Suppression of α-catenin and adherens junctions enhances epithelial cell proliferation and motility via TACE-mediated TGF-α autocrine/paracrine signaling

Eric Bunker, Graycen Wheeler, Douglas Chapnick, and Xuedong Liu

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Review Timeline:

| Event                      | Date       |
|----------------------------|------------|
| Submission Date            | 2019-08-27 |
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| Editorial Decision         | 2020-11-19 |
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Editor-in-Chief: David Drubin

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
RE: E19-08-0474
TITLE: Suppression of α-Catenin and Adherens Junctions Enhances Epithelial Cell Proliferation and Motility via Elevated TACE-Mediated TGF-α Autocrine/Paracrine Signaling

Dear Dr. Liu:

Your manuscript referenced above has been seen by two referees, whose verbatim comments are attached. As you can see from their comments, both referees felt that your findings would be of interest to our MBC readership. However, both also felt that there are important deficiencies in the story that need to be addressed before the paper can be accepted for publication. In particular, both reviewers indicated that a careful statistical analysis of all the data should be performed and included in the paper. Additional controls, such as those in shRNA studies, should be added to further strengthen the paper.

Based on their recommendation, I am afraid that I cannot recommend your paper to be accepted in the current form. However, I will be happy to receive a revised manuscript after you have carefully addressed all the concerns of the two reviewers. The revised paper will be sent back for re-review.

Kunxin Luo

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Dear Dr. Liu:

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made. Any specific areas to be addressed are outlined in the reviewer comments included below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision online please use the link below, and include a cover letter that details, point-by-point, how the Monitoring Editor's and reviewers comments have been addressed. When entering the author names online, enter them exactly as they appear on the manuscript title page. Please send only the latest revised manuscript. DO NOT resend any previous versions. Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers, when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

To prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision.
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Figure Size. Prepare figures at the size they are to be published.

1 column wide: Figure width should be 4.23-8.47 cm
1 to 1.5 columns wide: Figure width should be 10.16-13.3 cm
2 columns wide: Figure width should be 14.4-17.57 cm

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Resolution and Color Mode.
All images should be submitted at a minimum of 300dpi.
Save all color figures in RGB mode at 8 bits/channel.
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File Size. Final figures should be <10 MB in size. Figures larger than 10 MB are likely to be returned for modification. Tips for managing file sizes:
1. crop out all extraneous white space
2. RGB color mode for color images, Grayscale for images not containing color
3. avoid excessive use of imbedded color
4. select the LZW compression option when saving tif files in Photoshop, this is a lossless compression mechanism

Locants and Labels. Locants and labels can be between 1.5 and 2 mm high. Wherever possible, place locants and labels within the figures.

Line Images. Prepare line drawings at one-column width (less than 8.47 cm) or less if the graph or histogram is relatively simple. Symbols should be at least 1 mm high and large enough to be distinguishable from the lines connecting them.

To submit the cover letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,
Reviewer #1 (Remarks to the Author):

In this manuscript that authors investigate the role of the metalloprotease ADAM17/TACE in regulating EGFR signaling and downstream biology in keratinocytes. They demonstrate that depletion of α-catenin perturbs adherens junctions in keratinocytes resulting in increased cell proliferation and motility, largely independent of exogenous growth factors. They go on to demonstrate that depletion of α-catenin increases autocrine/paracrine release of the EGFR ligand, TGF-α, in a TACE-dependent manner. Further they demonstrate using TGF-α shedding and ERK biosensors that they have developed, that proliferating keratinocyte epithelial sheets and cell clusters have waves of oscillatory ERK activity, which is abrogated by TACE CRISPR knockout. The authors conclude that these waves of oscillatory ERK activity depend on autocrine/paracrine signals produced by TACE to control cell proliferation and cell migration. These results are of interest and are largely well done.

My main concern is the data are lacking in statistical analysis to assess virtually all results. In addition, the shRNA studies are done without appropriate controls for off-target effects.

Additional specific concern
Figure 7D. What are the effects of loss of TACE on cell migration and ERK activation induced by depletion of α-catenin? Are these effects rescued in the TACE rescue?

Reviewer #2 (Remarks to the Author):

Bunker, et al. detail a series of experiments examining TACE regulation of TGFα release, downstream ERK signaling, and eventual impacts on proliferation/migration of HaCaT keratinocytes. The experiments appear to have been conducted with appropriate rigor, and the findings are consistent with observations in other cell types (namely that TACE regulates TGFα release and TGFα stimulation leads to ERK pulsing). The authors also examine the regulation of TACE in response to lose of cell adhesion junctions. These experiments identify a relationship between α-catenin and TACE, but do not dig into the mechanism linking these two pathways.

The paper is difficult to follow in some places due to these two stories, which are not connected as clearly as they could perhaps be. Additionally, there are several conclusions stated that are not fully supported by the data (primarily because complementary experiments to rule out alternate hypotheses are not included).

Major Concerns
1. Statistical tests do not appear to be used. N's presented are not always clear (in the case of technical replicates + biological replicates, what average is presented, etc)
2. The presented studies do not clearly link ERK to proliferation/migration as no other pathways are invested and no downstream inhibition is used as a test. If the focus is on the upstream regulation, this is fine but there are multiple statements to the effect that TACE/TGF leads to ERK leads to proliferation, without a test of the last 'leads to' independent of the first part of the statement (and therefore these statements should be removed or actually tested).
3. ERK pulsing and migration were not examined for TACE rescue cells - this information would strengthen the claim that TACE leads to pulsing ERK and ultimately migration (whether through ERK or another path, since that is not established by any experiment shown in the paper)
4. The conclusion that an effect was seen with TACE inhibitor means that all other MMPs are not involved is unsupported by any experiment and should be removed.
5. The major novel findings seem to be related to the impact of loss of a-catenin on TACE, but it is not clear if this impacts TACE levels (qRT-PCR/WB) or localization or some other element of activity. Speculation of the mechanism makes up the majority of the discussion.
6. There is a lot of mention of ERK feedback to TACE, but there do not appear to be experiments examining this possibility (e.g. adding MEK inhibitor at some point, etc). This makes the story very confusing, as the finding that TACE leads to ERK pulsing does not automatically require that ERK feeds back to TACE itself.
7. The new biosensor is not completely validated - do the TGFa cleavage rates correspond to the normal cells (see for example Joslin J Cell Science 2007 for methods). Are the cells also producing endogenous TGFa? If so, does the increase in TGFa levels impact trafficking/signaling/behavior?
8. The introduction and discussion state that proliferation is essential for wound closure. This statement is not supported by data demonstrating normal/elevated proliferation in non-healing wounds (Usui J Histochem Cytochem 2008), proliferation is not observed in the early days of normal wound healing (Usui Wound Repair Regen 2005), and that inhibition of proliferation does not prevent collective closure in vitro (Wickert Sci Reports 2016)

Minor Concerns
1. Figure axis labels and other labels are very small, even when printed at full size.
2. Fig 5A legend says +/- TGFb but that doesn't appear to be what is shown.
3. Fig. 5B,C are unlabeled images so not 100% sure what they say
4. More detail is needed related to the pulse finding algorithm - the metrics used can dramatically alter the results obtained.
5. Fig. 6B,C aren't entirely clear - is that an image of a single time? Or a heat map of a timecourse? The latter would seem more appropriate? In general figure legends could be more detailed.
6. Cell line authentication/mycoplasma details not provided as required by journal
Response to reviewer’s comments

First, we want to thank the reviewers for your recognitions of the potential significance of our work. We are very grateful to the reviewers and the editorial team for their insightful comments, which have helped us strengthen this study. The detailed guidance is very much appreciated. Below is our point-by-point response to reviewers’ critiques.

Reviewer 1

My main concern is the data are lacking in statistical analysis to assess virtually all results. In addition, the shRNA studies are done without appropriate controls for off-target effects.

Answer: We have performed statistical student’s t-tests and included n-values for each graph included in this manuscript. We have included n-values for all experiments and performed student’s t-tests for data at the 6-hour timepoint. All shRNA studies were performed in parallel with non-targeting shRNA controls to account for off-target effects. Additionally, these knockdown experiments were complemented with chemical inhibition to confirm that TACE was required for the effects of the knockdowns.

Figure 7D. What are the effects of loss of TACE on cell migration and ERK activation induced by depletion of α-catenin? Are these effects rescued in the TACE rescue?

Answer: Depletion of α-catenin elevates intracellular ERK activity (Figure 5B) and speed of cell migration (Figure 5D). We also tested the effect of loss of α-catenin on cell migration and the requirement of ERK in TACE knockout cells. As shown in Figure S2, in TACE-null cells, depletion of α-catenin has little effect on cell migration. Inhibition of ERK activity by CI-1040 (5 µM) does not significantly change cell migration speed of TACE-null cells with or without α-catenin depletion. In TACE rescue cells, depletion of α-catenin increases cell migration speed compared with control shRNA and TACE null cells. The increase in cell migration requires ERK activation as CI-1040 treatment suppresses cell migration in TACE rescue cells. The measured average cell migration speed in Figure S2 is higher than Figure 5D. This is in part due to the inclusion of EGF and serum in the media to keep TACE-null cells viable and incomplete washout of EGF and serum prior to imaging cell migration.

Reviewer 2

The paper is difficult to follow in some places due to these two stories, which are not connected as clearly as they could perhaps be. Additionally, there are several conclusions stated that are not fully supported by the data (primarily because complementary experiments to rule out alternate hypotheses are not included).

Answer: Thanks for pointing out the issues about readability of the manuscript. We have revised the manuscript extensively to improve the focus, clarity, and cohesion of the stories. We have restructured our conclusions so that they are fully supported by the data.

Major Concerns

1. Statistical tests do not appear to be used. N’s presented are not always clear (in the case of technical replicates + biological replicates, what average is presented, etc)

Answer: We have included clearer n-values for each experiment and student’s t-tests where appropriate, clearly denoted in the legend for each figure and in the methods section.
2. The presented studies do not clearly link ERK to proliferation/migration as no other pathways are invested and no downstream inhibition is used as a test. If the focus is on the upstream regulation, this is fine but there are multiple statements to the effect that TACE/TGF leads to ERK leads to proliferation, without a test of the last 'leads to' independent of the first part of the statement (and therefore these statements should be removed or actually tested).

**Answer:** The reviewer is correct. The focus of our studies is on the upstream regulation. The linkage between ERK and cell proliferation/migration has been probed extensively in the published literature. In our previous publication in MBoC (Chapnick and Liu, 2014), we established that ERK activation is necessary and sufficient to drive HaCaT cell migration using inducible constitutively active MEK and MEK inhibitors.

We have updated the manuscript to include more references to the existing body of research that relates ERK to proliferation and migration. We have included an experiment showing that the effect of TACE knockout on cell motility is dependent on the ERK signaling pathway (Figure S2).

3. **ERK pulsing and migration were not examined for TACE rescue cells - this information would strengthen the claim that TACE leads to pulsing ERK and ultimately migration (whether through ERK or another path, since that is not established by any experiment shown in the paper)**

**Answer:** Agreed. We have validated the TACE rescue cells using Western blot and cell proliferation assays and TACE inhibitor studies. In the revised manuscript, we have also included assays that measure cell motility in the TACE rescue cells (Figure 3D and Figure S2). Additionally, we have demonstrated that bulk ERK signal is restored upon TACE rescue (Figure 3C). Collectively, these studies affirm the specificity of TACE knockout. However, we have not been able to obtain compelling evidence on restoring ERK pulses in TACE-null cells due to technical problems. The TACE rescue cells were created by stably integration of TACE transgene in the knockout cells followed by drug resistance selection. The rescued cells are polyclonal and there are cell-cell variability in TACE expression levels. To measure ERK pulsing, we stably transfected EKAR expression vector in the TACE rescue cell pool. The sequential transfection process to create TACE null EKAR reporter cell line resulted in compounding variabilities in TACE and EKAR expression. When these cells were analyzed for bulk ERK activity, we saw higher bulk ERK activity in TACE rescue cells with Western blot and bulk live-cell imaging analyses. While WT and TACE-null cells are suitable for single cell analysis for ERK pulsing, we found it very challenging to analyze ERK pulse results computationally with highly heterogeneous cells to reach a robust conclusion. Given the specificity of TACE-null affirmed by several methods complemented by TACE inhibitor studies, I hope the reviewer will agree there is sufficient evidence to support our conclusion that TACE is required for the generation of ERK pulses.

4. **The conclusion that an effect was seen with TACE inhibitor means that all other MMPs are not involved is unsupported by any experiment and should be removed.**

**Answer:** The text has been updated to reflect this suggestion.

5. **The major novel findings seem to be related to the impact of loss of a-catenin on TACE, but it is not clear if this impacts TACE levels (qRT-PCR/WB) or localization or some other element of activity. Speculation of the mechanism makes up the majority of the discussion.**

**Answer:** We have added a reference in the manuscript to the western blot in Figure 7A, which demonstrates that TACE levels remain consistent after α-catenin knockdown despite the change in TACE activity. Following the advice of the reviewer, we have revised the discussion section to focus
on the novelty and limitations of our studies as well as what needs to be done in the future to address possible mechanisms.

6. There is a lot of mention of ERK feedback to TACE, but there do not appear to be experiments examining this possibility (e.g. adding MEK inhibitor at some point, etc). This makes the story very confusing, as the finding that TACE leads to ERK pulsing does not automatically require that ERK feeds back to TACE itself.

Answer: A previous study from our lab, Chapnick et al. Sci Signaling (2015), used MEK and p38 inhibitors to show that ERK activity is required for TACE activation in HaCaT cells. We have more clearly summarized the results of that study in the introduction and discussion sections of this manuscript.

7. The new biosensor is not completely validated - do the TGFe cleavage rates correspond to the normal cells (see for example Joslin J Cell Science 2007 for methods). Are the cells also producing endogenous TGFe? If so, does the increase in TGFe levels impact trafficking/signaling/behavior?

Answer: Thanks for the suggestion. Using a TGF-α ELISA kit, we determined the levels of TGF-α in the media from normal and sensor expressing cells. Our results show that the amount of TGF-α in the media of was not significantly different between normal and sensor cells (Figure 3F), suggesting that the expression of the TGF-α shedding sensor does not significantly affect levels of TGF-α in the media under when treated with EGF or an MMP inhibitor.

8. The introduction and discussion state that proliferation is essential for wound closure. This statement is not supported by data demonstrating normal/elevated proliferation in non-healing wounds (Usui J Histochem Cytochem 2008), proliferation is not observed in the early days of normal wound healing (Usui Wound Repair Regen 2005), and that inhibition of proliferation does not prevent collective closure in vitro (Wickert Sci Reports 2016)

Answer: The manuscript has been edited to reflect these references. We have emphasized that while proliferation is not required for wound closure, signaling along the ERK axis helps wounds close more efficiently even when proliferation is inhibited (Wickert Sci Reports 2016).

Minor Concerns
1. Figure axis labels and other labels are very small, even when printed at full size.

Answer: The figure labels have been enlarged to at least 8-pt and made scalable where possible.

2. Fig 5A legend says +/- TGFe but that doesn't appear to be what is shown.

Answer: The text has been changed to reflect that this experiment was +/- α-catenin.

3. Fig. 5B,C are unlabeled images so not 100% sure what they say

Answer: The images in these figures have been clearly labeled.

4. More detail is needed related to the pulse finding algorithm - the metrics used can dramatically alter the results obtained.

Answer: More detail about the pulse-finding script has been added to the methods section. We are happy to make our code available either as supplementary material or upon request.
5. Fig. 6B,C aren't entirely clear - is that an image of a single time? Or a heat map of a timecourse? The latter would seem more appropriate? In general figure legends could be more detailed.

Answer: We have altered the figure legends and the methods section to reflect this feedback.

6. Cell line authentication/mycoplasma details not provided as required by journal

Answer: We have submitted our cell lines to University of Arizona Genetics Core for cell line authentication (https://uagc.arizona.edu/services/complete-solutions/cell-line-authentication). The result of cell line authentication is included in the supplemental Figure S3. During experimentation, we subjected these cells to the monthly mycoplasma testing provided by our cell culture core facility. The manuscript now reflects this.
Dear Dr. Liu:

Your revised manuscript has been seen again by the original reviewers. The reviewer 1 is completely satisfied with your response and reviewer 2 listed a few formatting issues with the figures. Please correct these issues accordingly. I will be happy to accept the final revised manuscript.

Sincerely,
Kunxin Luo
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Liu,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor’s decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor’s and reviewers’ comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

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In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

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published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

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Reviewer #1 (Remarks to the Author):

All concerns have been addressed.

Reviewer #2 (Remarks to the Author):

The revised manuscript is greatly improved, and clearly lays out the experimental data that is presented. There remain some concerns with the figures/figure legends:

1) Figure 1A and 1B are clearly the same plot
2) Figure 1 legend refers to A-G, but the paper and figure have only A-F
3) Potential pseudoreplication - Figure 2C and 5F have more dots than the number of biological replicates that are noted in the legend
4) Figure 5D doesn't have a clear n
5) In several figures the n is referred to as quantified sites (3C, 4F, 5B-C). It is unclear if this is appropriate - if all the sites are from the same biological replicate they are not truly independent 'n'. If the sites are different frames within a set of wells, then the 'n' is pseudoreplicated
6) Conversely, single cell data variation in 3D, 7B,C appears to be collapsed to an average for a well, which is not the common way to consider this day (in reality 5B,C may also be more appropriate to include all of the cells as independent measures if that is what was actually done)
7) Figure 7C doesn't appear to have a top/bottom as described in the legend wording
Response to reviewer’s comments

First, we would like to thank the reviewers for your thoughtful reading of our work. We greatly appreciate the careful consideration of data representation and statistical rigor, which we have addressed in our revisions. Below is our point-by-point response to reviewers’ critiques.

Reviewer 2

*Figure 1A and 1B are clearly the same plot*

**Answer:** We sincerely apologize for this error – Figure 1B was mistakenly covered up by a duplicated Figure 1A. The correct TACE FRET sensor plot (Figure 1B), which was in the original manuscript, has been restored.

*Figure 1 legend refers to A-G, but the paper and figure have only A-F.*

**Answer:** The figure legend has been corrected to reflect the final arrangement of the panels.

*Potential pseudoreplication - Figure 2C and 5F have more dots than the number of biological replicates that are noted in the legend*

**Answer:** We have eliminated the technical replicates from these plots and used only the first technical replicate for each biological replicate. We re-performed the t-tests and found that the p-value remained significant.

*Figure 5D doesn’t have a clear n*

**Answer:** Since 5D is a similar plot to 3D, we re-plotted it with data points for the average motility of each cell, re-performed the t-test, and included clear n-values in the caption.

*In several figures the n is referred to as quantified sites (3C, 4F, 5B-C). It is unclear if this is appropriate - if all the sites are from the same biological replicate they are not truly independent 'n'. If the sites are different frames within a set of wells, then the 'n' is pseudoreplicated.*

**Answer:** We re-analyzed figures 3C, 4F, and 5B-C using a slightly modified version of our original MATLAB script. The new analysis averages together the imaging sites with comparable cell densities within wells then finds the average and error between wells containing such sites. Each data point now represents a well average. We have updated the n-values, reperformed the t-tests, and updated the analysis descriptions for each of these experiments.

*Conversely, single cell data variation in 3D, 7B,C appears to be collapsed to an average*
for a well, which is not the common way to consider this day (in reality 5B,C may also be more appropriate to include all of the cells as independent measures if that is what was actually done)

**Answer:** We have re-plotted 3D and 5D and re-performed the t-tests using data points for the average motility of each cell over all timepoints.

For 7B-C, the analysis we use counts full ERK pulses in individual cells across the imaging period, resulting in quantized data. We fully agree that representing the single-cell data is important, which is why we include random samplings of raw single-cell ERK pulses as heatmaps (as seen in Sparta et al. *J Biol Chem*, 2015 and other publications). We have re-plotted our boxplots and re-performed t-tests using single-cell data rather than well averages but have not displayed the individual data points, as we feel that a box plot with quantized data points is an unhelpful visual and that the heat maps are a better representation of ERK pulsing in single cells. N-values and p-values have been updated.

Figure 5B-C measures bulk ERK or TACE activity at a single time point. We have improved our analysis as described above with multiple wells and multiple sites.

*Figure 7C doesn't appear to have a top/bottom as described in the legend wording*

**Answer:** The legend has been edited to reflect the final arrangement of the panels.
Dear Dr. Liu:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Congratulations!

Sincerely,
Kunxin Luo
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Liu:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org
