Three-dimensional niche stiffness synergizes with Wnt7a to modulate the extent of satellite cell symmetric self-renewal divisions

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RE: Manuscript #E20-01-0078
TITLE: Three-dimensional niche stiffness synergizes with Wnt7a to modulate the extent of satellite cell symmetric self-renewal divisions

Dear Professor Gilbert:

Thank you very much for your submission to the special 'Forces' issue of Molecular Biology of the Cell, the main society journal of ASCB. Two reviewers with deep expertise in muscle cell biology from a forces/bioeng'g perspective have provided comments and questions that we all hope you can address. Although the reviewers and I otherwise seem enthusiastic about the submission, I agree that a revision that suitably addresses the issues raised is necessary (and preferably within the text or figures).

Sincerely,

Dennis Discher
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Gilbert,

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, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

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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,

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

Reviewer #1 (Remarks to the Author):

The manuscript by Moyle et. al. investigates the role of niche stiffness in regulating satellite cell self-renewal. The results show damaged fibers have increased stiffness and that increased niche stiffness favors planar divisions. However, those planar divisions do not lead to increased self-renewal of the satellite cell niche as previous unless Wnt7a is added to the system. The paper is well written and investigates a pertinent stem cell mechanosensitivity. The embedding of isolated fibers in a controlled 3D mechanical environment is an asset over traditional free floating or fibers attached to a 2D rigid substrate. While this work has the potential to add valuable insight into satellite cell mechanobiology, there are some concerns. The initial data (Fig 1) purports to investigate the niche stiffness, yet when variable stiffness is applied to the cells it is an order of magnitude higher. Further explanation of how these data fit together and how the mechanical measurement apply to an in vivo context would be beneficial. The manuscript focuses on the symmetric vs. asymmetric satellite cell divisions, however it seems to assume all symmetric division results in expansion of the stem cell pool while symmetric depletion where both daughters are myogenic has also been described. It is not clear how this is considered or explored within this paper. The orientation of satellite cell division is a major component of this work, but identifying 3D orientation from 2D brightfield images can be a challenge. Further description or validation of the method would enhance the paper. Individual comments are detailed below:

Intro: The "dystrophin of the fiber sarcolemma" may more appropriately be the "dystroglycan-complex" as dystrophin itself does to enter the sarcolemma but integrates the components of the complex that do.

Intro: The citation of Webster et. al. 2016 to highlight the distinction between in vitro and in vivo contexts is useful. However, Webster et. al. describes nearly exclusively planar divisions in vivo. A statement of the different mechanical factors at play here or relation to the current study in the discussion would be helpful.

Fig 1C: The Youngs Modulus for fibers is quite low here compared to other reports. Given that this is used to make the gels this could be important. Is there a reason why the young's modulus for an intact fiber is measured at) 0.2 kPa compared to other reports of fiber stiffness measured with AFM (Ogneva IV, et. al. Biophys J. 2010).

Fig 1: Is the increased intensity of laminin due to increased expression, or could it be that the remaining laminin is condensed to a higher concentration (with higher stiffness) due to the decrease in fiber size at D4 of regeneration.

Fig 2: A more complete explanation of why the bulk tissue stiffness where chosen to be mimicked rather than the cellular level stiffness measured in Fig 1.

Fig 2B: It may be helpful to include the GFP signal to visualize overlap between the ethidium homodimer signal on Pax7+ cells on left and the presence of live satellite cells on right distinct from myonuclei.

Fig 2F: While somewhat tangential co-labeling of Edu with Ethidium homodimer would be of interest. Are the non-dividing cells mostly dead cells or might replication in a stressful environment elicit cell death?

Fig 3: Approximately how long does it take after an apical basal division for one or both of the cells to migrate a cell diameter? As the cells are fairly motile is it possible that the 30 min interval leads to an underestimation of apicobasal division? Given that many cells are more motile on stiff substrates would that account for some of the shift in planar division with stiff gels?

Fig 3: The 2D images make it somewhat difficult to obviously discern apicobasal vs. planar divisions. It is clear in 3A where the division takes place on the lateral edge of the fiber, but how obvious is it that in 3C at 48hrs those cells are on the same plane? A statement of blinding process for calling which type of division took place would enhance confidence.

Fig 4A: For Edu a color other than white would provide more contrast on top of a brightfield image

Fig 4: It may also be stated that division orientation alone does not impact cell state based on this data.

Fig 4: Is there any evidence of symmetric depletion during these assays? Edu+ cells that no longer express Pax7, but only MyoD? Could this fate be altered by stiffness?

Fig 4: The specificity for Wnt7a for stiff gels lends confidence to the result as would a similar specificity for soft.

Fig 4: Is it possible within apical basal divisions to determine if the cell that remains in contact with the fiber is indeed the more prominent MyoD+ cell? Is that polarity impacted in anyway by the relatively stiff external substrate?

Fig 4D: It isn't clear that WNT7a is associated with the emergence of nascent muscle fibers. It appears to be the highest in control healthy muscle.

Reviewer #2 (Remarks to the Author):

Three-dimensional niche stiffness synergizes with Wnt7a to modulate the extent of satellite cell symmetric self-renewal divisions

This study by Moyle and authors elegantly show that stiffness of the muscle stem cell niche can impact the plane of stem cell division. While modulating stiffness did not have any impact on satellite cell fate, the authors found that niche stiffness did synergize with WNT7a, a ligand that is known to stimulate symmetric satellite stem cell divisions to increase the self-renewing stem cell population. It will be interesting to apply these findings to degenerative conditions and assess how modulation of the niche may restore impaired regeneration due to imbalances between symmetric and asymmetric stem cell expansion.

I have a few comments and suggestions that I feel would improve the manuscript:

- The authors have omitted this reference that I feel is directly relevant to their work: Gurevich et al., Science 2016

- In the initial experiments using atomic force microscopy to measure niche stiffness during injury, the authors should explain why the TA muscle was injured with BaCl2, but the EDL muscle was used for fiber isolation and analysis.

- In Figure 1F, there is quite a large spread in the stiffness between control samples (non-Plasmin treated fibers), which makes it difficult to assess the validity of the conclusion that Plasmin treatment reduced niche stiffness. This experiment should be repeated with a larger n size.

- It is not clear to me if the experiments performed in Figure 3 to track the orientation of satellite cell divisions were performed in the Pax7-ZsGreen mice? If not, how were satellite cells identified?

- In Figure 4A the authors show representative immunostainings of EdU+ satellite cells stained for PAX7 and MYOD. The images shown are single satellite cells. How can the authors determine if these are cells that have already undergone a cell division and have migrated away from their sister cell, or if these are cells about to enter cell division?

- Le Grand et al. 2009 show that WNT7a increases the proportion of symmetric cell divisions. Do the authors see a further increase in planar divisions with WNT7a treatment? Currently, their only read out following WNT7a treatment is PAX7/MYOD staining.

- It is intriguing that although modulating stiffness affects planar vs. apicobasal divisions, there is no impact on cell fate in the absence of WNT7a treatment. Does this mean that fate is already pre-determined and is not altered by changing the mechanical properties of the niche? Or is cell fate independent of the orientation of division? The authors should discuss the implications of their findings.

- The authors refer to VANGL2 polarization multiple times and mention that mechanical stimuli can regulate VANGL2 localization. The authors should examine this in their system and perform immunostaining for VANGL2 in fibers embedded in 0.5 and 3% agarose (in the absence and presence of WNT7A) to see if localization of VANGL2 is affected.

Point-by-point rebuttal to reviewer's comments

We would like to thank the reviewers for their time spent assessing our manuscript 'Three-dimensional niche stiffness synergizes with Wnt7a to modulate the extent of satellite cell symmetric self-renewal divisions' for MBoC's special issue 'Forces within cells', and for the thoughtful suggested revisions. Due to the ongoing COVID-19 pandemic causing university closures worldwide (the University of Toronto campus closed the week of 16th March 2020), we were unable to perform all of the requested additional experiments within the required timeframe to be included in the Special Issue. However, we agree that our manuscript would be improved with the addition of further data and we are keen to have the opportunity to complete the suggested experiments and provide an updated manuscript at a later date, should the editors be amenable. If the result of any future experiments changes the conclusions of the manuscript, we will ensure to address them appropriately. Additionally, in order to address the editorial changes and expand our discussion of the results, we have reformatted the manuscript from a 'brief report' to an 'article'. This format will also enable us to add future experimental data.

Below we have included a point-by-point response to the comments outlined, including the proposed experiments that we will perform when given the opportunity to continue the work when the laboratory re-opens.

Reviewer #1 (Remarks to the Author):

The manuscript by Moyle et. al. investigates the role of niche stiffness in regulating satellite cell self-renewal. The results show damaged fibers have increased stiffness and that increased niche stiffness favors planar divisions. However, those planar divisions do not lead to increased self-renewal of the satellite cell niche as previous unless Wnt7a is added to the system. The paper is well written and investigates a pertinent stem cell mechanosensitivity. The embedding of isolated fibers in a controlled 3D mechanical environment is an asset over traditional free floating or fibers attached to a 2D rigid substrate. While this work has the potential to add valuable insight into satellite cell mechanobiology, there are some concerns. The initial data (Fig 1) purports to investigate the niche stiffness, yet when variable stiffness is applied to the cells it is an order of magnitude higher. Further explanation of how these data fit together and how the mechanical measurement apply to an in vivo context would be beneficial. The manuscript focuses on the symmetric vs. asymmetric satellite cell divisions, however it seems to assume all symmetric division results in expansion of the stem cell pool while symmetric depletion where both daughters are myogenic has also been described. It is not clear how this is considered or explored within this paper. The orientation of satellite cell division is a major component of this work, but identifying 3D orientation from 2D brightfield images can be a challenge. Further description or validation of the method would enhance the paper. Individual comments are detailed below:

1. The initial data (Fig 1) purports to investigate the niche stiffness, yet when variable stiffness is applied to the cells it is an order of magnitude higher. Further explanation of how these data fit together and how the mechanical measurement apply to an in vivo context would be beneficial.

We thank the reviewer for highlighting this point. The local MuSC niche AFM measurements were performed to understand whether the niche itself alters in stiffness following an injury, and in the experiments shown in Figure 1 we show that they do increase, due in part to increased basal lamina ECM deposition. However, we expect that niche stresses experienced by the cell differ when the basal lamina is integrated with the collagen rich interstitium just beyond the basal lamina. By virtue of the processing procedure; in isolating single myofibers we have removed most of the connective tissue layers which are normally present in muscle interstitium and that likely contribute to niche stiffness. For this reason, we determined to conduct studies in the context of agarose gels with Young' modulus similar to the apparent modulus of the tissue (rather than that of the MuSC niche basal lamina) as was measured and presented in Safee et al. 2017 and Gilbert et al. 2010. We have amended our manuscript to more clearly highlight our rationale, as shown below:

'To understand whether mechanical stiffness directly affects SC activation and fate, we developed a methodology to embed dissociated muscle fibers in 3D artificial niches using agarose gels of varying concentrations that mimic measurements of bulk apparent moduli acquired by measuring healthy and regenerating native tissues (Figure 2A, Supplemental Figure S1)) (Engler et al., 2004; Gilbert et al., 2010; Urciuolo et al., 2013; Lacraz et al., 2015; Safaee et al., 2017). These stiffness' were chosen as they more accurately recapitulate the stiffness across live muscles when all connective tissue layers are present, which is not the case in single fiber preparations.'

2. The manuscript focuses on the symmetric vs. asymmetric satellite cell divisions, however it seems to assume all symmetric division results in expansion of the stem cell pool while symmetric depletion where both daughters are myogenic has also been described. It is not clear how this is considered or explored within this paper.

We agree with your statement that a symmetric division can result in 2 Pax7+/MyoD+ progenitor cells in comparison to a symmetric self-renewal division, and we have added the following lines to the introduction to clarify that symmetric divisions can also occur to expand the progenitor pool:

'*Expansion of the SC stem cell pool requires symmetric division of PAX7^{+}/MYOD^{-} cells, whereas asymmetric division of DNA and segregation of cell fate determinants gives rise to one stem cell and one transient-amplifying myoblast. The PAX7^{+}/MYOD^{+} myoblast then produces commitment-determined daughter cells via subsequent symmetric divisions that contribute to muscle regeneration (Shinin et al., 2006; Conboy et al., 2007; Kuang et al., 2007; Rocheteau et al., 2012; Dumont et al., 2015b)*'

'In self-renewing SC divisions, this is regulated by Wnt family member 7a (WNT7a) via the non-canonical Wnt/planar cell polarity (PCP) pathway (Le Grand et al., 2009). Notably, Le Grand el al. offer evidence that WNT7a binding the frizzled 7 (FZD7) receptor in the myogenic factor 5 (MYF5)$^{-}$ SC subpopulation polarizes vang-like protein 2 (VANGL2) expression to opposite planar poles, thereby increasing planar divisions and maintaining the stem cell pool (Le Grand et al., 2009). However, this does not explain all symmetric division choices…'

Because there are currently no multi-gene reporter tools for real-time cell fate tracking in SCs, and time-lapse videos show that activated SCs can interact with unrelated 'strangers' (see Seigel et al. 2009 + supplementary videos), we chose to perform a retrospective analysis of SC fates (EdU+ Pax7+/MyoD- 'stem' cells and EdU+ Pax7+/MyoD+ 'progenitor's) across the total cell population, rather than performing lineage tracing. By performing this at 48 hours post-isolation when vast majority of cells have undergone 1 division event (Zammit et al. 2004, Siegel et al. 2011), we were able to distinguish whether the proportion of symmetric self-renewing divisions (resulting in 2 $Pax7^+/MyoD^-$ cells) was altered, but we could not directly determine whether $Pax7^+/MyoD^+$ cells were a result of asymmetric or symmetric progenitor divisions. However, at this time-point the vast majority of SCs are $PAX7^+$, with $PAX7^-$ cells appearing in single fiber cultures from 72 hours post-isolation (Zammit et al. 2004). Therefore, to probe the question of whether symmetric depletion events (where SCs are $PAX7^-$) are altered in a stiff niche, we would need to extend our culture time and co-stain with MYOGENIN. We would be happy to do this future analysis, should our model permit it.

To highlight these limitations, we have added the following section:

'Furthermore, our analysis of cell fate was retrospective, due to a lack of multi-gene reporter mouse strains to track cell fate in real-time. At this timepoint (48 hours) it is possible that a second SC division may have occurred in a small proportion of cells (Siegel et al. 2011), although the number of SCs per fiber did not alter between conditions (Figure 2E). Consequently, we were unable to track lineage relationships to quantitatively determine whether rates of asymmetric and symmetric progenitor cell divisions were affected by niche constraint. This would be an interesting avenue of future research.'

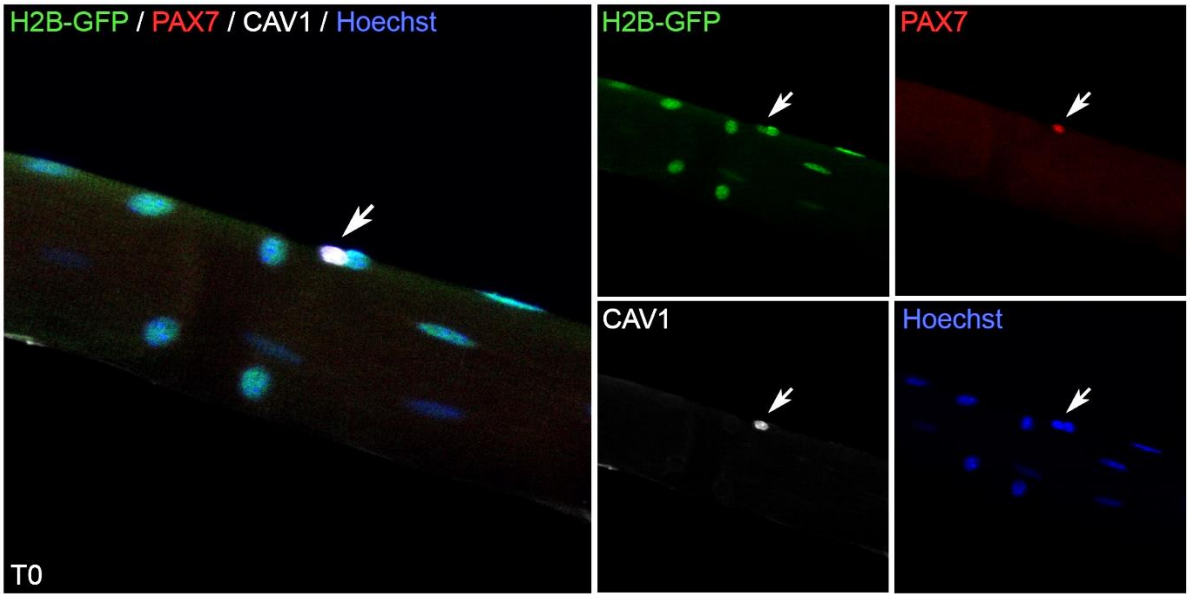
3. The orientation of satellite cell division is a major component of this work, but identifying 3D orientation from 2D brightfield images can be a challenge. Further description or validation of the method would enhance the paper.

We agree that understanding the orientation of the division is extremely important for the conclusions that we make. To ensure that we were certain of the orientation of the division, we analyzed SCs that were perpendicular to the fiber for time-lapse microscopy analysis, and imaging was taken on a confocal microscope, which enabled us to check orientation relative to the fiber by looking through the Z-stack. Additionally, a subset of experiments were quantified by 2 independent researchers to ensure reproducibility. We have clarified these points to the methodology and results sections (please see in red below):

'Images were obtained at 40X magnification and were arranged in sequential order using ImageJ software (NIH.gov) and exported as videos or still image files. Confocal Z-stack images were taken to aid in orienting the division to the myofiber. Division orientation was assessed manually by watching videos by two independent researchers to ensure reproducibility.'
'In contrast, planar division orientation, where both mother and daughter cells retain contact with the fiber, were more prevalent in stiff niches (28.1 % soft and 84.3 % stiff, p=0.0002) (Figure 3D and Supplemental Videos), as determined by two independent investigators.'

However, as this is indeed a major component of our work, we have taken several steps on the path to further characterize the orientation of SC division to the muscle fiber. We first

obtained Tg:Centrin2-GFP mice, which enable the orientation of the centrosome to be visualised during division (Higginbotham et al. 2003, Transgenic Research). However, despite optimization of time-lapse videos, we found it difficult to quantify orientation via the centrosome on live cells embedded within gels. An alternative mouse strain is Tg:H2B-GFP, which ubiquitously express GFP tagged to histone 2B, identifying the chromatin in all stages of the cell cycle (Hadjantonakis and Papaioannou, 2004, BMC Biotechnology). We have obtained this line and confirmed the expression of H2B-GFP in SCs on single fibers:

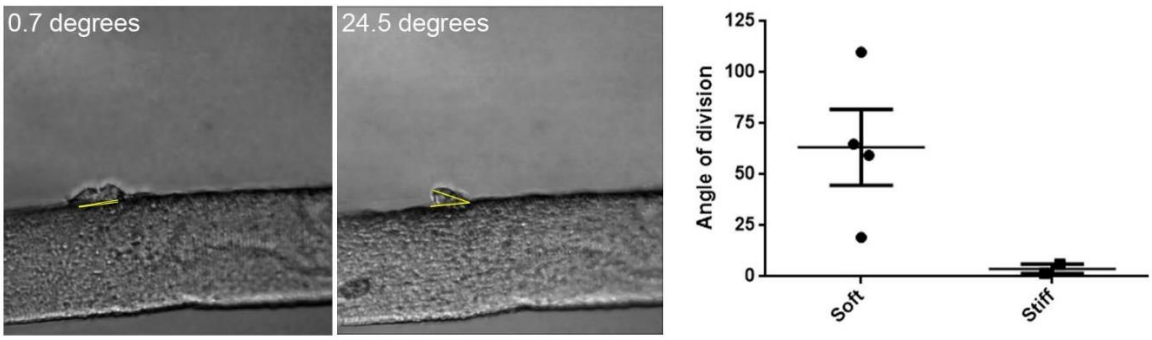


Using this transgenic mouse with time-lapse confocal microscopy will enable us to identify the exact orientation of the chromatin during division relative to the myofiber in our artificial niche model. Whilst the H2B-GFP line does not enable us to be 100% sure of SC origin, any contaminating cells in single fiber preparations (e.g. fibroblasts) are rare and appear larger in size than the quiescent SCs.

In parallel, we have also obtained a third transgenic strain, Tg:Pax7nGFP (Samastivan et al. 2009 Dev Cell) which will enable us to identify the Pax7+ SCs on the myofiber and by virtue of the nuclear localization of the GFP signal, may aid in further quantifying the angle of division orientation relative to the myofiber. Furthermore, these strains of mice are on a different genetic backgrounds (H2B-GFP is on CD1, Pax7zsGreen mice are C57/Bl6 and Pax7nGFP are C57/BL10ScSn), which will enable us to establish whether this is a widely observed phenomenon.

As a final measure to address this concern and to more quantitatively measure the orientation of divisions, in preliminary studies we have measured the angle of division from time-lapse frames using the imageJ angle measurement plug-in. This is the same tool used by Webster et al. 2016 to quantitatively measure division orientation in intravital imaging experiments. In this analysis, a 0° angle of division would be completely parallel to the myofiber (i.e. a planar division), with the angle increasing as the orientation moves perpendicular to the myofiber (i.e. an apicobasal division). Measurements were taken in the first brightfield image where two distinct cells were observed. We have included here a preliminary analysis of this methodology performed on samples from one experimental time-lapse experiment used in the manuscript (see below figure for examples and measurements, highlighted in yellow), showing that a stiffer niche is associated with a smaller angle of

division. In other words, a stiff niche resulted in more planar oriented divisions, validating the results in Figure 3 that bins divisions as 'planar' or 'apicobasal'.



Whilst we are content that this method is robust, we would like to perform a more thorough analysis using the H2B-GFP and Pax7-nGFP strains that we have recently obtained, to more accurately identify the orientation of the chromatin upon division, rather than relying on brightfield images.

Minor concerns:

1. Intro: The "dystrophin of the fiber sarcolemma" may more appropriately be the "dystroglycan-complex" as dystrophin itself does to enter the sarcolemma but integrates the components of the complex that do.

We have made the amendment as follows:
'Indeed, extracellular matrix (ECM) proteins of the basal lamina juxtapose with cadherins and the dystrophin glycoprotein complex of the myofiber, resulting in the differential expression of adhesion proteins on the basal and apical interfaces of the SC (Feige et al., 2018).'

2. Intro: The citation of Webster et. al. 2016 to highlight the distinction between in vitro and in vivo contexts is useful. However, Webster et. al. describes nearly exclusively planar divisions in vivo. A statement of the different mechanical factors at play here or relation to the current study in the discussion would be helpful.

This is an important point and we thank the reviewer for bringing it up, as we feel that it is a major advantage of our system. In Webster et al. 2016, they note that over 80% of divisions are planar orientation, with under 10% fully apicobasal (deviating over 70% from the myofiber), as quoted below:

'We measured division angles of H2B-GFP-labeled anaphase chromosomes relative to collagen and found over 80% of divisions occurred within 45° of the x-axis, while less than 10% deviated > 70° (Figures 2D–2G; Figure S2C). A similar division angle distribution was recorded with eYFP+ MPs (Figure S2D).' (Webster et al. 2016, Cell Stem Cell)

This is very similar to the 84.3% we observed in the stiff niche (Figure 3D). We have added two additional statements to our discussion to more fully describe how mechanical factors may alter in our system compared to the native niche.

'Using an artificial niche model to recapitulate altered mechanical properties, we observed that a stiff niche vastly increased the proportion of planar SC divisions (Figure 3D), with rates comparable to those reported during in vivo regeneration (Webster et al., 2016), in contrast to observations of division orientation in unconstrained floating fiber cultures (Le Grand et al., 2009) or fibers embedded in 2 mg / mL type I collagen (Siegel et al., 2009, 2011), which is much softer (0.5 kPa for collagen gels (Joshi et al., 2018)).'

'It is possible that SC behavior may alter from the native context in our system as a consequence of collagenase digestion of the basal lamina during fiber isolation, or the continual stiffness of a gel artificial niche compared to the mechanically dynamic native environment, which alters during contraction and relaxation. However, we believe that embedding SC-associated fibers in artificial 3D niches recapitulates physiologically-relevant biophysical cues, as evidenced by comparable levels of planar divisions (over 80%) in the stiff niche to in vivo intravital imaging (Webster et al., 2016). Consequently, the effect of biophysical cues is a variable that should be taken into consideration when assessing SC fate in in vitro studies.'

3. Fig 1C: The Youngs Modulus for fibers is quite low here compared to other reports. Given that this is used to make the gels this could be important. Is there a reason why the young's modulus for an intact fiber is measured at) 0.2 kPa compared to other reports of fiber stiffness measured with AFM (Ogneva IV, et. al. Biophys J. 2010).

This is an interesting point. One reason might be a species or muscle-specific difference in force, as the Ogneva et al. experiments were performed on rat soleus myofibers, whereas we quantified murine extensor digitorum longus (EDL) myofibers. Furthermore, the processing of the myofibers varied greatly; we isolated single live myofibers via collagenase digestion, in a process which separates the myofiber from the tendon tissue. In Ogneva et al. the whole muscle (including the tendon) was chemically skinned, frozen in a glycerol relaxing solution and treated with the detergent Triton-X or other permeabilising factors. All of these steps will likely alter the subsequent AFM measurements, compared to live myofibers with intact cell membranes. We chose the methodology of taking live measurements as we believe it to be more representative to the native stiffness.

To our knowledge, the only AFM experiments performed on live young murine EDL myofibers are from the Grenier laboratory (Trenz et al. 2015, Skeletal Muscle and Lacraz et al. 2015, Plos ONE), who found it to be a similar Young's Modulus of approximately 0.4 kPa when compared to our report. To our knowledge, these measurements were designed to quantify the myofiber, rather than the basal lamina close to the MuSC niche (as was the goal in our study design). We have added the following line to reference these papers:

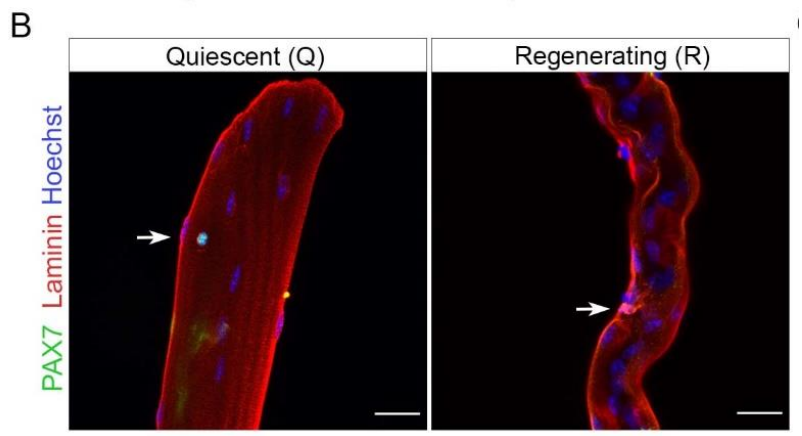
'Atomic force microscopy (AFM) measurements revealed an apparent Young's modulus of 0.2 kPa for healthy muscle fibers, similar to previous observations (Lacraz et al., 2015; Trensz et al., 2015). This increased to 0.56 kPa for regenerating myofibers (Figure 1C; p=0.0057), corresponding to a 2.86-fold increase in localized stiffness in the regenerating niche relative to the uninjured control (Figure 1D; p=0.0004).

Furthermore, we have included an acknowledgement of Ogneva et al. in the discussion to explain why our measurements may differ:

'*Our AFM measurements of 0.2 kPa at the SC niche in healthy live murine muscle fibers are similar to previous observations (Lacraz et al., 2015; Trensz et al., 2015), although stiffer values have been observed in rat fibers with alternative processing (Ogneva et al., 2010).*'

4. Fig 1: Is the increased intensity of laminin due to increased expression, or could it be that the remaining laminin is condensed to a higher concentration (with higher stiffness) due to the decrease in fiber size at D4 of regeneration.

Another interesting point. Our analysis of laminin a2 immunostained fibers at 7dpi shows a localized increase in laminin a2 expression at the SC niche. We have added arrowheads to Figure 1B to highlight the location of SCs and clarify this point:



Increased expression of several laminin isoforms has been measured by quantitative PCR in regenerating muscle (4dpi) compared to uninjured, which suggests that laminin is specifically increased as opposed to expressed in a condensed region (Rayagiri et al., 2018 Nat. Comms). We mention this publication in the text:

'This correlated with a localized increase in Laminin α2 expression (Figure 1B), in line with previous reports showing increased expression of other laminin isoforms (Rayagiri et al., 2018) in regenerating muscle.'

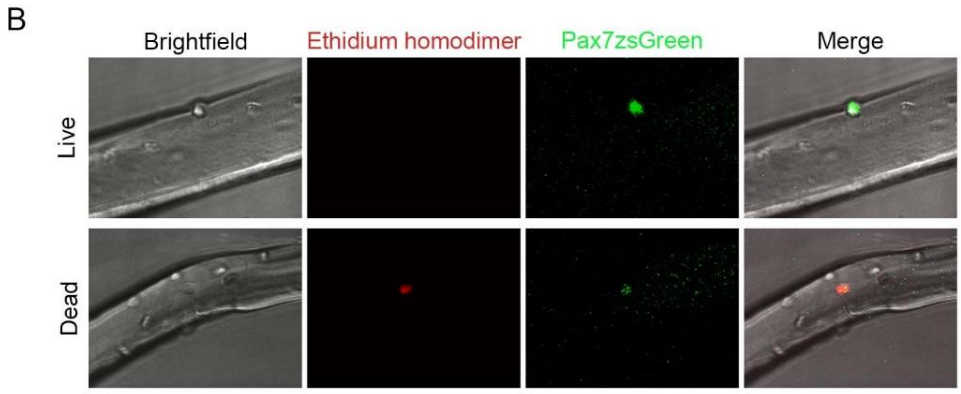
Whilst a further experiment normalising laminin a2 expression (by immunofluorescence or western blot) to cross-sectional area of myofibers at day 7 post-injury could answer whether laminin is condensed into a smaller fiber area (hence appearing increased), the analysis would include the myofiber as a whole, rather than specifically looking at the SC niche. As this is the focus of our manuscript, we believe our current single fiber analysis is more appropriate.

5. Fig 2: A more complete explanation of why the bulk tissue stiffness where chosen to be mimicked rather than the cellular level stiffness measured in Fig 1.

We believe we have addressed this point following your Comment #1 above.

6. Fig 2B: It may be helpful to include the GFP signal to visualize overlap between the ethidium homodimer signal on Pax7+ cells on left and the presence of live satellite cells on right distinct from myonuclei.

We have included the GFP channel and a merge image to Figure 2 for clarity:



7. Fig 2F: While somewhat tangential co-labeling of Edu with Ethidium homodimer would be of interest. Are the non-dividing cells mostly dead cells or might replication in a stressful environment elicit cell death?

We would expect that the small number of cells which do undergo cell death are a consequence of the isolation and/or embedding process rather than deaths being induced as they divide within the gels, as if it was due to the stress then we would expect to see higher Ethidium homodimer uptake in the stiffer gel, which we do not (see Figure 2D). However, it would be a useful control to have and we would be happy to perform this analysis at a future opportunity.

8. Fig 3: Approximately how long does it take after an apical basal division for one or both of the cells to migrate a cell diameter? As the cells are fairly motile is it possible that the 30 min interval leads to an underestimation of apicobasal division? Given that many cells are more motile on stiff substrates would that account for some of the shift in planar division with stiff gels?

This is an interesting point. Indeed, in vitro cultured prospectively isolated muscle stem cells were seen to be much more motile on stiffer substrates, when 1 x 10^6 kPa substrates were compared to 12kPa (Gilbert et al. 2010, Science). In our artificial niche system the differences in stiffness are much lower (soft gels are 5.9 kPa, stiff 21.7 kPa), and we have yet to compare the relative velocity. However, SCs were in fact motile within the gels, as can be observed in our supplementary time-lapse video.

The 30-minute interval between frames was chosen to allow for a greater number of fibers to be included per experiment. Whilst we acknowledge that it is possible that a change in motility speed could potentially bias the division orientation scores if the separation time post-division was quicker, our preliminary analysis of time to separation did not suggest this. In response to this query, we used the ImageJ measurement tool to measure individual cell lengths and noted the number of frames for the cells to separate 1 cell distance following a

division. Preliminary analysis of our time-lapse experiments found that cells had separated 1 cell's length apart on average 1.17 hours after the division on soft, and 2hrs in stiff gels (n=8 divisions).

However, to further ensure that we did not miss anything, in future division orientation experiments in H2B-GFP and Pax7nGFP mice (outlined in Comment #3 above) we shall decrease the time between frames to 10 minutes.

9. Fig 3: The 2D images make it somewhat difficult to obviously discern apicobasal vs. planar divisions. It is clear in 3A where the division takes place on the lateral edge of the fiber, but how obvious is it that in 3C at 48hrs those cells are on the same plane? A statement of blinding process for calling which type of division took place would enhance confidence.

We appreciate that in 2D images, some division orientations are clearly determinable, whereas other are more ambiguous. However, when viewing as a time-lapse video the plane of cell division could be determined (please see supplementary videos of the division orientations represented in Figure 4). Confocal stacks were also taken on many time-lapse videos to help determine the plane. To ensure that the quantifications determined were reproducible, two researchers independently assessed the same videos and quantified whether they were planar or apicobasal. We have added the following line to the methodology to clarify this point and enhance confidence

'Confocal Z-stack images were taken to aid in orienting the division to the myofiber. Division orientation was assessed manually by watching videos by two independent researchers to ensure reproducibility.'

Additionally, in the future experiments that we have set out in Comment #3, we shall quantify the angle of division relative to the fiber using the methodology used by Webster et al. 2016 by analyzing time-lapse videos with confocal stacks.

10. Fig 4A: For Edu a color other than white would provide more contrast on top of a brightfield image

We have edited the image to make EdU more visible.

11. Fig 4: It may also be stated that division orientation alone does not impact cell state based on this data.

We absolutely agree! We have added the following line to the discussion to highlight this point:

'Surprisingly, division orientation alone did not affect the proportion of stem ($PAX7^+/MYOD^-$) and progenitor ($PAX7^+/MYOD^+$) cell fates, suggesting that division orientation per say does not directly determine cell fate, with regards to PAX7/MYOD expression.'

12. Fig 4: Is there any evidence of symmetric depletion during these assays? Edu+ cells that no longer express Pax7, but only MyoD? Could this fate be altered by stiffness?

We did not observe any EdU+ cells that were Pax7- at this timepoint. However, the staining experiment was completed 48 hours post-isolation, when the majority of cells have completed 1-2 divisions (Siegel et al 2011) and Pax7 is expressed is over 97% of SCs (Zammit et al. 2004 J Cell Biol.). A longer-term study might provide these answers, although in this study we have *focused on earlier cell fate decisions. Should our system permit it, we would be happy to perform an analysis of Pax7/MyoD/MyoG expression at 72 hours post-isolation, when SCs adopt more divergent fates and Pax7$^-$ cells become more prevalent (Zammit et al. 2004 J Cell Biol.). As an alternative strategy, we could perform our future time-lapse analyses on fibers from our recently established colony of Pax7nGFP mice. As nuclear-localised GFP is driven from the Pax7 promoter, we would be able to assess Pax7 expression in real time.*

At this stage, based on our finding that stiffness alone did not alter cell fate, we would expect that this would not change following a further day of culture.

13. Fig 4: The specificity for Wnt7a for stiff gels lends confidence to the result as would a similar specificity for soft.

The expression of WNT7a increased the proportion of EdU+ SCs which expressed a Pax7+/MyoD- stem signature, both in soft and stiff gels (Figure 4C, columns 1-4 compared to 4B). However, in a stiff niche the relative proportion of Pax7+/MyoD- cells was further increased compared to soft (Figure 4c, columns 2 and 4). This suggests that niche stiffness provides an environment that promotes the expansion of the uncommitted population of SCs.

14. Fig 4: Is it possible within apical basal divisions to determine if the cell that remains in contact with the fiber is indeed the more prominent MyoD+ cell? Is that polarity impacted in anyway by the relatively stiff external substrate?

As mentioned above in Comment #2, although an interesting point, to our knowledge there are no tools to visualize PAX7 and MYOD expression in time-lapse analyses, and as such, in our analysis following fixation we looked at the population as a whole rather than assuming that the nearest cells were daughters or making conclusions from rare cells which were fixed exactly during an apicobasal division. Nevertheless, we do aim to look at polarity in further detail in the following experiments:

1) *Perform time-lapse analysis on fibers from Tg:H2B-GFP mice, in order to assess the orientation of chromatin during division relative to the myofiber*
2) *Perform immunofluorescent staining using an antibody against the polarising factor VANGL2, to confirm whether expression is localised to planar poles, and if expression is increased in a stiffer niche.*

15. Fig 4D: It isn't clear that WNT7a is associated with the emergence of nascent muscle fibers. It appears to be the highest in control healthy muscle.

We agree that WNT7a is highest in the uninjured muscle, as this ligand is produced by differentiated myofibers (LeGrand et al. 2009). Following injury, WNT7a re-appears at time-

points consistent with myofiber differentiation and is continually expressed (day 4/5 onwards) where it can affect cell fate choice. Indeed, loss of WNT7a from myofibers is associated with a reduced SC pool (Le Grand et al. 2009). With the western blot data in Figure 4, we are confirming that there is a local source of WNT7a at a time when the local SC niche is stiff (based on our AFM analysis) which our studies suggest would promote a dividing SC to undergo a symmetric self-renewal division to re-populate to stem cell pool. This is explained in the following section

'Therefore, since we (Figure 4D) and others (Le Grand et al., 2009) find that WNT7a protein is expressed at time-points associated with the emergence of nascent muscle fibers, we propose that biophysical cues within the regenerative SC niche (Figure 1) may synergize with WNT7a/PCP signaling to elicit a symmetric self-renewal division event prior to niche repopulation, a conclusion consistent with the increased number of quiescent SCs observed in muscle following a repair cycle (Shea et al., 2010).'

Reviewer #2 (Remarks to the Author):

This study by Moyle and authors elegantly show that stiffness of the muscle stem cell niche can impact the plane of stem cell division. While modulating stiffness did not have any impact on satellite cell fate, the authors found that niche stiffness did synergize with WNT7a, a ligand that is known to stimulate symmetric satellite stem cell divisions to increase the self-renewing stem cell population. It will be interesting to apply these findings to degenerative conditions and assess how modulation of the niche may restore impaired regeneration due to imbalances between symmetric and asymmetric stem cell expansion.
I have a few comments and suggestions that I feel would improve the manuscript:

1. The authors have omitted this reference that I feel is directly relevant to their work: Gurevich et al., Science 2016

We thank the reviewer for bringing this important publication to our attention and have added it as a reference in the introduction and discussion, as shown below:

'However, this does not explain all symmetric division choices, and division orientation has not consistently been linked to fate choice in vivo (Gurevich et al., 2016).'
'Whilst we acknowledge that this is in contrast to some reports (reviewed in (Feige et al., 2018)), there is evidence that asymmetric SC divisions are not orientation-specific to the myofiber in vivo during zebrafish muscle development (Gurevich et al., 2016).'

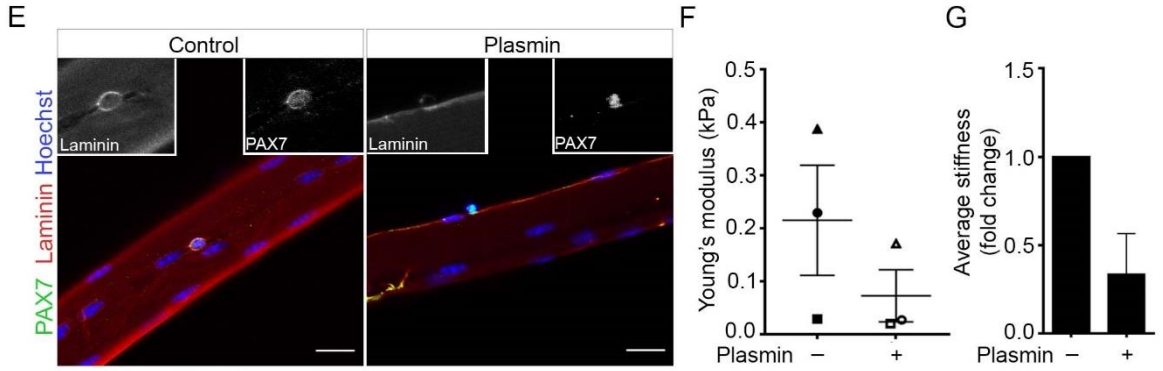
2. In the initial experiments using atomic force microscopy to measure niche stiffness during injury, the authors should explain why the TA muscle was injured with BaCl2, but the EDL muscle was used for fiber isolation and analysis.

We apologise if this wasn't clear. As the EDL is adjacent to the TA it also gets injured and undergoes regeneration. The EDL is ideal to isolate single fibres from as compared to the TA, as it can be dissected with intact tendons which limits myofibre damage and thereby maximizes the number of high quality fibers available for analysis. We have included a further clarification in the text, as shown below:

'To characterize changes of the SC niche during regeneration, we injected barium chloride ($BaCl_2$) intramuscularly into the tibialis anterior (TA) muscle, and isolated single fibers from the adjacent extensor digitorum longus (EDL), which also undergoes regeneration, 7 days post-injury (Figure 1A-B).'

3. In Figure 1F, there is quite a large spread in the stiffness between control samples (non-Plasmin treated fibers), which makes it difficult to assess the validity of the conclusion that Plasmin treatment reduced niche stiffness. This experiment should be repeated with a larger n size.

The data were presented to show the average of 3 experiments, and each experiment was the average of 1 – 2 fibers that were measured in multiple regions. We agree that the spread is large and therefore looked at plotting individual fiber data to compare the spread. In re-assessing our raw AFM data sets we noticed a mistake in one datapoint and have since amended this. However, the result of this is that the stiffness is no longer significantly decreased with plasmin treatment, although the trend still remains. We have updated the manuscript and figure (below) for the time being. However, we would be very keen to repeat this experiment and increase our N number once we have the opportunity.



4. It is not clear to me if the experiments performed in Figure 3 to track the orientation of satellite cell divisions were performed in the Pax7-ZsGreen mice? If not, how were satellite cells identified?

Yes, these experiments were performed in Pax7-zsGreen mice. We have included the green channel in the updated Figure 2 panel (please see Reviewer 1, Comment #6).

5. In Figure 4A the authors show representative immunostainings of EdU+ satellite cells stained for PAX7 and MYOD. The images shown are single satellite cells. How can the authors determine if these are cells that have already undergone a cell division and have migrated away from their sister cell, or if these are cells about to enter cell division?

We cannot and as addressed above to Reviewer #1, we have not looked directly at daughter cells, but rather how the population changes as a whole. However, by quantifying only EdU+ cells we aimed to identify only the ones who had replicated their DNA (as EdU is incorporated in the synthesis, or 'S' phase) and thus made a cell fate choice. There is the

possibility that a very small number of cells analysed had undergone S phase but not divided, as you allude to. We cannot separate these cells out within the limitations of our assay.

6. Le Grand et al. 2009 show that WNT7a increases the proportion of symmetric cell divisions. Do the authors see a further increase in planar divisions with WNT7a treatment? Currently, their only read out following WNT7a treatment is PAX7/MYOD staining.

This is a very interesting point. We do notice an increase of the proportion of PAX7+/MYOD- self-renewing stem cell divisions in a stiff niche, which is further increased with the addition specifically of WNT7A (not WNT3A or WNT5A). however, we did not measure whether the orientation angle was altered with WNT7A in soft or stiff niches. With 84% of divisions in planar orientation in a stiff niche (Figure 3) any change would be slight; however, it would be a good control and we would be happy to perform this experiment when we have the opportunity.

7. It is intriguing that although modulating stiffness affects planar vs. apicobasal divisions, there is no impact on cell fate in the absence of WNT7a treatment. Does this mean that fate is already pre-determined and is not altered by changing the mechanical properties of the niche? Or is cell fate independent of the orientation of division? The authors should discuss the implications of their findings.

We agree that this was an intriguing finding. From the data we have so far, we believe that fate is carefully managed by a combination of mechanical and biochemical cues which occur at specific times during the process of regeneration to ensure that muscle regeneration and SC pool replenishment are optimal. Both expression of WNT7A and local SC niche stiffness are dynamically regulated in vivo, which cannot be recapitulated in this model. However, we do show that when stiffness in increased (as occurs in vivo) the cell is 'primed' to make a symmetric division. The nature of this 'priming' is to be determined. The expression of WNT7A then provides a biochemical cue to alter fate. We do believe however, that the orientation of divisions captured in floating fiber cultures are not representative of division orientation in vivo. We would like the opportunity to further reinforce our findings using the H2B-GFP and Pax7nGFP lines and angle analysis, as outlined above (Reviewer 1, comment #3) and will update our manuscript accordingly. We have also added the following sentiments to the discussion section:

'…Surprisingly, division orientation alone did not affect the proportion of stem ($PAX7^+/MYOD^-$) and progenitor ($PAX7^+/MYOD^+$) cell fates, suggesting that division orientation per say does not directly determine cell fate, with regards to PAX7/MYOD expression. Whilst we acknowledge that this is in contrast to some reports (reviewed in (Feige et al., 2018)), there is evidence that asymmetric SC divisions are not orientation-specific to the myofiber in vivo during zebrafish muscle development (Gurevich et al., 2016).'

'…we reasoned that increased niche stiffness might prime SCs for WNT7a-regulated fate decisions. Indeed, we demonstrate that the combination of a stiff niche and WNT7a augmented an increase to the proportion of symmetric self-renewing divisions (Figures 3 and 4), suggesting that constraint of cells within the SC niche is a biologically important feature of

muscle regeneration. Notably, our finding that ECM protein deposition increases mechanical stiffness to prime SCs for symmetric cell divisions aligns with a recent study showing that deposition of laminin-111 at the basal lamina specifically induced planar-oriented symmetric self-renewal divisions (Rayagiri et al., 2018). Collectively, this demonstrates that remodeling of the niche provides both mechanical and biochemical cues that contribute to maintenance of the SC pool.'

8. The authors refer to VANGL2 polarization multiple times and mention that mechanical stimuli can regulate VANGL2 localization. The authors should examine this in their system and perform immunostaining for VANGL2 in fibers embedded in 0.5 and 3% agarose (in the absence and presence of WNT7A) to see if localization of VANGL2 is affected.

We agree and we would be happy to perform this experiment at a future date.

RE: Manuscript #E20-01-0078R
TITLE: "Three-dimensional niche stiffness synergizes with Wnt7a to modulate the extent of satellite cell symmetric self-renewal divisions"

Dear Dr. Gilbert:

Thank you for submitting responsive revision. I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

As discussed, please also indicate your willingness to publish online the reviews and your response. This should help the community to better understand your work.

Thank you for publishing in MBoC, the society journal of the ASCB.

Sincerely,
Dennis Discher
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Gilbert:

Congratulations on the acceptance of your manuscript.

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