Single-cell analysis reveals Comma-1D as a unique cell model for mammary gland development and breast cancer

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Original submission

First decision letter

MS ID#: JOCES/2021/259329

MS TITLE: Single-cell analysis reveals Comma-1D as a unique cell model for mammary gland development and breast cancer

AUTHORS: Rachel Werner, Erin Nekritz, Koon-Kiu Yan, Bensheng Ju, Bridget Shaner, Jonh Easton, Jiyang Yu, and jose silva
ARTICLE TYPE: Tools and Resources

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 share enthusiasm for the study and raise a number of substantial criticisms that prevent me from accepting the paper at this stage. In addition to a number of focused points, they emphasize that the biological significance of these subpopulations would be greatly clarified if their in vivo behavior after transplantation and in vitro response to milk-stimulating hormones were defined (please let me know if you need a reference for in vitro hormone conditions).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.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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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

The single cell analysis of Comma-1D cell line provides a detailed characterization of these cells as exponentially growing cells on a plastic substratum and nicely complements what is already known in the literature from several decades of research using this cell line. On the whole the studies are carefully performed and provide some new insights.

Comments for the author

However, the authors somewhat overstate the significance of their studies when they state, “Overall, these results reveal that the heterogeneity of Comma-1D cells can be used to study how the response to cancer-promoting mutations is influenced by the cellular fingerprint of cell subtypes and how different alterations impact the tumor phenotype.” One key omission in this manuscript were transplantation studies of the isolated cell populations back into the cleared mammary fat pad to determine their morphological and differentiation potential in vivo. Do these isolated populations form ductal or alveolar outgrowths and how do they respond to hormonal stimulation? For example, the Comma-1D cells have been studied extensively with respect to the induction of milk protein gene expression, but no information about this terminal differentiation process was included in this study. When the isolated cell population are cultured as organoids have the authors looked at hormonal regulation of milk protein gene expression. Continual passage of these cell lines has been reported to cause them to lose their hormonal responsiveness. Accordingly to the Methods these cells were obtained from the ATCC. No information about passage number was included. For the studies of activated Her2 the authors need to demonstrate that these “transformed” cells form mammary adenocarcinomas in vivo and compare them to their histology and gene expression with published results for the mouse and human Her2 tumors. In some previous studies transplanted Comma-1D cells formed sarcomas and not adenocarcinomas.

Reviewer 2

Advance summary and potential significance to field

This manuscript explores the heterogeneity of Comma-1D mouse mammary epithelial cell line by single-cell sequencing, uncovering three cell populations. They appear to possess different stemness features, cellular and molecular activities, as well as sensitivities to oncogenic transformation.
The authors claimed that this tissue-derived cell line is a suitable experimental model to investigate mammary epithelial differentiation and tumorigenesis. This claim would be better supported if the following points can be addressed.

Comments for the author

Major points:
1. In Fig. 1A, how are cell identities annotated? There is small cell cluster distinct from either “C1D-L” or “C1D-B”. According to the tSNE plot it is a mixture of “C1D-L” and “C1D-B” cells. Is it possible to examine its basal and luminal scores as well? Could it be a fourth population”?
2. In Fig. 2B, please include the gene expression heatmap, not just the hierarchical clustering structure.

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3. Related to Fig. 2E in the sorted C1D-L population, please explain why the flow cytometry results are different between 1 week in culture versus 2 weeks in culture?
4. The flow cytometry chart in Fig. 3A is the same as Fig. 2A.
5. Is the unsorted tSNE plot on the left-hand side in Fig. 3A the same dataset as in Fig. 1A? If so, please describe how it was generated following a different process.
6. Why is the percentage of C1D-bi population so low in the new sorted dataset in Fig. 3A? Did FACS achieve expected purity?

Minor points:
1. Since Sca-1 is used for FACS, it would be helpful to include its feature plot as well in either Fig. 1 or Fig. 2.
2. In Fig. 2B, is "EpCAM_L, Sca-1_H" mislabeled as "EpCAM_L, CD49f_H"?
3. In Fig. 2D, please indicate sample size (n) in each Matrigel organoids category and perform a statistical test.
4. Describe the process where you combine the two unsorted and sorted datasets (Fig. 3A). And did you see any batch effects?
5. Please describe how you perform gene set enrichment analysis (Fig. 5D) in the experimental procedures section.

First revision

Author response to reviewers' comments

Dear Editor,
First, we would like to thank the reviewers and the editorial office for their insightful and constructive comments. Following their suggestions, we provide a revised manuscript.
The reviewers agreed that our single-cell analysis of Comma-1D cell line provides a detailed characterization of these cells and uncovers distinct populations possessing different stemness features, cellular and molecular activities, as well as sensitivities to oncogenic transformation. To strengthen the manuscript, the main comment was to provide additional biological significance of these subpopulations in vivo. We have now included new figures including in vivo transplantation experiments supporting our previous finding in vitro and demonstrating that these populations have different behavior in vivo.

Bellow, we summarize a point-by-point response to each of the reviewers.

Point by point response to reviewers

Reviewer 1:

1- The authors somewhat overstate the significance of their studies when they state, "Overall, these results reveal that the heterogeneity of Comma-1D cells…"
Response: We have rephrased some of the statements to avoid overstatements.

2- One key omission in this manuscript were transplantation studies of the isolated cell populations back into the cleared mammary fat pad to determine their morphological and differentiation potential in vivo.
Response: We have performed additional experiments where individual populations were transplanted after FACS purification. Supporting our previous data, we observed that only the C1D-bi can fully phenocopy the reconstitution ability of the parental cultures. In contrast, isolated C1D-L and C1D-B presented a more limited reconstitution ability (see new Fig. 2F).

3- No information about passage number was included.
Response: Comma-1D cells were obtained years ago from Dr. Greg Hannon. After receiving these cells, they were used for an average of 10-15 passes before thawing a new batch. This information has been included in the updated material and method. We have also updated the acknowledgment section.

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4- For the studies of activated Her2 the authors need to demonstrate that these “transformed” cells form mammary adenocarcinomas in vivo…
Response: Epithelial tumor growth in vivo of Comma-1D cells transformed with HER2 has been previously reported (Xiang B et al. PNAS August 26, 2008 105 (34) 12463-1246). We have compared the tumor growth of fat pad transplanted unsorted parental Comma-1D and C1D-L expressing oncogenic HER2 (described in the main text) and both variants developed tumors compatible with epithelial poorly differentiated mammary cancers. This information has been included in the main text and in a new Fig. S5A).

5- ….and compare them to their histology and gene expression with published results for the mouse and human Her2 tumors.
Response: Comparison of C1D overexpressing oncogenic HER2 with published data sets have now been included in Fig. 5E-5F and Supp. Fig. 5B. The new results show that overexpression of oncogenic HER2 changes the transcriptome of C1D-L cells closer to HER2+ human and mouse tumor profiles. This effect is not seen in the other Comma-1D populations.

Reviewer 2:

1. In Fig. 1A, how are cell identities annotated? There is a small cell cluster distinct from either “C1D-L” or “C1D-B”. According to the tSNE plot, it is a mixture of “C1D-L” and “C1D-B” cells. Is it possible to examine its basal and luminal scores as well?
Response: The clusters were generated using Seurat based on high dimensional single-cell expression data. The major clusters are defined based on the well-known basal/luminal markers. Cells with similar expression profiles are clustered together. This approach identified the small group of cells mentioned by the reviewer as part of the largest luminal and basal clusters. The slight differences are based on the expression of genes non-related with basal and luminal differentiation and the scores of the different cells in the cluster (blue vs red) represent average scores observed in the corresponding larger luminal or basal populations.

2. In Fig. 2B, please include the gene expression heatmap, not just the hierarchical clustering structure.
Response: This has been included as supplementary material.

3. Related to Fig. 2E in the sorted C1D-L population, please explain why the flow cytometry results are different between 1 week in culture versus 2 weeks in culture?
Response: If the reviewer is referring to the difference in the percentage of the individual populations between weeks the reason is that this figure represents the evolution of individual FACS purified populations through time. It is expected that they present differences regarding the percentage of cells among the populations, especially in the case of C1D-bi as they generate the additional C1D-L and C1D-B.
If the reviewer is referring to the slight shift of the C1D-B, there are several potential reasons. The evolution of the populations until reaching equilibrium again may slightly affect the levels of expression of the markers used. Additionally, slight compensation/staining differences can bring the lower C1D-B population closer or farther to the upper ones. However, this does not affect the ability to clearly identify high vs low expressions of EpCAM.

4. The flow cytometry chart in Fig. 3A is the same as Fig. 2A.
Response: That is correct. This is because a complete set of experiments to obtain data for the two figures was run from the same batch to avoid variability.

5. Is the unsorted tSNE plot on the left-hand side in Fig. 3A the same dataset as in Fig. 1A? If so, please describe how it was generated following a different process.
Response: That is correct. Both tSNE plots were generated using the tool Seurat. Even though the tSNE plot in Fig. 3A was generated by a slightly different process (see below the response to Minor point 4), the difference between the two layouts is just because Fig 1A was generated using the unsorted cells alone. In Fig. 3 unsorted cells and sorted cells were pulled together in the pipeline to generate the plot on the right. The unsorted cells were then subsetted in the same layout (left) to facilitate comparison between left and right.

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6. Why is the percentage of C1D-bi population so low in the new sorted dataset in Fig. 3A? Did FACS achieve expected purity?
Response: The percentage of C1D-bi cells ranges between 1% up to 4% with the main part of the sortings having between 1 to 2%. When considering all the populations, this does not represent major variations, but it may mean a few folds in the number of C1d-bi obtained between experiments. Additionally, the separation between C1D-b and the rest is very profound, but the separation between C1D-bi and C1D-L is not such clear cut and slight technical variations between the experiments may affect the recovery of low percentage populations such as C1D-bi.

Minor points:
1. Since Sca-1 is used for FACS, it would be helpful to include its feature plot as well in either Fig. 1 or Fig. 2.
Response: The expression plot of both Sca1 genes expressed (Ly-6A and E) is included in Fig. Suppl. 1B.

2. In Fig. 2B, is “EpCAM_L, Sca-1_H” mislabeled as “EpCAM_L, CD49f_H”?
Response: This was a typo, thanks for bringing this to our attention. It has been corrected.

3. In Fig. 2D, please indicate sample size (n) in each Matrigel organoids category and perform a statistical test.
Response: The information has been now included in the legend of the figure. The result indicates the combination of n=3 different experimental replicates with >100 spheroids counted for each population in each of the replicates. The p-value was calculated by Anova.

4. Describe the process where you combine the two unsorted and sorted datasets (Fig. 3A). And did you see any batch effects?
Response: The unsorted and sorted datasets were combined using the scRNA-seq integration pipeline described in the tool Seurat. The pipeline matches and aligns shared cell populations across datasets and corrects for batch effects by identifying a set of anchors. As seen from the right panel of Fig. 3A, the proper alignment of cell types shows that batch effects were corrected.

5. Please describe how you perform gene set enrichment analysis (Fig. 5D) in the experimental procedures section.
Response: Differential expression for each of the 3 HER2-transformed cell populations and their corresponding controls was estimated using the tool GFOLD. The enrichment of up-regulated genes and down-regulated genes among the MSigDB gene sets (v7.1) were examined using the tool fgsea (https://github.com/ctlab/fgsea). Gene sets enriched with the up (down) -regulated genes will receive positive (negative) normalized enrichment score (NES) with significant P-values. This information has been included in the updated material and methods.

Second decision letter

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AUTHORS: Rachel Werner, Erin Nekritz, Koon-Kiu Yan, Bensheng Ju, Bridget Shaner, Jonh Easton, Jiyang Yu, and jose silva
ARTICLE TYPE: Tools and Resources

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.)

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As you will see, Reviewer 2 is fully satisfied and Reviewer 1 raises two points. The first is that Dr. Medina originally isolated this line; I am confused by that comment as you clearly cite Dr. Medina as establishing the line. You can ignore that comment. I think the point that cell lines can diverge over time and so this scRNA-seq analysis is most useful if others can access the specific cells you have. The only revision I would like to see is a plan for dissemination of these cells, preferably through a public repository such as ATCC. I hope that you will be able to carry these out because I would like to be able to accept your paper. Please explain this plan in the manuscript and the cover letter so that I can assess it directly, without returning to 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

The main advance here is the scRNA characterization of the COMMA-D cell line. There is some useful information here. Whether this cell line will provide a useful tool for studying oncogene effects is less clear and the Her2 experiments added little novelty to this study.

Comments for the author

The authors have performed the necessary transplantation experiments with the different COMMA-D sublines, but unfortunately they only looked at the whole mounts for alveolar development during pregnancy. This is not sufficient to determine the hormonal effects on differentiation including milk protein and lipid gene expression. The presence of an alveolar structure per se is not sufficient to conclude which of the cells have differentiation potential.
The other major concern is the source and availability of these cells.
Early studies from the Bissell laboratory isolated sublines of COMMA-D cells by differential plating and looked at casein expression. Hormonal regulation in these cells is quite variable as they are passaged, so it is critical to maintain early passage lines for reproducibility of these experiments.
The Medina and not the Hannon laboratory was not the developer of the Comma-D cells. If other investigators are to benefit from these studies, these cells need to be available from a repository like ATCC. This is critical if other investigators are to use these cells.

Reviewer 2

Advance summary and potential significance to field

The authors have adequately addressed my previous points.

Comments for the author

N/A

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Second revision

Author response to reviewers' comments

Reviewer 1:
Q1- "…they only looked at the whole mounts for alveolar development during pregnancy…. The presence of an alveolar structure per se is not sufficient to conclude which of the cells have differentiation potential".
Response: In Supplementary Fig.2C we also provide direct visualization of the transplanted fat pads clearly showing the white appearance of the alveoli. This appearance is characteristic because it is filled with milk. This is unambiguous evidence of the differentiation potential of some of the C-1D transplanted subpopulations in vivo. We have now added a line in the legend of the figure highlighting this fact.

Q2- "…The Medina and not the Hannon laboratory was the developer of the Comma-D cells. If other investigators are to benefit from these studies, these cells need to be available…"
Response: I obtained this cell line through Dr Hannon (I was a postdoc in his laboratory) during a collaboration with Dr. Muthuswamy. As described in Cell. 2008 Nov 28; 135(5): 865–878, Dr. Muthuswamy got the cell line directly from the Medina laboratory. Thus, this is the original line described by Dr. Medina.
As this is the original Medina line, any decision regarding vendors is for him to make, but we are happy to share completely free our sc-RNA-seq-characterized culture stocks with any investigator that requests it through collaborations. The full origin in Medina's laboratory of the cell line and our willingness to share the line through direct collaborations is now explained in the material and method section.

Third decision letter

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MS TITLE: Single-cell analysis reveals Comma-1D as a unique cell model for mammary gland development and breast cancer

AUTHORS: Rachel Werner, Erin Nekritz, Koon-Kiu Yan, Bensheng Ju, Bridget Shaner, Jonh Easton, Jiyang Yu, and jose silva
ARTICLE TYPE: Tools and Resources

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

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