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Mammary mechanobiology - investigating roles for mechanically activated ion channels in lactation and involution

Teneale A. Stewart, Katherine Hughes, Alexander J. Stevenson, Natascia Marino, Adler L. Ju, Michael Morehead and Felicity M. Davis
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MS TITLE: Mammary mechanobiology: Investigating roles for mechanically-activated ion channels in lactation and involution

AUTHORS: Teneale Stewart, Katherine Hughes, Alexander Stevenson, Natascia Marino, Adler Ju, Michael Morehead, and Felicity Davis
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers share enthusiasm for the manuscript and also raise a number of substantial criticisms that prevent me from accepting the paper at this stage. These concerns largely relate to quantification of the data and presentation and discussion of the results. 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 currently be unable to access the lab to undertake experimental revisions. 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.

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

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

Reviewer 1

Advance summary and potential significance to field

This very elegant study of force during mammary gland development is well presented, thorough and very interesting. Understanding how force drives lactation and involution is crucial to understanding mammary gland biology and potentially development of cancer.

Comments for the author

Only minor revisions are encouraged prior to acceptance.
1. Perhaps a discussion of how force during mammary involution could contribute to tumorigenesis in recently pregnant women or to protection in later parous women would be interesting and add some clinical relevance to the pathways identified. Are these pathways mis-regulated in cancers?

2. Similarly, could the authors include in the discussion more detail on how other organisms can respond to loss of PIEZO and discuss how these mechanisms may be at play in the mammary gland?

Reviewer 2

Advance summary and potential significance to field

The manuscript “Mammary mechanobiology: Investigating roles for mechanically-activated ion channels in lactation and involution”, aimed to characterize the force landscape in mammary glands during lactation and involution, and the molecular pathways associated with such response, using a series of mouse models and normal-like mammary cell lines. The authors provided incredibly beautiful videos that support their claims, and their molecular analysis revealed that PIEZO1, a mechanically-activated ion channel is not essential during luminal cells mechano-sensing.

However, lack of proper image quantification in all figures, and the over interpretation of some results, take some of the enthusiasm away from the results and their significance.

A fully revised draft of the manuscript would be better suited for publication at JOCES.

Comments for the author

Major points:

1) The current abstract and introduction sessions present some background about the topic, but barely describes the findings or their implications.

2) Currently most of figure panels lack data quantification. These should be provided to support the author’s conclusions.

Minor points

1) On Fig1b, the authors mentioned “basal cell contraction during feeding” Was the tissue imaged during suckling? Or was the tissue harvested right after feeding and then image? Please clarify. Also, please indicate on text or figure the definition of SA, currently only explained on figure legend

2) On line 76 – 77 “making these epithelial cell contractions comparable in magnitude to those of cardiomyocytes and intestinal smooth muscle cells (36, 37).” – what kind of comparison? Morphology? Contraction? How comparable?

3) On Fig1c, the authors refer to the results as “warping during feeding”, but again it is unclear whether the images/videos were collected during active feeling (live imaging) or from isolated tissue during lactation. Also please provide quantification to support enhanced warping

4) line 81 “substantial stochastic deformations to alveolar structures as a consequence of repetitive basal cell” – please provide quantification to support the claims

5) Fig1d, the authors claim the present of milk in H&E stained tissue sections from lactating glands, which isn't clear. Please provide a staining that would specific detect milk proteins to support the original claim.

6) line 89 – 90 – the authors claim that “The sustained overextension of the alveolar epithelium during early involution causes apical cell shedding (Fig. 1D, arrow)”, however cellular shedding can also be observed in histological sessions of lactating mammary glands. A proper quantification of shedding during lactation and involution would perhaps support the authors claims.

7) Still on Fig1D - Given that immune cells are known to be carried with milk to the infant, the authors should provide a specific staining (CD45, KRT8, KRT18, etc.) to demonstrated that cells inside the duct’s lumen are indeed epithelial cells.

8) Line 99 – 100 – “Apical projections were absent in tissue amples collected during phase 1 involution (Fig. 2A)” – please provide quantification to support the claim

9) Line 101- 102 “Luminal cell length and area were reduced by more than 50% by the end of the first phase of involution (Fig. 2B).” this is well quantified, but it lacks an explanation of how the measurement was performed, or why it is important result.

10) Line 108 – 109 – “protrusions often lacked near-plasmalemmal organelles (Fig. 2C, asterisk” – please provide quantification to support the claim

11) Line 112 – 113 “Consistent with IHC and SBEM imaging, luminal cell length was reduced during involution (Fig. 2D, (i) vs (iv)).” – please provide quantification to support the claims

12) Line 116 – this part of the result session seems to be missing an overall conclusion that discusses the meaning of presented results

13) Line 126 – 128 “A fraction of non-differentiated HC11 cells responded to shear stress via a transient increase in intracellular calcium (340/380 ratio) (Fig. 3C and Supplementary Movie 6).” Please provide quantification to support statement

14) Line 144-145 “operated calcium entry subunit Orai1 were significantly enriched in lactating mammary tissue (Fig. 4A)” – provide difference in fold change or percentage to support the claim

15) Line 146 – 147 “and the epithelial sodium channel Enac were unchanged or were significantly reduced in lactating samples (Fig. 4A).” provide difference in fold change or percentage to support the claim

16) Fig.3c, d – include key to indicate what the colors representation means

17) Fig.3d, bottom panel – this panel was not mentioned on the results session, or at least it wasn’t very obvious – so please include a description of these results or remove panel from figure

18) Fig. 5d – the authors must include a duct alveologenesis quantification to support the conclusion that KO displays normal mammary gland

19) The images presented on Fig. 5d and 5h top panel have poor resolution image (no clear definition of branching). Please provide higher/clear images to support the original conclusion

20) Please include pvalue and number of mice to Fig.5e, 5g, 5k, 5m, 5n

21) Line 184 – 186 “These data demonstrate that, unlike store-operated channels (12), mechanically-gated PIEZO1 ion channels are not essential for the basolateral flux of calcium that is required for milk calcium enrichment during lactation (11)” This conclusion can only be drawn to described the effects of PIEZO1 KO is not essential in luminal cells, which express WAP. Thus, is it possible the PIEZO1 expression in basal cells may be the reason for why the phenotype was not stronger?

22) Line 193 – 196 “Gross morphology of mammary glands stained with the histochemical stain methyl green (54) and analysis of mammary tissue sections showed no discernable differences between control and Piezo1fl/fl;WAPCre mice at either 24 h involution (reversible phase; Fig. 5H)” – what kind of differences were analyzed? Provide quantification to support the claim.

Reviewer 3

Advance summary and potential significance to field

This manuscript contributed significantly in the field in the areas of understanding the mechanobiology of the mammary gland in lactation and involution critical aspects for understanding normal biology of the development and maturation of breast tissue. This information is greatly needed to not only contribute to our understanding of healthy physiology, but for laying the basis for understanding disease processes, such as cancer development, in this context. The linking together of the molecular, cellular and organism level biology of breast lactation and involution contributes greatly to the field.

Comments for the author

This manuscript was very well written and covered a breadth of techniques to address the central questions. There was a tremendous amount of data in the manuscript and supplementary information that was presented well. I felt the discussion section was short and had the opportunity to expand a bit more on the bigger picture of the studies and implications relevant to breast health and pathology.

Although the focus of the manuscript was on mechanobiology, there was very little detail on the mechanical aspects. Page 12 line 327: "shear" stress. Lines 335-337. More detail is needed. What was the shear stress? Specifics of the device the cells were exposed to shear in. Were these levels in the physiological range?

What about the potential role of mechanosensors beyond ion channels? The cell flow stimulation experiments were very short. Longer term mechanical stimulation is required for other mechanosensors to be upregulated. Some comment or statement regarding the possibility of involvement of other non-ion channel mechanosensors may benefit the discussion.

Justification for using HC11 cells. How might results be similar or different using primary cells? How might results be the same or different in humans?

Researcher to be aware of in the area for potential citations: Pepper Schedin OHSU.

First revision

Author response to reviewers' comments

Reviewer 1
Reviewer 1 described the study as a very elegant study of force druing mammary gland development that is well presented, thorough and very interesting.

Minor revisions:
1. Perhaps a discussion of how force during mammary involution could contribute to tumorigenesis in recently pregnant women or to protection in later parous women would be interesting and add some clinical relevance to the pathways identified. Are these pathways mis-regulated in cancers?
We thank the Reviewer for this suggestion. We have included some discussion around breast cancer and a study examining PIEZO1 in MCF7 breast cancer cells (Introduction pg. 3 lines 55-59; Discussion pg. 10 lines 286-294).

2. Similarly, could the authors include in the discussion more detail on how other organisms can respond to loss of PIEZO and discuss how these mechanisms may be at play in the mammary gland?
We agree with this suggestion and have now incorporated this into the Discussion (pg. 11, lines 300-304).

Reviewer 2
Reviewer 2 noted that the study included "incredibly beautiful videos to support the claims" in addition to molecular analyses. Additional quantification was requested. Although quantification of the main experimental findings of this manuscript had been provided (e.g., in original figures 1B, 2B, 3A, 3E, 4A, 4D, 4E, 5C, 5E, 5G, 5K, 5M, 5N, S5, S6B, S7 and S9C-D), our endeavours to add to these quantitative analyses are outlined below (see minor points).

Major points:
1. The current abstract and introduction sessions present some background about the topic, but barely describes the findings or their implications.
We have now updated our abstract (working within the 180-word JCS requirement) to provide a summary of the major findings (pg. 2, lines 31-34).

2. Currently most of figure panels lack data quantification. These should be provided to support the author's conclusions.
Specific comments are addressed below.

Minor points:
1. On Fig1b, the authors mentioned "basal cell contraction during feeding" Was the tissue imaged during suckling? Or was the tissue harvested right after feeding and then image? Please clarify. Also, please indicate on text or figure the definition of SA, currently only explained on figure legend.
We agree that this was not clearly defined and have now amended to "basal cell contraction (oxytocin stimulation)". We have also clarified in the figure legend that this is ex vivo imaging and pointed to the Methods for further information. We have removed the term "SA".

2. On line 76 – 77 "making these epithelial cell contractions comparable in magnitude to those of cardiomyocytes and intestinal smooth muscle cells (36, 37)." – what kind of comparison? Morphology? Contraction? How comparable?
As stated (pg. 4, lines 101-103), the comparison is contraction and magnitude. The references have been provided.

3. On Fig1c, the authors refer to the results as "warping during feeding", but again it is unclear whether the images/videos were collected during active feeling (live imaging) or from isolated tissue during lactation. Also please provide quantification to support enhanced warping.
This has now been changed to "alveolar warping (oxytocin stimulation)". Alveolar warping (Feret's diameter) is now quantified (pg. 4, lines 108-109).

4. Line 81 "substantial stochastic deformations to alveolar structures as a consequence of repetitive basal cell" - please provide quantification to support the claims.
See minor point #3.

5. Fig1d, the authors claim the present of milk in H&E stained tissue sections from lactating glands, which isn't clear. Please provide a staining that would specific detect milk proteins to support the original claim.

Milk accumulation in the mammary gland during involution is a widely-appreciated phenomenon (Hughes et al., 2012; Marti et al., 1997; Stein et al., 2007; VanHouten et al., 2010; Watson, 2006) and no claim of novelty has been made. It also does appeal to common sense that if milk can no longer be ejected, it transiently accumulates in the mammary gland. Staining for milk proteins, which are often lost during processing [discussed in Ref (Palmer et al., 2006)], adds no value to this figure nor does it alter its conclusions. We quantify the extent of secretory cell distension in Fig. 2B. To improve clarity around this point, we have provided additional references in the text and re-worded the title of this figure panel.

6. Line 89 – 90 – the authors claim that "The sustained overextension of the alveolar epithelium during early involution causes apical cell shedding (Fig. 1D, arrow)", however cellular shedding can also be observed in histological sessions of lactating mammary glands. A proper quantification of shedding during lactation and involution would perhaps support the authors claims.
This is again a very well-established phenomenon and no claim of novelty has been made. To make this more clear we added to the number of references already cited here, in particular a review by Christine Watson (Watson, 2006), which discusses this in depth. Shed cells are most commonly detected by conventional morphological criteria (as discussed in Ref (Lund et al., 1996), with staining for apoptotoic cells (later performed and quantified in this manuscript in Fig 5J-K).

To provide perspective on the number of shed cells, these have been counted and are now presented in (new Fig. S1B). As per Methods, cell counting was limited to Lac (d10), Inv (24 h) and Inv (48 h). Cells were not counted at Inv (96 h), when many alveolar units had already collapsed and no longer had discernable lumens.

7. Still on Fig1D - Given that immune cells are known to be carried with milk to the infant, the authors should provide a specific staining (CD45, KRT8, KRT18, etc.) to demonstrated that cells inside the duct's lumen are indeed epithelial cells.
This has been addressed above.

8. Line 99 – 100 – "Apical projections were absent in tissue amples collected during phase 1 involution (Fig. 2A)" – please provide quantification to support the claim.
Cell length, width and area have already been quantified in Fig. 2B and discussed in the Results.

9. Line 101- 102 "Luminal cell length and area were reduced by more than 50% by the end of the first phase of involution (Fig. 2B)." this is well quantified, but it lacks an explanation of how the measurement was performed, or why it is important result.
Details on luminal cell measurements are provided in the Methods section (pg. 21, lines 591-593) and also in the figure legend itself. The rationale and interpretation of these experiments are given in the manuscript (pg. 5, lines 121-132 and now expanded pg. 6 lines 144-147).

10. Line 108 – 109 – "protrusions often lacked near-plasmalemmal organelles (Fig. 2C, asterisk" – please provide quantification to support the claim.
Serial block face EM images provide an alternative view of data shown and quantified in Fig. 2A-B. Each image sequence represents terabytes of data and as such pipelines for processing and analysis are still in development by the field and are beyond the scope of this manuscript. Although we consider organelle distribution an interesting observation that may spur future research, we have now removed the comment.

11. Line 112 – 113 "Consistent with IHC and SBEM imaging, luminal cell length was reduced during involution (Fig. 2D, (i) vs (iv))." – please provide quantification to support the claims
Quantification of IHC images is already given in Fig. 2B.

12. Line 116 – this part of the result session seems to be missing an overall conclusion that discusses the meaning of presented results.
We have now reiterated the conclusion at the end of this results section (pg. 6, lines 144-147).

13. Line 126 – 128 "A fraction of non-differentiated HC11 cells responded to shear stress via a transient increase in intracellular calcium (340/380 ratio) (Fig. 3C and Supplementary Movie 6)." Please provide quantification to support statement

We have quantified f/f0 (340/380 ratio) in Figure 3. To add to this quantification with a more traditional comparison of two means using a student's t-test, we have added a new supplementary figure (Fig. S2A).

14. Line 144-145 "operated calcium entry subunit Orai1 were significantly enriched in lactating mammary tissue (Fig. 4A)" - provide difference in fold change or percentage to support the claim.
Fold change with statistical analysis is already provided in the original Fig. 4A.

15. Line 146 – 147 "and the epithelial sodium channel Enac were unchanged or were significantly reduced in lactating samples (Fig. 4A)." provide difference in fold change or percentage to support the claim.
As above, fold change with statistical analysis is already provided in the original Fig. 4A.

16. Fig.3c, d – include key to indicate what the colors representation means
A legend has already been provided in the origianl Fig. 3.

17. Fig.3d, bottom panel – this panel was not mentioned on the results session, or at least it wasn't very obvious – so please include a description of these results or remove panel from figure
We have now made this a new sub-panel and described it more clearly in the figure legend.

18. Fig. 5d – the authors must include a duct alveologenesis quantification to support the conclusion that KO displays normal mammary gland
Histological sections were analyzed by a American College of Veterinary Pathologists board certified pathologist experienced in mouse mammary histopathology and blinded to mouse genotype. Evaluation utilised whole slide images and digital microscopy. Histological assessment included all aspects of glandular morphology, including consideration of both the epithelial and stromal compartments, taking into account the proportion of alveolar units, the degree of expansion/collapse of alveolar units, lumen size, epithelial cell morphology, and degree of adipocyte infiltration. We have now modified the Methods section to better describe the method for histological evaluation.

19. The images presented on Fig. 5d and 5h top panel have poor resolution image (no clear definition of branching). Please provide higher/clear images to support the original conclusion
The images presented in Fig. 5D, H and I are mammary gland wholemounts. As lactation progresses, the number and size of alveolar units increase and it becomes impossible to see individual structures in wholemount images (in contrast to ductal morphogenesis, gestation, early lactation or later stages of involution, where some stuctures are more discernable). Nevertheless, wholemount images are not intended to provide information about microscopic structure, they are intended to provide a macroscopic view, which, in our case, has been combined with high resolution histology (analysed blinded by a veterinary pathologist, as above).

This is, of course, common to all lactation studies (see, for example, Andrecheck and Nevins PMID: 18550711 ;Pollard and Hennighausen PMID: 7937762; Chen and Chodosh PMID: 20849614). We have recently written an invited methods paper on mammary gland wholemounts during lactation for Journal of Mammary Gland Biology and Neoplasia, which we can cite here, pending acceptance dates.

20. Please include pvalue and number of mice to Fig.5e, 5g, 5k, 5m, 5n
This is very clearly articulated in the figure legend.

21. Line 184 – 186 "These data demonstrate that, unlike store-operated channels (12), mechanically-gated PIEZO1 ion channels are not essential for the basolateral flux of calcium that is required for milk calcium enrichment during lactation (11)" This conclusion can only be drawn to described the effects of PIEZO1 KO is not essential in luminal cells, which express WAP. Thus, is it possible the PIEZO1 expression in basal cells may be the reason for why the phenotype was not stronger?
The conclusion can be drawn for the following reasons. Luminal cells (not basal/myoepithelial) are responsible for milk calcium enrichment. We have confirmed and quantified knockdown in this population. Related to this, we have also demonstrated, using the PIEZO1 agonist Yoda1, that functional PIEZO1 channels are not expressed in basal cells (Fig. 4).

22. Line 193 – 196 "Gross morphology of mammary glands stained with the histochemical stain methyl green (54) and analysis of mammary tissue sections showed no discernable differences between control and Piezo1fl/fl;WAPCre mice at either 24 h involution (reversible phase; Fig. 5H)" – what kind of differences were analyzed? Provide quantification to support the claim.
This has been addressed above.

Reviewer 3
Reviewer 3 noted the significant contribution of the manuscript, the breadth of techniques and the volume of data. Specific comments are addressed below.

1. Although the focus of the manuscript was on mechanobiology, there was very little detail on the mechanical aspects. Page 12 line 327: "shear" stress. Lines 335-337. More detail is needed. What was the shear stress? Specifics of the device the cells were exposed to shear in. Were these levels in the physiological range?
Detail has now been added to the Methods as requested (Pg. 14). Although some studies have attempted to explore fluid interactions of milk during breastfeeding in human patients (e.g., Ref (Azarnoosh and Hassanipour, 2020)), to our knowledge the physiological range of milk fluid shear stress in mice is not yet known.

2. What about the potential role of mechanosensors beyond ion channels? The cell flow stimulation experiments were very short. Longer term mechanical stimulation is required for other mechanosensors to be upregulated. Some comment or statement regarding the possibility of involvement of other non-ion channel mechanosensors may benefit the discussion.
We agree with this comment. We have now noted the transient nature of the flow experiments (e.g., pg. 14, line 407). We have also noted our particular focus on ion channels and discussed the potential involvement of non-ion channel mechanosensors (Abstract pg. 2; pg. 3, lines 75-77; pg. 8 lines 228-229)

3) Justification for using HC11 cells. How might results be similar or different using primary cells?
HC11 cells were used as this is a well established in vitro tool to study lactation. Primary cells isolated from a lactating animal are difficult to culture. However, we appreciate the limitations associated with in vitro, immortalized cell-based models and, for this reason, moved on to mouse models (discussed pg. 6 153-154 and 165-167)

4) How might results be the same or different in humans?
We have now discussed species differences in other systems (pg. 11 lines 300-307).

5) Researcher to be aware of in the area for potential citations: Pepper Schedin, OHSU.
Thank you and apologies for the oversight. We have now added citations from the Schedin group (McDaniel et al., 2006; Schedin and Keely, 2011; Schedin et al., 2004; Lyons et al., 2011).

We feel that we have been able to address the majority of comments raised by the Reviewers. We have also modified our manuscript to comply with the formatting guidelines and have provided images that may be considered as cover art for this issue (with additional e.g., SEM images, available on request). We hope you agree that the manuscript is now suitable for publication in Journal of Cell Science and we very much look forward to hearing from you.

Sincerely,
Felicity Davis, PhD
Group Leader
Mater Research Institute-The University of Queensland

Second decision letter

MS ID#: JOCES/2020/248849

MS TITLE: Mammary mechanobiology: Investigating roles for mechanically-activated ion channels in lactation and involution

AUTHORS: Teneale Stewart, Katherine Hughes, Alexander Stevenson, Natascia Marino, Adler Ju, Michael Morehead, and Felicity M Davis
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript. I apologize for the delay; we had one reviewer drop out late.

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, one reviewer is satisfied and the other remaining reviewer raises two issues of quantification and image clarity that should be readily addressable. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

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.

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 findings in the paper are significant and will advance our knowledge of mechanosignaling in the mammary gland that ensures successful lactation in mammals.

Comments for the author

My suggestions have been addressed I recommend the manuscript for publication.
Traci R Lyons

Reviewer 2

Advance summary and potential significance to field

The authors of the manuscript entitled "Mammary mechanobiology: Investigating roles for mechanically-activated ion channels in lactation and involution" carefully addressed to most of my concerns referent to data quantification and accurate data description. There are minor comments that I would like to further discuss before fully endorsing this manuscript.

Comments for the author

a) Regarding my original point #18, it is great that the authors utilized an specialized and double blinded strategy to call for mammary gland abnormalities in the Piezo1 KO model. However the histology images provided on Fig.5D show differences across WT and KO. Given that the authors have these H&E images available, a duct quantification and lumen area quantification would provide a stronger rationale to support the lack of a phenotype.

b) still in regards to Fig.5 - the point of providing whole-mount images is to demonstrate the fullness of the tissue, which cannot be appreciated in the dark-green stained images. If the authors judge that such images are important to backup their claims, they must provide views that allow for macroscopic structures to be visualized.

Second revision

Author response to reviewers' comments

Thank you for providing us with the opportunity to address the remaining two comments from Reviewer 2. These comments are now fully addressed and are described below.

a) Regarding my original point #18, it is great that the authors utilized an specialized and double blinded strategy to call for mammary gland abnormalities in the Piezo1 KO model. However the histology images provided on Fig.5D show differences across WT and KO. Given that the authors have these H&E images available, a duct quantification and lumen area quantification would provide a stronger rationale to support the lack of a phenotype.

This remaining concern by R2 is fully addressed in the revised manuscript. We have quantified the relative area of stroma vs epithelial and lumen area in H&E stained histological sections from lactating control and Piezo1fl/fl;WAPCre animals. This is provided in the new Fig. S3C-D). These data, support the conclusions from the rigorous and blinded analyses from the veterinary pathologist (American College of Veterinary Pathologists).

b) still in regards to Fig.5 - the point of providing whole-mount images is to demonstrate the fullness of the tissue, which cannot be appreciated in the dark-green stained images. If the authors judge that such images are important to backup their claims, they must provide views that allow for macroscopic structures to be visualized.

This remaining concern by R2 is fully addressed in the revised manuscript. Additional wholemount images of control and Piezo1fl/fl;WAPCre mammary gland, which allow for identification of macroscopic structures within the lactating tissue, have now been provided (Fig. S3B).

We hope you find the manuscript suitable for timely publication in Journal of Cell Science. We have also uploaded some suggestions for cover art for the issue.

Third decision letter

MS ID#: JOCES/2020/248849

MS TITLE: Mammary mechanobiology: Investigating roles for mechanically-activated ion channels in lactation and involution

AUTHORS: Teneale Stewart, Katherine Hughes, Alexander Stevenson, Natascia Marino, Adler Ju, Michael Morehead, and Felicity M Davis
ARTICLE TYPE: Research Article

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

Reviewer 2

Advance summary and potential significance to field

The authors have addressed all of my concerns.

Comments for the author

The authors have addressed all of my concerns.