the strength of the BLI signal. Analysis of cleared, whole tissues substantiated these conclusions. GFP⁺ tumor cells colonized a sizeable percentage of the lung (8.6%) when compared to uninoculated controls (**Figure 1h**). Interestingly, we also found that GFP⁺ tumor cells occupy a non-zero percentage of skeletal muscle (1.2% of the TA, 0.16% of the quadriceps, or 0.08% of the gastrocnemius; **Figure 1h**), again compared to uninoculated controls.

Thus, even upon spontaneous dissemination, the arrival and persistence of breast tumor cells within multiple skeletal muscle sites is not a rare event. This further motivates the need to understand the mechanisms by which skeletal muscle suppresses metastasis. We now include details of the study design and pertinent results from this orthotopic model in **Figure 1**. Results from the experimental metastasis model, previously Figure 1, have been moved to **Supplementary Figure 2**.

2. Tumor cells identified within muscle were non proliferating (Ki67⁻). Were they Ki67⁺ in other organs?

The short answer is that in the MDA-MB-231 model, we identify a mixture of Ki67-positive and -negative cells in other organs, but have only detected Ki67-negative cells within skeletal muscle. We add new panels to **Supplementary Figure 2 (Panel f)** to better highlight the Ki67 status of disseminated tumor cells and metastases in many of the organs surveyed following intracardiac injection.

We acknowledge that analysis of cell cycle status at a fixed timepoint may obscure events/transitions between inoculation and observation. In fact, in a recent manuscript (Dai *et al.*, *Nature Cancer*, In press), we conducted intravital imaging to map the fate of MDA-MB-231 once they entered the brain. What we found was that a large percentage of MDA-MB-231 cells that successfully extravasated into the brain remain as single cells throughout the course of the study. But a minority proliferate into small micrometastatic clusters, the majority of which regress back to the single cell level. Therefore, while the vast majority of DTCs in an organ that does not harbor metastasis may be Ki67⁻, transitions occur that yield Ki67⁺ cells. Ostensibly, these transitions occur more frequently and more successfully in organs like the lung.

This all said, in the present work we find that the majority of single DTCs in lung, brain and bone marrow are Ki67⁺, whereas micrometastatic clusters harbor Ki67⁺ subsets. It is also worth noting that we are able to find single Ki67⁺ DTCs in both brain and lung. However, all single DTCs residing in skeletal muscle were Ki67⁻ and no multi-cellular clusters were identified in this model.

3. It would be important to evaluate GSH metabolism, including GSH:GSSG ratio, in metastatic (4T1) and non-metastatic cell lines before i.c. injection and after isolating the injected cells from metastatic lesions.

We agree that the comparison of the metabolic profiles of both the parental (4T1) and skeletal muscle metastatic (4T1-SkM) sublines pre- and post-isolation could yield a more complete picture of metabolic adaptations undergone and sustained by 4T1 cells as they gain the capacity to colonize skeletal muscle. We re-ran our previous sample set (isolated skeletal muscle and lung metastases, healthy skeletal muscle and lung) with the parental 4T1 cells and 4T1-SkM isolated from tissue culture. The goal, as suggested by the reviewer, was to better compare changes in GSH metabolism as 4T1 cells become more capable of colonizing skeletal muscle.

These new data reveal that the metabolic profiles of parental 4T1 and 4T1-SkM cell lines on plastic are significantly different from each other and from metastases excised from skeletal muscle tissue (**Supplementary Figure 3a**). Principal component analysis reveals that 4T1-SkM drift towards 4T1 skeletal metastases, which are highly similar metabolically to healthy skeletal muscle, and quite disparate from lung tissue and lung metastases (which are statistically indistinct from each other). These data now better capture 4T1-SkM cells on their trajectory towards “muscle-like”, in line with theories about metabolic adaptation/metastatic fitness (Dupuy *et al.*, Cell Metabolism 2015; Schild *et al.*, Cancer Cell 2018).

With respect to the GSH:GSSG ratio – In the tissue culture setting, we found that 4T1-parental cells had 1.67-fold higher GSH:GSSG ratio than 4T1-SkM cells (**Supplementary Fig. 3e**). Our interpretation of this datum is that the metabolic programs that benefit 4T1-SkM cells in skeletal muscle only become apparent and/or advantageous *in vivo*.

4. Figure 3. It is interesting to note that the GSH metabolite sets behave in a specular manner in metastasis SkM/healthy SkM and metastasis lung/healthy lung. How do metabolites compare in healthy SkM and healthy lung?

In **Figure 3**, we present a comparison that is meant to draw out metabolic adaptations undergone by tumor cells as they colonize skeletal muscle, specifically. Here, glutathione metabolism as a metabolite set had an enrichment ratio of 7.5, with a p -value <0.001 (MetaboAnalyst). Now in response to this question, we examine how GSH metabolites (as defined by KEGG's glutathione metabolism set) compare between healthy skeletal muscle and healthy lung. This comparison revealed that these two sites do not differ dramatically with respect to glutathione metabolism (**Supplementary Figure 3b-c**: enrichment ratio of 4 but p -value >0.2). This supports the interpretation that the GSH metabolite set is indeed enriched in skeletal muscle metastases.

5. How did the growth of 4T1 cells compare on SkM and LF cultures?

Great question. Under standard tissue culture conditions, 4T1-parental and 4T1-SkM cell lines double at the same rate. This suggests no inherent proliferative bias/advantage for either cell line. However, on organotypic culture, 4T1-parental and 4T1-SkM cell lines recapitulate their *in vivo* phenotypes. Whereas the 4T1-parental cell line grows poorly in the skeletal muscle niche, the 4T1-SkM successfully outgrows, and ultimately achieves a dramatic >400-fold increase in outgrowth on the skeletal muscle niche when compared to the parental line. 4T1-SkM cells also display a growth advantage, albeit far less dramatic (2-fold instead of 400-fold) on lung fibroblasts.

These data are presented in a separate manuscript we are putting together focusing on the progression series itself. Therefore, we include these data here for the reviewer only (**Figure R1**).

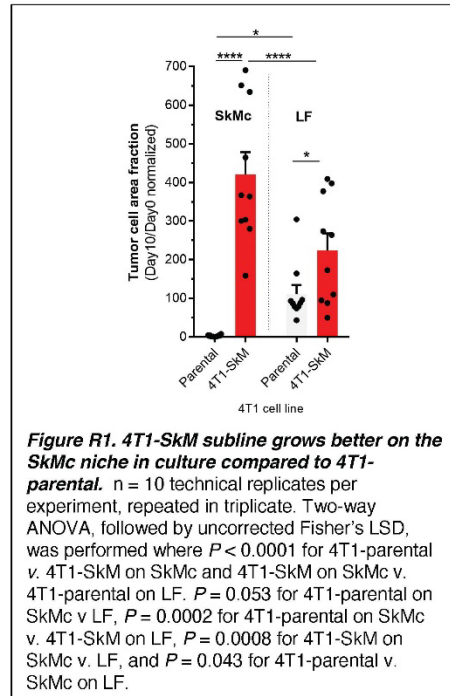
6. HyPer3-DAAO expression in alanine-treated LFs may decrease MDA231 metastases. Has this been evaluated?

It has not but this was a great suggestion and complimented a question about mCAT hosts made by Reviewer 3 quite nicely, so we decided to address this question. We hypothesized that incorporating the DAAO vector into lung fibroblasts (LFs) would result in suppression of breast tumor cell outgrowth if we were able to approximate the levels of hydrogen peroxide (H_2O_2) measured on niches comprised of differentiated skeletal muscle myocytes.

To determine the amount of D-alanine necessary to achieve this level of H_2O_2 induction, we cultured wild-type LFs (LF-WT) and LFs expressing the DAAO vector (LF-DAAO) on tissue culture plastic and conducted dose-response experiments with D-alanine, using Amplex Red measurement of H_2O_2 in the culture medium as the output. Testing a range of D-alanine spanning 0 to 100 mM, we determined that that 30 mM D-alanine most closely approximated the amount of H_2O_2 measured on the skeletal muscle myocyte niche ($\sim 1 \mu M$). Therefore, in our studies aiming to determine whether this level of H_2O_2 induction by LF-DAAO suppressed breast tumor cell outgrowth, we co-cultured MDA-MB-231 cancer cells on LF-WT, LF-DAAO or SkMc niches and administered 0, 30 or 50 mM D-alanine daily.

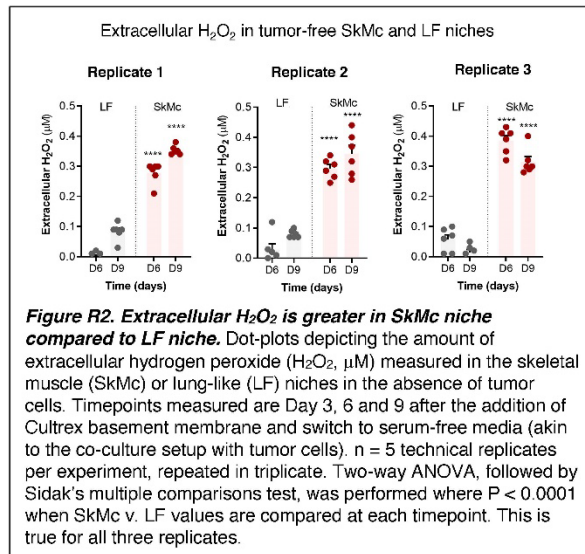
MDA-MB-231 outgrowth was significantly suppressed in the LF-DAAO niche primed with 30 mM D-alanine (**Figure 5j**). In fact, the level of suppression was profound enough that burden on the LF-DAAO niches was statistically indistinguishable from tumor burden on SkMc niches. Importantly, outgrowth of controls (treatment of LF-WT or SkMcs with 30 mM D-alanine) was not impacted.

From these new data we conclude that recreating the oxidative environment constituted by skeletal muscle myocytes in the LF niche inhibits MDA-MB-231 outgrowth. These data support the conclusion that the microenvironment does significantly contribute to the redox state of the tumor cells, and influences a tumor cell's outgrowth potential. These new data are included in **Figure 5** and validation/dosing studies are displayed in **Supplementary Figure 4**.



7. Figure 4. How was extracellular H₂O₂ production in SkMc and LF niches without MDA-MB-231 co-culture?

Good question. To address it, we employed the Amplex Red assay to measure extracellular H₂O₂ production in skeletal muscle (SkMc) and lung-like (LF) niches following culture in differentiation medium and in the absence of MDA-MB-231 tumor cells. Comparing the amount of H₂O₂ between the SkMc and LF niches, we observed a similar trend as when MDA-MB-231 cells were present – there is approximately 3-4 times more H₂O₂ in the SkMc niche than the LF niche (Figure R2). Ostensibly, tumor cells experience these elevated H₂O₂ concentrations from the start and the Amplex Red and Grx1-roGFP2 data presented in Figure 4 suggest that these initial differences are only accentuated thereafter, resulting in a robust, sustained oxidative stress that suppresses outgrowth of cells on that niche.



We note also that while the trend remains in the absence of tumor cells, the total levels of H₂O₂ are reduced in the tumor cell-free SkMc and LF niches compared to the niches co-cultured with MDA-MB-231 cells (see Figure 4g). These data suggest that either i) MDA-MB-231 tumor cells contribute to the total extracellular H₂O₂ pool and/or ii) SkMc and LF niche cells respond to the presence of MDA-MB-231 by shifts in metabolism that result in increased ROS production. Regardless, these data demonstrate that the SkMc niche is more oxidative than the LF niche with or without MDA-MB-231 cells present.

8. How comparable were tumors (in growth and size) generated by MDA-MB-231-mCAT/cCAT cells to those generated by 4T1 cells?

In Table 1 (please see the following page) we display approximate tumor volumes for: i) 4T1-SkM metastases in skeletal muscle, ii) the one MDA-MB-231-mCAT tumor that arose in skeletal muscle, and iii) EO771-Ctl/mCAT tumors. Our original outputs were not based on tumor volume measurements. Therefore, to address this question, we approximated tumor volume by using a scale bar on i) mCherry fluorescent images (AxioZoom) taken *ex vivo* and/or ii) BLI images (IVIS) taken *ex vivo* to approximate the width and length of the tumor. To approximate the volume of a given lesion, we used the following formula for tumor volume: $V = 0.5Lw^2$, where w is the shorter dimension.

We caution against making one-to-one comparisons between these measurements, especially between conditions, because the routes of inoculation resulted in either a single cell nucleating metastasis or a bolus of injected cells giving rise to a skeletal muscle lesion following intramuscular inoculation. Moreover, measurements based on estimates of tumor fluorescence or bioluminescence are more likely to be inaccurate than not, in our experience, given differences in depth and reporter intensity from mouse to mouse.

Table 1.

4T1-SkM line	Ex vivo size (mCherry)	Volume (mm ³)	Average volume (mm ³)	
1	1 x 1 mm	0.5	2 mm ³	4T1-SkM (i.c.)
2	2 lesions next to each other: 1 x 1 mm, 2.5 x 1.5 mm	2.8		
3	1.5 x 2.5 mm	2.8		
4	1.5 x 2 mm	2.3		
MDA-MB-231 line	Ex vivo size (BLI)	Volume (mm ³)	Average volume (mm ³)	
mCAT	4 x 12 mm	96	48 mm ³	MDA231-mCAT (i.m.)
EO771 line / Mouse strain				
	Ex vivo size (BLI)	Volume (mm ³)	Average volume (mm ³)	
mCAT/ WT	21 x 21 mm	4631	2120 mm ³	EO771-mCAT (i.m., WT mice)
mCAT/ WT	16 x 15 mm	1800		
mCAT/ WT	15 x 11.5 mm	992		
mCAT/WT	14 x 15 mm	1470		
mCAT/WT	18 x 15 mm	2025		
mCAT/WT	15 x 16 mm	1800	2616 mm ³	EO771-mCAT (i.m., MCAT mice)
mCAT/MCAT	21 x 18 mm	3402		
mCAT/MCAT	13 x 15 mm	1268		
mCAT/MCAT	17 x 22 mm	3179	3402 mm ³	EO771-Ctl (i.m., MCAT mice)
Ctl/MCAT	18 x 21 mm	3402		
Ctl/MCAT	18 x 21 mm	3402		
Ctl/MCAT	17 x 20 mm	2890	908 mm ³	EO771-Ctl (i.m., WT mice)
Ctl/WT	11 x 15 mm	908		

Reviewer #2:

Remarks to the Author:

Crist and colleagues addressed the question of whether the tissue environment limits the outgrowth of disseminated breast cancer cells thus contributing to organotropism. They relied on intracardiac injections and measured the number of disseminated breast cancer cells in different tissues. They find in multiple tissues including skeletal muscle disseminated breast cancer cells. Yet, in skeletal muscle the disseminated breast cancer cells were barely growing into metastasis. Based on metabolomics analysis and breast cancer cells evolved to grow metastases in skeletal muscle they find that a highly oxidative environment of the muscle prevented metastasis outgrowth. Overexpression of catalase increased the capacity of breast cancer cells to outgrow in skeletal muscle. **In principle the study addressed an important and very interesting question.** We thank you for your kind words about our study, and for your thoughtful and constructive feedback, which we address below.

The following points should be considered:

1) The manuscript contains many generalizing statements such as "...revealing that breast cancer cells can and do traffic to skeletal muscle." This and other generalized statements should be rephrased so that they include the model systems and cell lines for which the conclusions can be drawn.

Thank you for making this suggestion. We have revised the manuscript to be more specific in our conclusions and to eliminate over-generalizing statements. For example, we now reference specific cancer line(s) used rather than the generalized term "tumor cell" more often. Several examples of this are:

- page 11, line 267: "Disseminated **MDA-MB-231** tumour cells exhibit oxidative stress in vivo."
- page 12, line 280: "Increased oxidative stress suppresses **MDA-MB-231** tumour cell outgrowth on a permissive niche"
- page 15, line 389: "**MDA-MB-231** and **EO771** tumour cells do not benefit from enhanced redox capacity in lung"
- page 17, line 441: "**MDA-MB-231** and **EO771** tumour cells have a higher mitochondrial ROS burden when cultured in the skeletal muscle niche"

2) A major limitation of the study is that it does not include spontaneous models of metastasis. This is a limitation because as shown previously, the circulation is a highly oxidative environment. Thus, cancer cells that survive the circulation may already be selected to have a high ROS defense and thus may not be limited by the oxidative environment of skeletal muscle but some other particularities of this environment. The authors should exclude this possibility.

Thank you for this comment. We realize now that we were not explicit enough in the reasons behind our choice of model, and have revised our manuscript to be more clear.

As the reviewer notes, there are indeed multiple ways to model metastasis in mice. Each have advantages and disadvantages. In our original submission, we employed an experimental model of metastasis to quantitatively assess trafficking and residence of MDA-MB-231 cells within multiple skeletal muscles. A major impetus was our desire to normalize input in order to determine whether failure of tumor cells to colonize skeletal muscle was due to poor trafficking to the tissue, a failure to survive within the tissue, or an inability proliferate within the skeletal muscle microenvironment. Therefore, we adopted a quantitative genomic assay (AluYb8 PCR) and examined three different skeletal muscles and several other more common metastatic sites at an early (Day 3) and later (Day 42/Week 7) timepoint. This experiment revealed that MDA-MB-231 human breast cancer cells

traffic to and persist within each of the skeletal muscles surveyed (**Supplementary Figure 2a-e**). It also allowed us to make the important point that the failure of tumor cells to colonize the muscle manifests despite achieving an equal level of dissemination initially, as AluYb8 levels measured within the TA and the lung were equivalent at Day 3. Controlling input at the outset of the experiment was critical to reaching this conclusion.

It is important to note also that delivering cells via intracardiac injection ensures a hematogenous route; cells are injected directly into blood and then pumped throughout the body. Therefore, breast cancer cells delivered via this method are constrained by the same hematogenous pressures whether they wind up in the lung or in the skeletal muscle. We would expect that breast cancer cells that arrive to skeletal muscle, lung or any other tissue after intracardiac injection are no more or less adapted to an oxidative environment than another.

However – while even the best controlled spontaneous models do not allow us to normalize input in the same fashion due to heterogeneity of primary tumor growth and dissemination between even inbred hosts – the reviewer correctly points out that such models capture the full spectrum of the metastatic cascade. By exclusively relying on experimental metastasis models, we could not rule out that invasion and intravasation, for example, select for a phenotype (such as EMT) that poorly navigates skeletal muscle endothelium and/or fails to survive within skeletal muscles.

To effectively rule this out, we now employ a model of spontaneous metastasis, as suggested by the reviewer, in **Figure 1**. We also substantiate our findings using Alu qPCR via tissue clearing and quantification of GFP⁺ DTCs. Because MDA-MB-231 exhibit more variable kinetics in NOD-SCID mice, we orthotopically injected MDA-MB-231-ffluc-eGFP into the inguinal mammary gland of female NSG mice. This provided a model with consistent progression kinetics, allowing us to analyze mice with similar tumor volumes at the same timepoint.

We allowed primary tumors to develop and resected them once they reached a tumor volume of ~200 mm³. Three weeks post-resection, bioluminescence revealed that all mice had substantial metastatic burden in their lungs. We then compared disseminated tumor cell burden in lung and skeletal muscle via Alu qPCR and by Ce3D (Li *et al.*, Nature Protocols 2019) whole tissue clearing with immunofluorescent microscopy on paired, contralateral tissue to obtain high resolution images of the entire lung lobes and skeletal muscles with GFP⁺ cancer cells.

This experiment revealed that MDA-MB-231 cells traffic to and persist within skeletal muscle sites after orthotopic injection. When compared to uninoculated controls, we observed a 7.5-fold increase of human AluYb8 DNA in lung, a 3.5-fold increase in the quadriceps, a 2.6-fold increase in the gastrocnemius, and a 2.3-fold increase in the TA (**Figure 1f**). Ce3D whole tissue clearing of lung and these three skeletal muscle sites substantiated our conclusions. GFP⁺ tumor cells colonized a sizeable percentage of lung (8.6%) when compared to uninoculated controls (**Figure 1h**). Interestingly, we also found that GFP⁺ tumor cells occupied a non-zero percentage of skeletal muscle (1.2% of the TA, 0.16% of the quadriceps, or 0.08% of the gastrocnemius; **Figure 1h**) compared to uninoculated controls. Thus, upon even spontaneous dissemination, the arrival and persistence of breast tumor cells within multiple skeletal muscle sites is not a rare event. This provides even more solid grounds to understand the mechanisms by which skeletal muscle suppresses metastasis.

We include details of the study design and pertinent results from this orthotopic model in **Figure 1**. Results from the experimental metastasis model, previously Figure 1, have been moved to **Supplementary Figure 2**.

3) Similarly, what is the evidence that breast cancer cells spontaneously travel to the skeletal muscle?

Great question. There is scattered evidence in the literature to suggest that tumor cells spontaneously disseminate to skeletal muscle. Clinical case reports show that breast cancer metastasis can emerge in skeletal muscle sites (Haygood et al, Skelet. Radiol 2012; Surov et al, Eur. Radiol 2010; Magee and Rosenthal, AJR Am J Roentgenol 2002; Ong et al, Clin Radiol 2019; Salemis, Breast Dis 2015). Additionally, autopsy studies from patients with metastatic disease indicate the presence of tumor cells or even micrometastases in skeletal muscle. However, these case studies were not necessarily convincing and the reviewer's question tempted us to try to determine whether we could document that DTCs exist in the skeletal muscle of human breast cancer patients.

We partnered with the BROCADE Rapid Autopsy Program to examine the skeletal muscle of two Stage IV breast cancer patients who had consented to rapid autopsy upon their death. Prior to the COVID-19 pandemic, we had successfully implemented a change to BROCADE IRB to include collection of skeletal muscle tissue from the tibialis anterior, quadriceps and gastrocnemius. Thus, while a reasonable criticism is that we only have data from two patients, it is important to note that: collection has been paused indefinitely due to the pandemic, and we do not have the timely ability to expand.

Both donors had been identified as harboring estrogen receptor-positive (ER⁺) and progesterone receptor-positive (PR⁺) disease. So, we utilized these molecular markers to locate breast tumor cells in skeletal muscle. First, we verified that this antibody cocktail worked in our hands by sampling ER/PR⁺ breast tumor (**Figure 1b**, **Supplementary Figure 1a**). We also ensured that this cocktail did not result in the identification of "DTCs" in tissue from donors with documented history of cancer (**Supplementary Figure 1b**). Finally, we surveyed 5 skeletal muscle slides per donor, containing 4 μ m-thick tissue sections collected across 200 μ m of the tibialis anterior, gastrocnemius and quadriceps muscles. To our great surprise, we found evidence of ER⁺/PR⁺/pan-cytokeratin⁺ tumor cells inside and outside of the vasculature, verified as breast cancer cells by a seasoned pathologist (Dr. Lawrence True). These data illustrate that breast tumor cells spontaneously disseminate to the skeletal muscle of humans. And, the identification of multiple DTCs in such a small amount of tissue suggests that this event may be much more common than previously assumed. We have included representative IHC images of these exciting new data in the revised **Figure 1** and **Supplementary Figure 1**.

The aforementioned experiments conducted in mice with a model of spontaneous MDA-MB-231 dissemination and metastasis allowed us to confirm using multiple methodologies that even spontaneous dissemination in this setting is not a rare event; all muscles surveyed (TA, gastrocnemius and quadriceps; same as those examined in humans) contained significantly elevated AluYb8 signal compared to uninoculated mice in multiple animals, and all muscles surveyed across mice contained GFP⁺ tumor cells when cleared tissue was examined (**Figure 1**).

Taken together, our data now reveal that disseminated breast tumor cells reside within the skeletal muscle of humans, that this is not a rare event in mice, and that even when represented equally at the outset following hematogenous dissemination tumor cells colonize the lung but fail to colonize the muscle. This provides a strong quantitative and qualitative basis to interrogate the mechanisms by which skeletal muscle suppresses breast cancer metastasis.

Reviewer #3:

Remarks to the Author:

The manuscript "Unchecked Oxidative Stress is an insurmountable barrier for tumor cells that disseminate to skeletal muscle" investigates the difference in the colonization of tumors to metastatic versus non-metastatic sites, with a focus on skeletal muscle as a non-metastatic tissue. The authors employ an innovative and high throughput approach for detecting low levels of disseminated tumor cells (DTCs) involving qPCR-based amplification of AluYb8 elements, which are only found in primates. Using this approach, the authors find that DTCs exist and survive in skeletal muscle, but do not colonize and proliferate due to an inhospitable oxidative environment. **These initial observations of examining the interaction between tumor and host tissue are innovative and timely, however, several points should be addressed to allow for full interpretation of the data.** Thank you for appreciating the importance of the question addressed in our manuscript, and for your thoughtful critique of our work. In response to the your suggestions, we have performed a number of additional experiments, which are presented below. The majority have been included in the revised manuscript.

1. The authors are able to detect DTCs in the skeletal muscle using qPCR-based amplification. Can the authors validate this finding using an alternative approach, such a qPCR of GFP over-expression in the tumor cells? Do the authors observe DTCs or increased oxidative stress in DTCs in the heart?

Please note that real-time qPCR amplification of human repetitive elements to accurately quantify small numbers of cells *in vivo* has been utilized successfully in a number of other studies (Schneider et al., Clin Exp Metastasis 2002; Campbell et al., Sci Rep 2015; Zijlstra et al., Cancer Research 2002; Walker et al., Analyt Chem 2003; Shiozawa et al., J Clin Invest 2011; Funakoshi et al., Nature 2019). Here, we chose to amplify the repetitive human AluYb8 element over an introduced expression construct like GFP for two main reasons: 1) Detecting rare populations of cells in bulk tissue requires high sensitivity, which is driven by the signal-to-noise ratio of the assay. For these purposes we used the repetitive nature of AluYb8 to our advantage, since there are >1800 copies of AluYb8 (Caroll et al., JBM 2001) to amplify human tumor cell signal above the technical noise. Infection with a lentivirus encoding GFP results in insertion of a single copy (or on the order of a single copy), which would not provide sufficient template to detect cells that are as rare as 1 in 1×10^6 . Another important facet of our methodology is that we shear DNA into 500 bp fragments to uncouple the repetitive human Alu sequences (300 bp), distributing the human sequences more evenly across the DNA suspension. We believe that this also enables higher detection sensitivity per reaction.

We agree however that more wholesale validation of the conclusions gleaned from Alu qPCR data would strengthen the quantitative evidence of DTC abundance in skeletal muscle. Therefore, as we conducted new experiments to determine whether breast tumor cells disseminate spontaneously to skeletal muscles such as the quadriceps, tibialis anterior (TA) and gastrocnemius, we paired Ce3D whole tissue clearing and imaging of GFP⁺ cells with AluYb8 qPCR on tissues collected from a spontaneous metastasis model (human MDA-MB-231 injected orthotopically into NSG mice). Ce3D is a tissue clearing method that achieves excellent transparency, preserves cell morphology and tissue architecture, and is compatible with immunofluorescent staining (Li et al., Nature Protocols 2019). In this model we can now visualize extensive metastases in Ce3D lungs, in line with measurements made using AluYb8 qPCR. AluYb8 qPCR amplifies signal in all three muscles probed in multiple mice (compared to uninoculated controls), which we substantiate further by imaging cleared whole contralateral muscles and documenting DTCs throughout. In all, these data now allow us to state with more certainty that dissemination and persistence within skeletal muscle is not a rare event and this better motivates the study of how skeletal muscle suppresses metastasis. We include the results from this analysis in **Figure 1** and **Supplementary Figure 1**.

To your final point, we did not examine the heart in great detail in this study, but this is indeed an intriguing question and one we look forward to addressing in the future.

2. In Figure 2, the authors observe that live myotubes suppress tumor cell outgrowth. Does the oxygen level affect this phenotype?

We have addressed this question in our response to your third point, immediately below.

3. By several measures, including GSH:GSSH and the use of a HyPer3-DAAO sensitive chemogenetic reporter, DTCs in the skeletal muscle are highly oxidized due to the H₂O₂ levels in the environment and endogenous ROS. Using a culture set up the authors demonstrate that skeletal muscle is a more oxidizing environment compared with lung. However, these conditions do not accurately reflect the vast difference in oxygen in these organs *in vivo*. How do these levels compare in physiological oxygen levels, which one would expect the lung to be even more oxygenated compared with the skeletal muscle?

These are both great points. Oxygen tension in the lung is as high as atmospheric levels (17-21 kPa *in vivo* versus 20-21% atmospheric O₂) in regions surrounding the airway epithelium (Keely and Mann, *Physiol Rev* 2019; Cieslar *et al*, *Magn Reson Med* 2007). These are akin to oxygen tension (18-20% O₂) inside of a standard tissue culture incubator. However, oxygen tension ranges from 1-10% in skeletal muscles *in vivo* (Csete *et al*, *J Cell Physiol* 2001; Li *et al*, *Med Hypotheses* 2007; Chakravarthy *et al*, *Cell Mol Life Sci* 2001, Launay *et al*, *Cell Prolif* 2010; Yun *et al*, *Mol Cell Biol* 2005). Therefore, the reviewer's concerns about recapitulating the metabolic microenvironment in muscle when culturing in what are indeed hyperoxic conditions warranted further investigation.

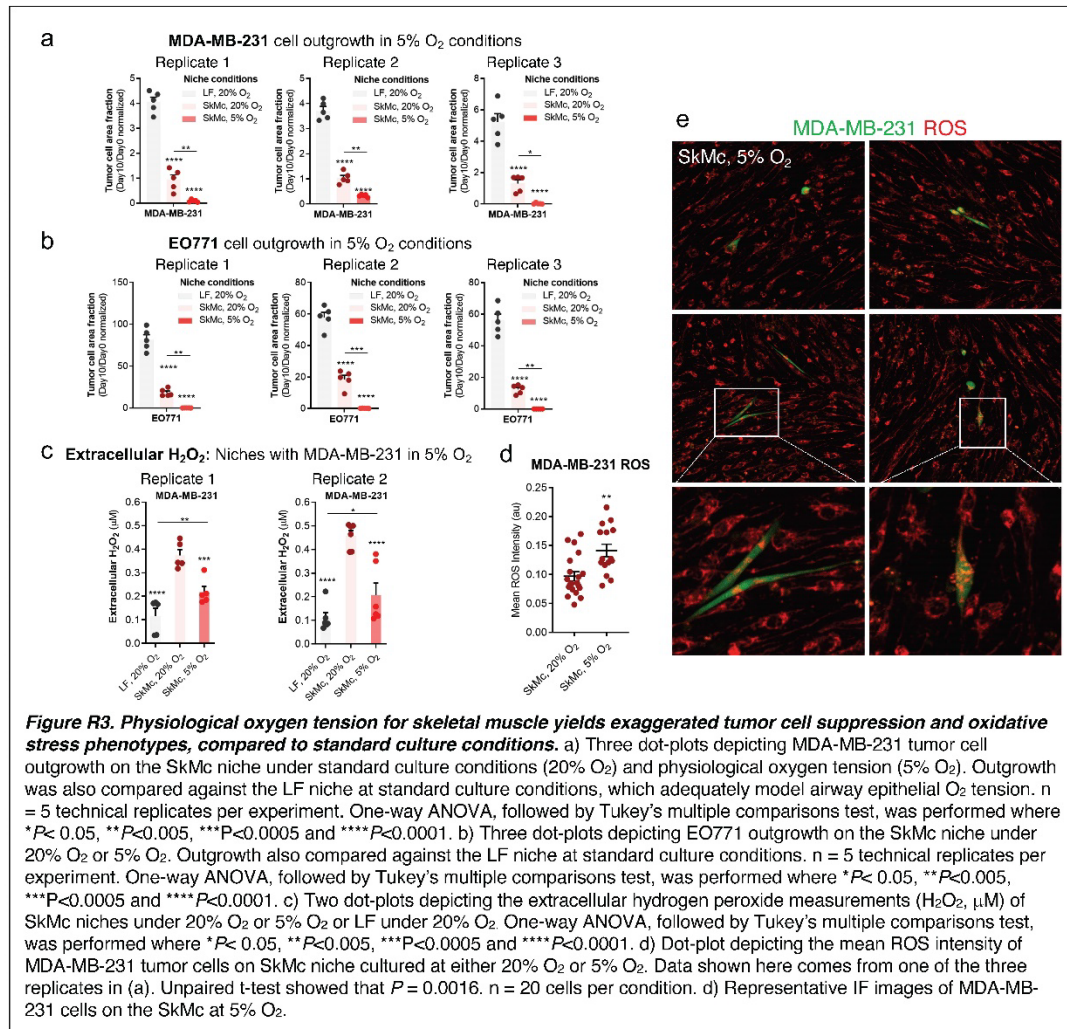
Therefore, we compared three key parameters (tumor cell outgrowth, H₂O₂ generation, and tumor cell ROS) on lung-like (lung fibroblast or LF) niches at 20% O₂ vs. skeletal muscle niches (differentiated skeletal muscle myocytes or SkMc) at both 20% O₂ and 5% O₂. Normoxia (5% O₂) was achieved using a Coy O₂ Control InVitro Glove Box, which allows for incubation and culture maintenance at a pre-determined oxygen tension.

We chose 5% O₂ for recreating physiological skeletal muscle because this falls at the midpoint of the reporter oxygen tension in skeletal muscle and also because proliferation, differentiation, and viability of human, mouse and rat myoblasts are preserved at this oxygen tension (Sellathurai *et al*, *PLoS One* 2016; Lees *et al*, *Cell Prolif* 2008; Chakravarthy *et al*, *Cell Mol Life Sci* 2001; Csete *et al*, *J Cell Physiol* 2001, Martin *et al*, *Cytotechnology* 2009). 96-well plates housing the skeletal muscle co-cultures remained in the hypoxic chamber for the duration of the 10-day experiment and were only removed at the endpoint. What we uncovered for each measured parameter is summarized below and displayed in **Figure R3**, also below.

- **Tumor cell outgrowth** - MDA-MB-231 and EO771 tumor cells survive at lowered oxygen tension but are further suppressed when cultured on SkMcs at 5% O₂ compared to SkMcs at 20% O₂ (**Figure R3a-b**).
- **Extracellular H₂O₂** - We measured the level of extracellular H₂O₂ (Amplex Red) in the SkMc or LF co-cultures containing MDA-MB-231 when grown under 5% oxygen tension, and compared against standard culture conditions (20% O₂). In this series of experiments, conditioned media was collected at the Day 10 endpoint. We observed a modest reduction in extracellular H₂O₂ when the tumor cell-containing skeletal muscle niches were cultured in 5% O₂ compared with 20% O₂ (**Figure R3c**). This

was still significantly above that measured on LFs at 20% O₂. These levels may be influenced by reduced burden of MDA-MB-231 cells at 5% O₂ (please see [Reviewer 1/Reponse 7](#)).

- **Tumor cell ROS (as a proxy for oxidative stress)** – We measured ROS abundance in the SkMc niche when cultured in 5% O₂ using the CellROX647 fluorescent ROS dye. Tumor cells in the SkMc niche maintain high concentrations of ROS, despite the change in oxygenation (**Figure R3d-e**). Interestingly, quantification of tumoral ROS in the skeletal muscle niche revealed that culture at 5% O₂ actually increased the ROS burden compared to culture at 20% O₂ (**Figure R3d-e**). These data demonstrate that MDA-MB-231 tumor cells cultured on skeletal muscle experience enhanced oxidative stress even



under physiological oxygen conditions and that this enhanced oxidative stress is associated with further suppression of tumor cell outgrowth on these niches.

These findings support our original conclusions and mirror our observations made *in vivo* (inherently under physiologic oxygen tensions) where we stabilized the oxidation state of tumor cells in lung and skeletal muscle via thiol fixation (N-ethylmaleimide) then processed and ratiometrically imaged tissues according to Fujikawa *et al.* (Science Signaling, 2016). In line with our culture findings, we observed that single tumor cells in skeletal muscle and lung are highly oxidized, implying high concentrations of intracellular ROS. DTCs in lung were able to relieve this stress as they progressed; however, DTCs in skeletal muscle did not relieve this stress and failed to progress beyond the single cell state (**Fig. 4**).

In sum, while every model can be tweaked to better approximate the complex physiology of the phenomenon being studied, it is notable that in this case culturing at physiological oxygen tension magnifies the phenotypes we documented thoroughly in our manuscript.

4. The authors find that catalase overexpression promotes tumor outgrowth and even colonization in a skeletal muscle environment. Does overexpression of catalase in the skeletal muscle versus lung cells have a similar effect?

Fantastic question. The short answer is: yes. We took advantage of the MCAT transgenic mouse model to test if overexpressed catalase in the skeletal muscle or lung would recapitulate the pro-growth (muscle) or growth-restrictive (lung) phenotypes we observed when catalase was expressed intratumorally. The MCAT mouse expresses mitochondrial-targeted catalase (akin to our system) to reduce mitochondrial H_2O_2 generation and is expressed throughout the host, with the highest MCAT enhancement in cardiac and skeletal muscle (Schriner *et al.*, Science 2005). Another group, Kim *et al.* (Free Rad. Biol Med 2016), demonstrated that the level of catalase overexpression in the lung (not measured by Schriner *et al.*) was comparable to that of the heart, motivating use of this mouse model to adequately test this question in a metastasis permissive tissue (lung) and a metastasis suppressive tissue (skeletal muscle, more specifically the TA).

We first designed a study to explore the metastasis-initiating capabilities and dynamics of the EO771-mCAT cell lines or empty vector controls (EO771-Ctl) when injected intramuscularly into C57BL/6 wildtype (WT) or MCAT mice (**Figure 7**). After six-weeks of tumor monitoring via bioluminescent imaging (BLI), we learned and/or confirmed several key pieces of information:

- i) In line with our previous results, reducing *tumoral* mitochondrial ROS stimulates skeletal muscle colonization. EO771-mCAT cell outgrowth was significantly increased in wildtype mice compared to EO771-Ctl cells (32% of mice developed muscle tumors with EO771-mCAT cells compared to the 5% of mice with EO771-Ctl cells; **Figure 7c-d**).
- ii) Reducing *microenvironmental* ROS also significantly enhanced tumor cell outgrowth in skeletal muscle. When transgenic MCAT mice - which have lower levels of H_2O_2 tissue-wide - were inoculated with EO771-Ctl cells, we observed that *one-third* of MCAT mice developed muscle tumors (compared to the 5% of mice that developed tumors with EO771-Ctl cells in WT mice; **Figure 7c, e**). These data suggest that the contribution of ROS from the environment is as important as the ROS generated intratumorally in response to an oxidative environment. Resolving either stressor is sufficient for DTCs to progress into proliferative lesions in skeletal muscle.
- iii) To our surprise, we did not measure an additive effect of tumoral and microenvironmental ROS suppression. When EO771-mCAT cells were injected into MCAT mice, only one-third of mice developed and sustained skeletal muscle tumors (**Figure 7f**). Based on the BLI kinetics over six weeks, it appears that increased proliferation occurred early on in 5/9 mice (55%), but two of the

lesions regressed. This suggests a fine-tuned range where DTC redox state must fall in order to stimulate colonization of skeletal muscle. This observation also nicely ties into the findings in lung, where tumoral mCAT expression hinders metastatic colonization rather than promoting it.

To keep fully with the theme of our study, we designed a parallel experiment to assess the impact of mitochondrial catalase expression on metastatic colonization of lung. Tumor cells were routed to the lung via tail vein injection of wild-type (WT) C57BL/6 or MCAT mice. Metastatic burden was quantified using BLI imaging and fluorescence microscopy *ex vivo*.

This experiment revealed that ectopic expression of catalase in the host mitochondria did not benefit lung colonization. In fact, BLI burden within lung was significantly reduced in MCAT mice inoculated with either EO771-Ctl or EO771-mCAT cells (**Figure 7h**). At endpoint (two weeks), lung bioluminescence *ex vivo* was significantly diminished in all cohorts when compared to EO771-Ctl cells inoculated in WT mice (**Figure 7i-j**). Quantification of metastatic lesions based solely on *ex vivo* fluorescence however suggests that tumoral mCAT is more deleterious to lung metastasis than expression of MCAT in the host (**Figure 7k-l**).

Nonetheless these data contribute the additional insight that promoting breakdown of mitochondrial ROS via ectopic catalase expression disproportionately benefits colonization of skeletal muscle – regardless of whether catalase is targeted to the mitochondria of the tumor or the host. This speaks further to tissue specificity and provides further insight into the role of microenvironment-derived ROS, which is a source of tumoral oxidative stress (see below) and is deleterious to colonization of the skeletal muscles.

5. A large gap in the study is the lack of mechanistic understanding in the source of ROS generated in DTCs in the skeletal muscle versus lung environment under physiological conditions. Is this due to fundamental differences in mitochondrial generation of ROS? This information is important to understand as it would provide additional nodes of regulation to examine *in vivo*. Further insight into this area would help to clarify the differences between permissive and non-permissive tissues.

We appreciate your recommendation for more mechanistic insight. Based on the specific question you raised, we focused our mechanistic efforts on whether the amount of the mitochondrial ROS differs between tumor cells in skeletal muscle or lung.

We found measurements of DTC ROS *in vivo* to be difficult and unreliable, though we did note differences in mitochondrial morphology between tumor cells in lung and skeletal muscle that we will follow up on in future studies (see below). So, to address your question in a quantitative manner, we settled with the organotypic culture model as a proxy especially in light of our hypoxia studies in response to the points raised earlier (Points 2 and 3, specifically) showing that suppression of tumor cell outgrowth, ROS production and tumoral oxidative stress were consistent with what was observed *in vivo* and in culture in normoxia.

First, we co-cultured human MDA-MB-231 (Ctl and mCAT) and mouse EO771 (Ctl and mCAT) tumor cells on human skeletal muscle (SkMc) and lung-like (LF) niches and stained the wells with the live cell mitochondrial dye (MitoTracker Green) in combination with CellROX DeepRed (ROS dye) to measure the level of ROS localized to the mitochondria. We measured substantially higher mitochondrial ROS burden for both MDA-MB-231 and EO771 tumor cells cultured in a SkMc niche compared to a LF niche (**Figure 8a, d**). Further, introduction of mitochondrial catalase (mCAT) into MDA-MB-231 and EO771 cells reduced mitochondrial ROS burden of cells cultured on SkMcs, as expected (**Figure 8a,d**), coinciding with a significant and substantial growth advantage in these conditions.

We also wanted to assess how mitochondrial ROS burden of tumor cells shifted when residing in a less oxidative skeletal muscle niche (the MCAT niche), given that we observed enhanced tumor colonization in MCAT hosts *in vivo*. So we derived two unique myoblast cultures from the skeletal muscle (gastrocnemius) of MCAT mice, as well as one unique myoblast culture from the gastrocnemius of a wild-type littermate. This allowed us to determine whether the differences in skeletal muscle colonization observed in MCAT mice were truly associated with reduced ROS burden within the myofiber/SkMc niche. We stained organotypic niches for mitochondrial ROS where i) only the tumor cell expressed mCAT or ii) only the muscle niche expressed MCAT. We found that the highest mitochondrial burden existed in the EO771-Ctl cells when cultured on the WT niche (**Figure 8h-i**). To our excitement, relieving oxidation within the skeletal muscle niche was sufficient to lower mitochondrial ROS levels intratumorally, resulting in enhanced outgrowth upon the mCAT SkMc niche (**Figure 8g**).

These new data suggest a direct role for mitochondrial ROS in suppressing breast/mammary tumor cell outgrowth within the skeletal muscle niche, and point to increased mitochondrial ROS in DTCs that reside in skeletal muscle due to both internal and external (environmental) stressors. This motivates a full inquiry into the mechanism(s) by which ROS impact DTC mitochondria within skeletal muscle to suppress outgrowth. We are very excited to pursue this in future studies – particularly in light of these new data generated in response to your suggestion.

6. How is the difference in the redox environment molecularly transmitted to the tumor?

Fascinating question, and one that is even more compelling with our new finding that reducing *host* mitochondrial ROS facilitates skeletal muscle colonization to the degree that reducing mitochondrial ROS in the implanted tumor cell does. This suggests that mitochondrial metabolism of a given tissue plays an important role in its capacity to suppress metastasis.

A major phenotypic output of directing catalase to the mitochondria is that MCAT mice exhibit decreased extracellular H₂O₂, particularly in skeletal muscles (Schriner *et al.*, Science 2005). Since we measured enhanced tumor colonization in this context, one provocative interpretation is that H₂O₂ molecules (or other ROS conjugates of H₂O₂) are 'transmitted' from the environment to the DTC(s) that reside within the tissue. Indeed, when we facilitate such a transfer from the lung environment via expression of chemogenetic DAAO system in lung fibroblasts, approximating the H₂O₂ levels measured in the SkMc niche is sufficient to convert the lung-like niche to one that is as suppressive as the skeletal muscle niche.

How, exactly, this results in elevated tumoral mitochondrial ROS remains an open question, as does the specific effect(s) ROS has within the mitochondria to suppress DTC proliferation. Addressing all of this will require extensive experimentation and could very well serve as a bedrock of several new studies. We are excited to follow up on these lines of questioning in the very near future.

Decision Letter, first revision:

Subject: Your manuscript, NCB-G45082A

Message:

Our ref: NCB-G45082A

19th December 2021

Dear Dr. Ghajar,

Thank you for submitting your revised manuscript "Unchecked oxidative stress is an insurmountable barrier for tumour cells that disseminate to skeletal muscle" (NCB-G45082A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Cell Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTeX)-- we can not proceed with PDFs at this stage.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Cell Biology Please do not hesitate to contact me if you have any questions.

Sincerely,

Zhe Wang, PhD
Senior Editor
Nature Cell Biology

Tel: +44 (0) 207 843 4924
email: zhe.wang@nature.com

Reviewer #1 (Remarks to the Author):

The authors have satisfactorily addressed my comments.

Reviewer #2 (Remarks to the Author):

The authors addressed all my questions.

Reviewer #3 (Remarks to the Author):

The revised manuscript addresses the majority of the points raised by the reviewers and provides new insights into breast cancer metastasis. It is somewhat disappointing that the revision did not include more insight into mechanisms that regulate ROS generation. In the authors words "How, exactly, this results in elevated tumoral mitochondrial ROS remains an open question, as does the specific effect(s) ROS has within the mitochondria to suppress DTC proliferation."

Decision letter, final requests:

Subject: NCB: Your manuscript, NCB-G45082A
Message: Our ref: NCB-G45082A

6th January 2022

Dear Dr. Ghajar,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Cell Biology manuscript, "Unchecked oxidative stress is an insurmountable barrier for tumour cells that disseminate to skeletal muscle" (NCB-G45082A). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within one week). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: <https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication> for details).

In recognition of the time and expertise our reviewers provide to Nature Cell Biology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Unchecked oxidative stress is an insurmountable barrier for tumour cells that disseminate to skeletal muscle". For those reviewers who give their assent, we will be publishing their names alongside the published article.

Nature Cell Biology offers a Transparent Peer Review option for new original research manuscripts submitted after December 1st, 2019. As part of this initiative, we encourage our authors to support increased transparency into the peer review process by agreeing to have the reviewer comments, author rebuttal letters, and editorial decision letters published as a Supplementary item. When you submit your final files please clearly state in your cover letter whether or not you would like to participate in this initiative. Please note that failure to state your preference will result in delays in accepting your manuscript for publication.

Cover suggestions

As you prepare your final files we encourage you to consider whether you have any images or illustrations that may be appropriate for use on the cover of Nature Cell Biology.

Covers should be both aesthetically appealing and scientifically relevant, and should be supplied at the best quality available. Due to the prominence of these images, we do not generally select images featuring faces, children, text, graphs, schematic drawings, or collages on our covers.

We accept TIFF, JPEG, PNG or PSD file formats (a layered PSD file would be ideal), and the image should be at least 300ppi resolution (preferably 600-1200 ppi), in CMYK colour mode.

If your image is selected, we may also use it on the journal website as a banner image, and may need to make artistic alterations to fit our journal style.

Please submit your suggestions, clearly labeled, along with your final files. We'll be in touch if more information is needed.

Nature Cell Biology has now transitioned to a unified Rights Collection system which will allow our Author Services team to quickly and easily collect the rights and permissions required to publish your work. Approximately 10 days after your paper is formally accepted, you will receive an email in providing you with a link to complete the grant of rights. If your paper is eligible for Open Access, our Author Services team will also be in touch regarding any additional information that may be required to arrange payment for your article.

Please note that Nature Cell Biology is a Transformative Journal (TJ). Authors may publish their research with us through the traditional subscription access route or make their paper immediately open access through payment of an article-processing charge (APC). Authors will not be required to make a final decision about access to their article until it has been accepted. Find out more about Transformative Journals

Authors may need to take specific actions to achieve compliance with funder and institutional open access mandates. For submissions from January 2021, if your research is supported by a funder that requires immediate open access (e.g. according to Plan S principles) then you should select the gold OA route, and we will direct you to the compliant route where possible. For authors selecting the subscription publication route our standard licensing terms will need to be accepted, including our self-archiving policies. Those standard licensing terms will supersede any other terms that the author or any third party may assert apply to any version of the manuscript.

Please note that you will not receive your proofs until the publishing agreement has been received through our system.

For information regarding our different publishing models please see our Transformative Journals page. If you have any questions about costs, Open Access requirements, or our legal forms, please contact ASJournals@springernature.com.

Please use the following link for uploading these materials:

[REDACTED]

If you have any further questions, please feel free to contact me.

Best regards,

Ziqian Li
Editorial Assistant
Nature Cell Biology

On behalf of

Zhe Wang, PhD
Senior Editor
Nature Cell Biology

Tel: +44 (0) 207 843 4924
email: zhe.wang@nature.com

Reviewer #1:

Remarks to the Author:

The authors have satisfactorily addressed my comments.

Reviewer #2:

Remarks to the Author:

The authors addressed all my questions.

Reviewer #3:

Remarks to the Author:

The revised manuscript addresses the majority of the points raised by the reviewers and provides new insights into breast cancer metastasis. It is somewhat disappointing that the revision did not include more insight into mechanisms that regulate ROS generation. In the authors words "How, exactly, this results in elevated tumoral mitochondrial ROS remains an open question, as does the specific effect(s) ROS has within the mitochondria to suppress DTC proliferation."

Final Decision Letter:

Subject: Decision on Nature Cell Biology submission NCB-G45082B

Message:

Dear Cyrus,

I'm writing on behalf of my colleague, Zhe Wang, who is out of the office.

I am pleased to inform you that your manuscript, "Unchecked oxidative stress in skeletal muscle prevents outgrowth of disseminated tumour cells", has now been accepted for publication in Nature Cell Biology.

Thank you for sending us the final manuscript files to be processed for print and online production, and for returning the manuscript checklists and other forms. Your manuscript will now be passed to our production team who will be in contact with you if there are any questions with the production quality of supplied figures and text.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Cell Biology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

After the grant of rights is completed, you will receive a link to your electronic proof via email with a request to make any corrections within 48 hours. If, when you receive your proof, you cannot meet this deadline, please inform us at rjsproduction@springernature.com immediately.

You will not receive your proofs until the publishing agreement has been received through our system.

Due to the importance of these deadlines, we ask that you please let us know now whether you will be difficult to contact over the next month. If this is the case, we ask you provide us with the contact information (email, phone and fax) of someone who will be able to check the proofs on your behalf, and who will be available to address any last-minute problems.

If you have any questions about our publishing options, costs, Open Access requirements, or our legal forms, please contact ASJournals@springernature.com

Once your paper has been scheduled for online publication, the Nature press office will be in touch to confirm the details. An online order form for reprints of your paper is available at <https://www.nature.com/reprints/author-reprints.html>. All co-authors, authors' institutions and authors' funding agencies can order reprints using the form appropriate to their geographical region.

Publication is conditional on the manuscript not being published elsewhere and on there being no announcement of this work to any media outlet until the online publication date in Nature Cell Biology.

Please note that Nature Cell Biology is a Transformative Journal (TJ). Authors may publish their research with us through the traditional subscription access route or make their paper immediately open access

through payment of an article-processing charge (APC). Authors will not be required to make a final decision about access to their article until it has been accepted. Find out more about Transformative Journals

Authors may need to take specific actions to achieve compliance with funder and institutional open access mandates. If your research is supported by a funder that requires immediate open access (e.g. according to Plan S principles) then you should select the gold OA route, and we will direct you to the compliant route where possible. For authors selecting the subscription publication route, the journal's standard licensing terms will need to be accepted, including self-archiving policies. Those licensing terms will supersede any other terms that the author or any third party may assert apply to any version of the manuscript.

To assist our authors in disseminating their research to the broader community, our SharedIt initiative provides you with a unique shareable link that will allow anyone (with or without a subscription) to read the published article. Recipients of the link with a subscription will also be able to download and print the PDF.

If your paper includes color figures, please be aware that in order to help cover some of the additional cost of four-color reproduction, Nature Research charges our authors a fee for the printing of their color figures. Please contact our offices for exact pricing and details.

As soon as your article is published, you will receive an automated email with your shareable link.

If you have not already done so, we strongly recommend that you upload the step-by-step protocols used in this manuscript to the Protocol Exchange (www.nature.com/protocolexchange), an open online resource established by Nature Protocols that allows researchers to share their detailed experimental know-how. All uploaded protocols are made freely available, assigned DOIs for ease of citation and are fully searchable through nature.com. Protocols and the Nature and Nature research journal papers in which they are used can be linked to one another, and this link is clearly and prominently visible in the online versions of both papers. Authors who performed the specific experiments can act as primary authors for the Protocol as they will be best placed to share the methodology details, but the Corresponding Author of the present research paper should be included as one of the authors. By uploading your Protocols to Protocol Exchange, you are enabling researchers to more readily reproduce or adapt the methodology you use, as well as increasing the visibility of your protocols and papers. You can also establish a dedicated page to collect your lab Protocols. Further information can be found at www.nature.com/protocolexchange/about

You can use a single sign-on for all your accounts, view the status of all your manuscript submissions and reviews, access usage statistics for your published articles and download a record of your refereeing activity for the Nature journals.

Please feel free to contact us if you have any questions.

All the best,

Christina

Christina Kary, PhD
Chief Editor
Nature Cell Biology
1 New York Plaza
Tel: +44 (0) 207 843 4924