Migration speed of captured breast cancer subpopulations correlates with metastatic fitness

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Original submission

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MS TITLE: Migration speed of captured breast cancer subpopulations predicts metastatic fitness

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ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This skillfully documented and nicely presented study addresses a very interesting question about whether the in vitro property of cell migration speed can predict the metastatic potential of cancer cells. The findings are statistically robust and convincing, though this reviewer has some concerns about the wording of their interpretations. Overall, the findings do support the notion that the migration speed of cancer cells can correlate at a population level with the extent of experimental metastasis, which is an interesting and useful conclusion. A key element that needs to be addressed, however, is whether this property is a key driver mechanistically, or whether it is a proxy or correlation but not a causal cellular property underlying metastasis. A discussion of the potential reservations listed below would strengthen this very solid and interesting paper.

Comments for the author

1. The authors should be commended for their in-depth quantification and careful conceptualization of experimental approaches in this manuscript, including the comparisons with random selection. It is very helpful for readers to be able to see detailed information about the underlying primary data, which importantly appear to indicate strongly that the conclusions are based on overall population behavior rather than identifying a clear mechanistic driver of metastasis especially for predicting behavior of each individual cell. In that regard, the title is rather objectionable in claiming that migration speed "predicts" metastatic fitness; is this true to any specific cell? A similar concern can be raised about the last sentence of the Abstract, stating that "a highly migratory phenotype alone confers increased fitness for metastasis." Basically, the authors appear to be conflating correlation with causation. It will be much safer and more accurate to use a term more like correlation then to basically claim that migration speed "alone" can predict metastatic fitness, which is a very complex phenomenon for which migration speed may be merely a correlated property or a proxy. As specific examples, the authors have identified but have not tested whether the significant cell size differences or especially the integrin expression differences could be the true drivers of metastatic capacity. These concerns do not require further experimentation if the authors would provide a more nuanced, scholarly discussion of the meaning of their findings.
2. The data presented show clearly that the authors are basing their conclusions on slight differences at a population level. Even though highly statistically significant at times due to the large number of cells evaluated, it seems quite clear from the curves and especially the violin plots that what is involved is a slight tendency rather than a clear mechanistic difference. That is, the "effect size" is quite small. It would be useful for the authors to indicate more clearly the differences in means or medians throughout the text, while including the two out of six selections in which there was no difference in subsequent migration speed. For example, the indication of a median 30% increase in average speed compares to a huge standard deviation. Again, these concerns could be resolved by a more explicit discussion of the findings.
Various minor points and questions:
3. On page 6, the authors refer to six replicates, but there may have actually been only two biologically independent replicates.
4. Were the differences in Figure 2d and e not statistically significant?
5. The authors normalized expression against alpha-tubulin, but was the level of this protein unaltered after selection, e.g., compared to GAPDH or ribosomal or other housekeeping proteins?
6. The finding of substantial elevation of the beta4 integrin is very interesting considering that the migration substrate was fibronectin, and its upregulation might be a key driver of enhanced metastasis. It is unfortunate that its role was not tested, but this reviewer would not require that for acceptance.
7. The average STED intensity was enhanced even though adhesion sizes were reduced. If standard confocal immunofluorescence is performed, is there a total reduction in paxillin staining?
8. Under Experimental procedures, please explain what "bootstrapping" is for naïve readers, and indicate which Triton-X was used.
9. Approximately how many cell passages occurred over the 36 days in Figure 1?
10. In terms of data availability, the authors feel that what they show in this manuscript is sufficient, but some might wish to see the primary data – are they available upon request?

Reviewer 2

Advance summary and potential significance to field

In this study the authors utilize single-cell magneto-optical capture to isolate migratory cells and profile them by RNAseq analysis.The authors did an analysis of the metastatic potential of these tumor cell population and identify several pathways upregulated in this population.

Comments for the author

There are main areas that need revision:
1) the authors claim that "very few attempts have been made to isolate metastatic cells". This is not the case, the authors obviate the extensive literature from the Condeelis lab that profile the migratory tumor cells from breast tumors in vivo and revealed key migratory pathways mediating metastasis. The authors have to revised that literature in depth and comment on that in the context on what has been already done. What are the new aspects that their study add to the previous study done? A discussion of those studies is important due to the nature of the paper they have done.
2) The study is largely descriptive. I will suggest the authors to perform functional experiments to further validate the phenotype. For example, can they KD any of the targets they found and reverse the phenotype? Can they transform the non motile population in a motile one by overexposing the integrins identified?
3) Images of lung lesions should be shown.
4) How many cells needs to be capture to generate the cell lines?

First revision

Author response to reviewers' comments

Dear editor,

We thank you and the reviewers for the constructive comments and criticisms that led to an improved version of our manuscript. We made changes in several sections of the text to nuance our statements, as recommended by the reviewers. Importantly, we have changed the title to address a concern regarding whether our main observation relating migration velocity and metastatic fitness can be claimed to be causal or rather is correlative. We also provided more information on size effects and comprehensive statistical information regarding population comparisons. We added several new references and discussion describing the work of by the Condeelis lab, as requested by the reviewers. Finally, we produced new data addressing the role of specific target genes identified in our analyses with respect to their importance in the *in vitro* migratory phenotype we observe.

A detailed, point-by-point response to the reviewer's comments follows.

Reviewer 1
1. The title is rather objectionable in claiming that migration speed "predicts" metastatic fitness; is this true to any specific cell? A similar concern can be raised about the last sentence of the Abstract, stating that "a highly migratory phenotype alone confers increased fitness for metastasis." Basically, the authors appear to be conflating correlation with causation. It will be much safer and more accurate to use a term more like correlation then to basically claim that migration speed "alone" can predict metastatic fitness, which is a very complex phenomenon for which migration speed may be merely a correlated property or a proxy. As specific examples, the authors have identified but have not tested whether the significant cell size differences or especially the integrin expression differences could be the true drivers of metastatic capacity.

These concerns do not require further experimentation if the authors would provide a more nuanced, scholarly discussion of the meaning of their findings.

We thank the reviewer for these comments. We have rephrased several sections of the revised manuscript to ensure that our observations are not overinterpreted as a proof of causation. Enhanced metastatic potential was observed after selecting cells solely based on a migration phenotype, but we do not claim that migration capacity alone confers fitness. The new title makes this more explicit, as suggested by the reviewer:

"*Migration speed of captured breast cancer subpopulations correlates with metastatic fitness"*

The last line of the abstract has also been modified accordingly:

"Thus, the subpopulations selected for their highly migratory phenotype demonstrated an increased fitness for metastasis.

Finally, we modified the 6th paragraph in the Discussion section to nuance our opinion

"Differences in cell migration phenotypes between captured and parental cells were associated with rapid turnover of smaller cell-matrix adhesions within the fast population resulting in average earlier FA maturation stage. […]"

2. The data presented show clearly that the authors are basing their conclusions on slight differences at a population level. Even though highly statistically significant at times due to the large number of cells evaluated, it seems quite clear from the curves and especially the violin plots that what is involved is a slight tendency rather than a clear mechanistic difference. That is, the "effect size" is quite small. It would be useful for the authors to indicate more clearly the differences in means or medians throughout the text, while including the two out of six selections in which there was no difference in subsequent migration speed. For example, the indication of a median 30% increase in average speed compares to a huge standard deviation. Again, these concerns could be resolved by a more explicit discussion of the findings.

In response to the reviewer's concerns, the effect size has now added throughout the Results section and calculated as follow: $\frac{m_1 - m_2}{std}$ where m_x stands for the median and *std,* the average of both standard deviations. Following the classification provided in (Sullivan & Feinn, 2012), the average speed and d_{max} differences between fast and parental populations would be classified as large effect sizes. An average median effect size of 0.51 was calculated for capture replicates for fast cells. The modifications are contained in the 2nd and 3rd paragraph of the Results section.

[…] In particular, the distribution's median for d_{max} and average speed remained 62% (ES = 0.82) and 77% (ES = 1.19) greater for the fast cells compared to the parental cells, respectively. […]

[…] While new cell populations displayed a wide distribution of velocities, a median 30% increase (ES = 0.51) in average speed remained consistent over 3 weeks in culture (Fig. 1E and Fig. S2A).

A similar exercise revealed a small effect size for the amount of paxillin clusters and distance for nearest neighbors, a medium effect size for tested intensity, and a very small effect size for the total confocal intensity per FA. This suggests that the total amount of paxillin engaged in an FA is independent of FA size, and hence its concentration increases. We thus modified the text from the 8th paragraph of the Results section:

[…] Immunofluorescence images of endogenous paxillin acquired with confocal microscopy revealed that fast cells possessed significantly smaller ($p < 0.0001$, ES = 0.40) adhesions than parental cells on fibronectin (~0.42 μm^2 compared to ~0.50 μm^2, respectively) and on laminin ($p < 0.0001$, ES = 0.52) (~0.60 μm^2 compared to ~0.65 μm^2, respectively). Focal adhesions were also significantly

smaller on fibronectin than on laminin ($p < 0.0001$, ES = 0.55). To resolve the size and structure of small, nascent adhesions, super-resolution stimulated emission depletion (STED) microscopy was employed (Fig. 3F and G). STED imaging of endogenous paxillin revealed that fast cells possessed 1.8 times fewer ($p < 0.0001$, ES = 0.2) paxillin clusters per focal adhesion area (~50 clusters μm^2 for fast cells and ~89 for parental cells) (Fig. 3H). Adhesion clusters in fast cells were on average 1.2 times closer to each other when compared to parental ($p < 0.0001$, ES = 0.42), with average nearest-neighbor distances of 0.14 µm in fast cells compared to 0.18 µm for parental cells (Fig. 3I). […] Also, the difference in total confocal intensities over all FAs was significantly higher for fast cells with a very small effect size ($p < 0.0001$, ES = 0.06), suggesting that the total amount of paxillin is independent of the FA size and that its concentration decreases when FA matures. Hence, fast cells rapid turnover do not allow complete maturation of FA.

Finally, small effect sizes were calculated for the difference of cell spread area and volume, while a medium effect size was found for the difference in aspect ratio (height / area). The results are available in the 9th paragraph of the Results section.

[…] Fast cells exhibited ~24% smaller volume (median of 4077 compared to 5338 μm^3, ES = 0.18, $p = 7 \times 10^{-3}$), 27% smaller contact area with the substrate (median of 593 compared to 809 μm^2, ES = 0.18, $p = 5 \times 10^{-3}$), and 28% greater aspect ratio (height/area) (ES = 0.55, $p = 9 \times 10^{-5}$) (Fig. 3M-O), suggesting that the fast cells are more contractile. […]

3. On page 6, the authors refer to six replicates, but there may have actually been only two biologically independent replicates.

We thank the reviewer for pointing out this error. The text was modified accordingly in the 4th paragraph of the Results section.

[…] We observed a high degree of concordance between the RNA-seq data generated from ~~six~~ two independent batches comprising three technical replicates of fast cell populations, confirming that the phenotype obtained after laser capture is stable. […]

4. Were the differences in Figure 2d and e not statistically significant?

We thank the reviewer for this question. According to the sequencing data available in Supplementary Table 1, all the differences presented in Figure 2d and e are all significant, and the figure has been modified accordingly in the revised manuscript.

5. The authors normalized expression against alpha-tubulin, but was the level of this protein unaltered after selection, e.g., compared to GAPDH or ribosomal or other housekeeping proteins?

In response to the reviewer's question, we tested three independent loading controls (a-Tubulin, GAPDH and a-Adaptin) and observed no difference in any of these proteins between the parental and fast-selected MDA-MB-231 cells.

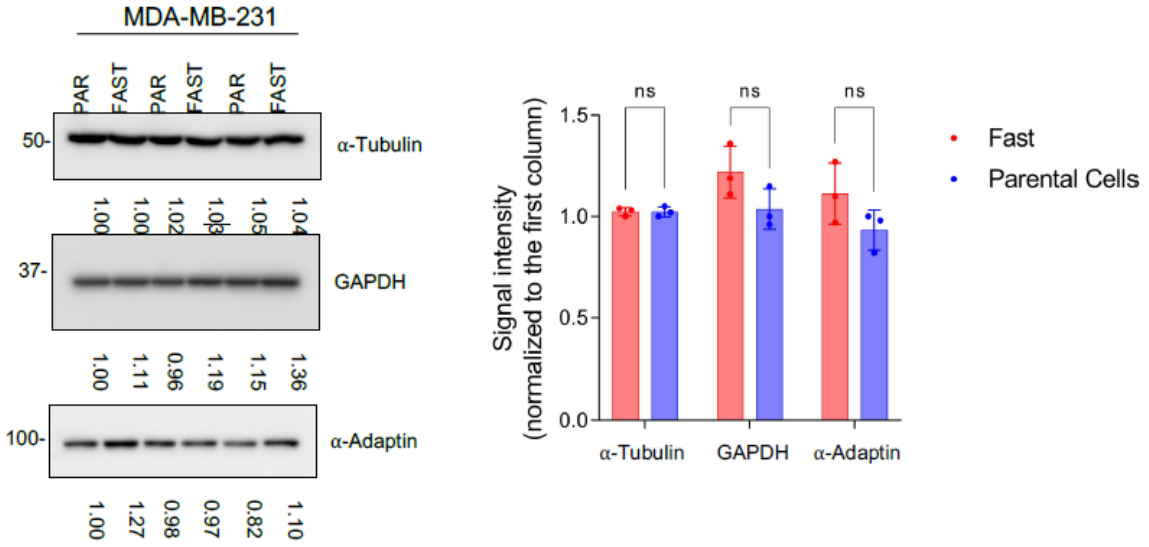

Figure 1:Housekeeping protein levels for parental and fast cells by immunoblot and its quantification.

6. The finding of substantial elevation of the beta4 integrin is very interesting considering that the migration substrate was fibronectin, and its upregulation might be a key driver of enhanced metastasis. It is unfortunate that its role was not tested, but this reviewer would not require that for acceptance.

The impact of integrin beta4 on *in vitro* motility was tested in response to another reviewer. Please refer to the response to point 2 for reviewer #2.

7. The average STED intensity was enhanced even though adhesion sizes were reduced. If standard confocal immunofluorescence is performed, is there a total reduction in paxillin staining?

We thank the reviewer for this question. The total confocal intensity over focal adhesions was indeed significantly higher, but the effect size was small for the fast MDA-MB-231 cells (2.5% interval of confidence at 0.41 after the difference on 10000 bootstrapped medians, effect size of 0.06). The images used for confocal quantification were acquired at the same time as the STED images. Figure 2 illustrates the process.

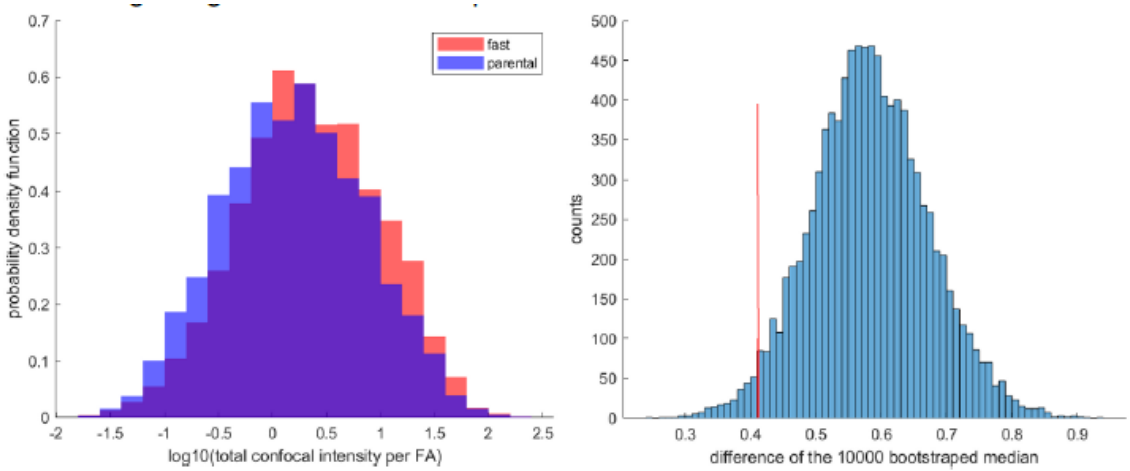

Figure 2 : Total confocal intensity over focal adhesions. A) Distribution of averages per cell B) distribution of the bootstraped difference over the medians. The red line indicates 2.5% of the distribution.

8. Under Experimental procedures, please explain what "bootstrapping" is for naïve readers, and indicate which Triton-X was used.

We apologize to the reviewer for the lack of a clear description for this term. The text has been modified accordingly in the 1st and 7th paragraph in the Experimental procedures section of the revised manuscript.

[…] Since each tracking experiment quantified about 8,000 cells per condition, we used bootstrapping to perform the statistical analysis, which consists of measuring the probability of the null hypothesis by numerically replicating the experiments 1,500 times using resampled datasets from the original data. In other words, the two populations were compared thousands of times by randomly labeling cells to assess whether differences observed experimentally were significant […]

[…] Cells were than fixed with PBS 3% PFA and 0.1% Triton X-100 and rinsed with PBS with 3.8 mg mL^{-1} of glycine. Cells were incubated overnight at 4°C with 1:200 purified mouse anti- paxillin (BD Transduction Laboratories) in 90% PBS, 10% FBS, 0.05% Triton X-100 and 10 mg mL^{-1} BSA. […]

9. Approximately how many cell passages occurred over the 36 days in Figure 1?

We thank the reviewer for this question. Considering a doubling time of about 40 hours and a passaging frequency of 2-3 days, we estimate approximately ~15 passages occurred during the 36 days. This information is now provided in the legend for Figure 1 in the revised manuscript.

[…] E, Expanded cells were characterized at several time points after capture and exhibited more than 1.3 times larger d_{max} ($p < 0.0001$, 1500 bootstrap samples) compared to the parental cell population. Captured cells were more than 2 times faster ($p < 0.0001$, 1500 bootstrap samples) and maintained their phenotype after several cell divisions over a period greater than a month (approximately 20 cell divisions and 14 passages occurred in 36 days).

10. In terms of data availability, the authors feel that what they show in this manuscript is sufficient, but some might wish to see the primary data – are they available upon request?

For a single tracking experiment, about 7.2 Go of data is produced and more than 10 tracking experiments were performed for the manuscript. It is hard to find a way to permanently share such a large amount of images in a public repository. However, the data is available upon request to the corresponding authors. We have added a comment regarding access to the single cell tracking data in the data availability section.

Reviewer 2

1. the authors claim that "very few attempts have been made to isolate metastatic cells". This is not the case, the authors obviate the extensive literature from the Condeelis lab that profile the migratory tumor cells from breast tumors in vivo and revealed key migratory pathways mediating metastasis. The authors have to revised that literature in depth and comment on that in the context on what has been already done. What are the new aspects that their study add to the previous study done? A discussion of those studies is important due to the nature of the paper they have done.

We thank the reviewer for this comment. We meant to indicate that few attempts have been made to isolate metastatic cancer cells solely based on *in vitro* phenotypes. As the reviewer points out, there is many examples in the literature where cellular interactions within the tumor microenvironment have been shown to induce cancer cell migration, invasion, and metastasis (work from the Condeelis' laboratory being an excellent example). We have modified the discussion section of the revised manuscript to address this point.

2. The study is largely descriptive. I will suggest the authors to perform functional experiments to further validate the phenotype. For example, can they KD any of the targets they found and reverse the phenotype? Can they transform the non motile population in a motile one by

overexposing the integrins identified?

We thank the reviewer for this important suggestion. To address this comment, we performed transient knockdowns of 6 targets in the fast-selected MDA-MB-231 cells and assessed the impact on cancer cell motility *in vitro*. The data is included in Figure 6 of the revised manuscript. Two of the six candidates (KLF4 and L1CAM) revealed an impairment in cell migration when their expression levels were reduced. These two targets (KLF4 and L1CAM) were then knocked down in the parental MDA-MB-231 cells, which failed to alter cell migration. This data is described in the results section, entitled '***Two targets revert the fast migration phenotype'***.

3. Images of lung lesions should be shown.

In response to the reviewer's request, we now show representative lung and liver metastatic lesions in the new Supplementary Fig. S6 of the revised manuscript (both H&E and vimentin- stained sections highlighting the metastatic lesions are provided). The reference has been added to the main text at page 10.

4. How many cells needs to be capture to generate the cell lines?

We thank the reviewer for this question. In order to improve the throughput of our approach, and due to plating efficiency, in this study, we captured tens to a few hundreds of cells to generate new cell populations. The cell lines we produced are thus not clonal. This may explain, in part, the wide distribution of migratory phenotypes of the new lines, but should ultimately improve the probability of finding pathways that are common to a larger number of cells, rather than serendipitous singularities. To clarify this, we added a line in the 2nd paragraph of the Results section in the revised manuscript:

To assess whether a fast-motility phenotype can be retained over time, highly motile cells were automatically detected, illuminated, captured, and expanded. A total of 100 to 250 cells were effectively captured when the experiment was replicated. After a period of 20 days in culture, several motility features were retained (Fig. 1D). […]

References

Sullivan, G. M., & Feinn, R. (2012). Using effect size—or why the P value is not enough. *Journal of graduate medical education*, *4*(3), 279-282.

Second decision letter

MS ID#: JOCES/2022/260835

MS TITLE: Migration speed of captured breast cancer subpopulations correlates with metastatic fitness

AUTHORS: Nicolas Desjardins-Lecavalier, Matthew Annis, Alex Nowakowski, Alexander Kiepas, Loic Binan, Joannie Roy, Graziana Modica, Steven Hebert, Claudia Laura Kleinman, Peter Siegel, and Santiago Costantino

ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks. Please see the comment from referee #2 about not seeing images of the lung and liver metastasis in the revised manuscript although they were mention to be in fig s6.

Reviewer 1

Advance summary and potential significance to field

See original review.

Comments for the author

The authors have responded fully and appropriately to my concerns and suggestions.
This paper now appears appropriate for acceptance for publication in JCS.

Reviewer 2

Advance summary and potential significance to field

The authors satisfactory answer my comments/ concerns. However, I was not able to find the images of the lung and liver metastasis in the revised manuscript although they were mention to be in fig s6.

I dont have any further questions. Congratulations on the great work!

Comments for the author

The authors satisfactory answer my comments/ concerns. However, I was not able to find the images of the lung and liver metastasis in the revised manuscript although they were mention to be in fig s6.

I dont have any further questions. Congratulations on the great work!