A mechanogenetic role for the actomyosin complex in branching morphogenesis of epithelial organs

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AUTHORS: Jin Man Kim, YoungJu Jo, Ju Won Jung, and Kyungpyo Park

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

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 attend to all of the reviewers' comments and 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. 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

In this paper, the authors examine the mechanical basis of branching morphogenesis. The main organ they use (in particular via culture systems) is salivary gland, although they seek to generalise it by looking at other organs, which provides scope and breadth for the paper. They then link the distribution of mechanical forces (actomyosin-based) to YAP/TAZ mechanotransduction. Thus, the paper covers an overall topical and interesting question. One issue however, that would need to be clarified for publication, is that it remains hard to understand exactly the physical mechanism proposed by the authors - for instance the relative contribution of central/outer regions vs ECM (see below for details and suggestions).

Comments for the author

A/ Clarity of presentation and proposed mechanism

The authors propose that “cleft formation is related to de-stressing of the concentrated force. ” (p8) from the aspect ratio, but then that “This effect could provide compelling evidence for a buckling-infolding model of cleft formation ”.

To me, these two sentences are contradictory: the classical buckling is due to accumulation of compressive stresses (so the reverse of destressing). Can the authors clarify what they mean?

On that note, as the authors mention in discussion, fibronectin and the role of matrix in general has been well-studied for clefting. But they don’t link this knowledge in the paper: for instance they modulate the ECM in the main text (p8-9), but only use this as a way to manipulate myosin structure. Can the authors exclude for instance that some of the phenotype/movements is due to matrix rigidity change itself?

Especially given the above question on buckling vs de-stressing, could it be instead that some forces (either from central cells or ECM-linked) effectively push/stretch cells locally at the cleft?

On that front, the summary schematic of FIg. 3H for instance mentions as a key first first that continuous cell proliferation increases internal stresses. But i don’t see it really discuss so much in the paper? (proliferation of inner cell would create pressure, thus stretching of the outer layer, whereas proliferation of outer layer alone would create compression in the outer layer - opposite effects again which are relevant i think for buckling vs de-stressing.)

B (more minor)/ Presentation/quantifications

I don’t quite understand the quantifications of Fig. 2B. Is it a ranked measurement of 50 randomly selected cells? i think that if the point is to convey variability, the authors should instead with dot or violin plots. If it’s to signify spatial distribution as they mention, then maybe the x axis should be position then, to show spatial trends? A schematic of cell shape (as quantified in S2D might help for addition to 1E or 1G) as a function of regions would also really help i think to make the data more explicit.

The correlation between traction forces and protrusions is not very easy to see/understand from FIg. 3E-F given variations and fluctuations, maybe the authors could average/show curves for these side by side?

Reviewer 2

Advance summary and potential significance to field

Here Kim et al. investigate the role of actomyosin contractility in branching morphogenesis of several developing branched epithelial organs, primarily focusing on the submandibular gland (SMG). They report that myosin mediated contractility in the epithelium is required for generating the physical forces to promote branching the SMG. They also show that YAP/TAZ is activated in the peripheral epithelial cells of the SMG and that nuclear translocation of YAP/TAZ in these cells is dependent on actomyosin contractility. Finally, they conclude that YAP/TAZ activation regulates epithelial cell fate in a myosin dependent manner. Taken together their data suggest a mechanical role for epithelial cell contractility in SMG cleft formation and contraction-mediated inhibition of YAP/TAZ to specify epithelial cell fate in the SMG.

Comments for the author

Major Comments:
The current state of the manuscript makes it challenging for the reader to understand what aspects of this work are novel. There are many reports on the role of the actomyosin machinery in epithelial cleft formation and branching morphogenesis in the submandibular gland (Joo, E. and Yamada, K., 2014; Kadoya, Y. and Yamashina, S., 2010; Daley, W. P. et al., 2009; Hsu et al., 2013; Harunaga, J. S. et al., 2014). There are also reports on the role of YAP/TAZ regulating SMG branching morphogenesis(Enger et al. 2013) and specifying cell fate in the developing SMG epithelium (Szymaniak et al., 2017). The authors highlight where their data either agrees or conflicts with many of these findings, but don't make it clear how their findings add to the field. The most interesting finding is that mesenchyme-free epiSMG cultures undergo buckling when myosin is inhibited. This suggests that when epithelial cell contractility is reduced, the tissue folds due to mechanical buckling. This buckling phenomenon is found in many epithelial organs (Nelson, C. M., 2016; Varner, V. D. et al. 2015; Shyer, A. E. et al. 2013), and to my knowledge has not been shown in the SMG prior to this work. The work would have more impact if the authors reframe their results in this context. Their finding of more buckling when actomyosin contractility is inhibited could add to our understanding of how compression leads to epithelial buckling by showing that tension caused by actomyosin contractility decreases buckling.

The authors switch between whole SMG explants and mesenchyme-free epiSMG frequently throughout the manuscript without rationalizing which model is used for a given experiment/analysis. This made interpreting the findings quite challenging. The authors need to more clearly state their questions and hypotheses for each set of data.

The authors try to extend their findings regarding the role of cell contractility in the SMG epithelium to include other branching organs. These data were confusing and not entirely conclusive. It would be best to focus the findings on the SMG and move all other organs to the supplement. The universal role of epithelial cell contractility in branching epithelial organs should also be de-emphasized in the text, as there is not much data to support these claims.

The authors do not address how fibronectin (FN) may be altered in blebbistatin treatments. Considering this is an important part of cleft formation in the SMG and could be produced by the epithelial explants, it is surprising that any analysis on FN staining/distribution was omitted.

Minor Comments:
Throughout the manuscript, all stages of each tissue should be listed in the respective figure panel (or at least in the figure legend) and the methods section.

In Figure 1A-C, the authors claim that the staining intensity of filamentous actin varies in certain regions of the tissues but the quantification appears to just show cells listed from dimmest to brightest instead of showing where in the organ the brighter or dimmer cells are. The authors also need to be more clear about where the staining was quantified within each cell: at the cortex or throughout the cytoplasm? Additionally, the presence of actomyosin complexes does not necessarily mean there is active force. Throughout the manuscript the authors need to weaken their claims

about force within epithelia since this was not directly measured and it is not clear whether the cell shape changes are the result of externally applied forces or active shape change of the cells.

Many of the staining patterns throughout the manuscript seem to have a lot of epithelial markers in mesenchymal tissue. For example, excessive mesenchymal staining of ZO-1 in Figure 2G and keratin staining in Figure 4I. Why does this happen? Several of the images throughout the paper are highly saturated (4I, 1A, 2G) adding to this difficulty of interpretation.

From Figure 2E and F, the authors conclude that cells expressing MRLC-AA change cell shape, similar to blebbistatin treatment. It is clear and obvious that peripheral epithelial cells change shape in blebbistatin treated explants. However, the images in Figure 2D indicate that the most peripheral epithelial cells maintain an elongated cell shape, despite expressing MRLC-AA. To compare similarities between these two experiments, it would be useful to quantify epithelial cell shape in the most peripheral layer versus interior epithelial cells in both conditions. Further, do the authors expect a single cell without myosin contractility within a sheet of normal cells to have similar shapes to cells in a blebbistatin treated explant? It is not clear that this should be the case.

Representative images used to quantify pMyo staining intensity in Figure 2J should be shown. The changes in ZO-1 distribution in Figure 2G and I should be more clearly annotated in the images.

The authors state the stiffness of synthetic ECM as defined by the manufacturer (Figure 2I). The authors should measure the elastic modulus of these gels if they want to list these values, as the stiffness of a gel can vary depending on how it is exactly prepared. Further, the authors do not address the fact that changing the matrigel concentration also changes the ligand density.

In Figure 3, the authors discuss force in their traction measurements. While bead displacement is related to force imparted on the matrix, force cannot be inferred without also measuring a zero-stress state. The authors should be more explicit in what the bead displacement shows. Also, according to the displacement fields shown in Fig 3E, bead displacement slows as clefts form. If force is driving cleft formation, and bead tracking is a measurement of force, then why does traction slow as clefts are forming?

Why are there no clefts forming in the blebbistatin examples in Figure 3F? This result seems inconsistent with data described in Figure 1F and 3B. If there are clefts, then the authors should annotate them in 3F as they did in 3E.

The authors show only one or two salivary glands in Figure 3 and the results appear variable from cleft to cleft. They need to quantify multiple clefts and summarize the results in a clearer graphical display to show their results are consistent across multiple explants, particularly for panels E, F, and G.

In Figure 3G the text implies the vectors are the tangential component of the bead displacements just outside the epithelium, however, the vectors are not tangential to the epithelium. It needs to be more clear what this panel adds, because panel D also shows that the vectors point in towards the clefts.

In Figure 4, the authors discuss the importance of Kit, however the volume plot where they identify Kit as a differentially expressed gene was listed in the supplement. This key finding would be better suited in the figures in the main text.

In Figure 4I, it is difficult to interpret K14/K19 staining patterns in a merged image. These channels need to be split and the staining intensity at the cellular level should be quantified. Similarly, it should be made clear how Kit staining intensities were quantified.

Having 4 panels of figure 4 showing gene ontology or pathway analysis does not add much to the story and the motivation for RNA-sequencing was not clear in the text. This figure might be improved by starting with the change in K14 and K19 distributions and using RNA-sequencing to understand the mechanism. Displaying changes in the RNA-seq data for K14, K19, and YAP/TAZ and target genes would then be very informative in this figure.

The authors claim that YAP is not phosphorylated in SMG peripheral epithelium. The representative images do not reflect this. Quantification of pYAP and LATS staining at the cellular level would aid in the reader's interpretation of these data.

The authors need to explain why different pathways might be acting upstream of YAP in their study compared to the study they mention which reported a decrease in K14 expression in Yap-cnull mice.

In the discussion, the authors comment on the structure of the actin network in their tissues, specifically stating that the epiSMG explants have a stress fiber-free circumferential cortex, however this was not quantified. The authors should clearly quantify the structure of the actin network to make this claim.

It is not clear what information is gained from the epiSMG ECM experiments. It seems that low ECM concentration corresponds to decreased pMyosin, which is plausible. Then the authors show an inverse relationship between YAP/TAZ activation and ECM stiffness. This is a surprising finding and it is not clear how to interpret this finding. How is YAP activated in a microenvironment with low stiffness while cells are also exhibiting features of low contractility? The authors' results suggest that if actomyosin machinery is inhibited, then the epiSMG explants buckle in culture. If this hypothesis is correct, culturing epiSMG explants within a stiff ECM in the presence of blebbistatin would enhance buckling. This experiment would be critical for understanding their data.

First revision

Author response to reviewers' comments

Reviewer 1 Comments for the Author:

A/ Clarity of presentation and proposed mechanism
The authors propose that "cleft formation is related to de-stressing of the concentrated force." (p8) from the aspect ratio, but then that "This effect could provide compelling evidence for a buckling- infolding model of cleft formation". To me, these two sentences are contradictory: the classical buckling is due to accumulation of compressive stresses (so the reverse of destressing). Can the authors clarify what they mean?
We apologize for the unclear description. More accurately, we suggest that accumulation of compressive stress induces epithelial buckling, and cleft formation progresses according to a buckling mechanism, resulting in de-stressing of the concentrated force in the epithelial layer. Therefore, de- stressing is the resultant event of the buckling process. The compelling evidence for the de-stressing of concentrated force is the single-cell geometry data and spatial actin pattern of the epithelial buds (Figure 1B and S2F), commonly indicating low force distribution in pericleft regions. We corrected the description in the revised manuscript (p7).

On that note, as the authors mention in discussion, fibronectin and the role of matrix in general has been well-studied for clefting. But they don't link this knowledge in the paper: for instance they modulate the ECM in the main text (p8-9), but only use this as a way to manipulate myosin structure. Can the authors exclude for instance that some of the phenotype/movements is due to matrix rigidity change itself?
We designed this experiment based on the previous methods that investigated the biological effects of matrix stiffness through changes in gel concentrations (Aragona et al. 2013; Paszek et al. 2005). As the reviewer pointed out, there is an intrinsic methodological limitation in the regulation of cell contractility by extracellular matrix (ECM) modulation - the method can induce mechanical effects on adjacent cells. To avoid this mechanically unwanted impact, we used this method only for investigating ductal differentiation and related genetic effects, which are relatively less affected by the intrinsic effect of matrix rigidity. The cellular parameters directly affected by matrix rigidity (e.g., cell morphology, migration) were excluded with this method. Additionally, we checked the pattern of fibronectin (FN) around developing epithelial buds to exclude the possible role of the

ECM in our experimental designs. The results showed no clear changes in FN patterns upon actomyosin perturbation (Figure S3).

Especially given the above question on buckling vs de-stressing, could it be instead that some forces (either from central cells or ECM-linked) effectively push/stretch cells locally at the cleft? On that front, the summary schematic of FIg. 3H for instance mentions as a key first that continuous cell proliferation increases internal stresses. But i don't see it really discuss so much in the paper? (proliferation of inner cell would create pressure, thus stretching of the outer layer, whereas proliferation of outer layer alone would create compression in the outer layer - opposite effects again which are relevant i think for buckling vs de-stressing.)

In developing submandibular glands, differential growth in epithelial buds was previously reported (Kim et al. 2018; Rebustini et al. 2012) (Response Fig. 1). In detail, the outer layer of epithelial cells presents higher mitotic activity than the inner cells based on the following data (Response Fig. 1). Such differential growth has been recognized as a crucial event for generating force that triggers morphogenesis of epithelial layers in diverse organs (Varner and Nelson 2014). Therefore, the proliferation discrepancy between the outer and inner parts of epithelial buds can support our key mechanism: stress concentration in the outer epithelial layers triggers cleft progression through a buckling mechanism

We have removed unpublished data provided for the referees in confidence.

Response Fig. 1. Removed

B (more minor)/ Presentation/quantifications
I don't quite understand the quantifications of Fig. 2B. Is it a ranked measurement of 50 randomly selected cells? i think that if the point is to convey variability, the authors should instead with dot or violin plots. If it's to signify spatial distribution as they mention, then maybe the x axis should be position then, to show spatial trends? A schematic of cell shape (as quantified in S2D might help for addition to 1E or 1G) as a function of regions would also really help i think to make the data more explicit.
We apologize for the unclear description of the quantification data. Figures 2B and 2C (Fig. 2C and 2D in the revised figure) are the quantified results of peripheral epithelial cells showing high F-actin intensity. We presented the scheme depicting the region for single-cell geometry analysis (Fig. 2B). To evaluate the spatial distribution of cell morphologies, we conducted comparative analysis of the aspect ratio between peripheral and central cells (Figure S2D and S2E).

The correlation between traction forces and protrusions is not very easy to see/understand from Fig. 3E-F given variations and fluctuations, maybe the authors could average/show curves for these side by side?
We thank the reviewer for their helpful suggestion. We would like to note that our visualization schemes in Figures 3E and 3F were inspired by the widely used "edge velocity" and "local activity" maps in cell-migration studies (for example, see Figure 7 in Yang et al. 2016). These plots efficiently summarize the complex spatiotemporal dynamics in two dimensions; however, tend to require some time to understand as the reviewer pointed out. A standard approach to correlate the two maps and to further reduce the dimensionality (to one dimension) is quantification of temporal correlation (see the right column in Fig. 7 in Yang et al. 2016). Unfortunately, in our case, the relationship between traction and protrusion involves not only temporal but also spatial correlations (i.e., increased traction force in the vicinity of the future cleft loci). Here the quantification of spatiotemporal correlation resulted in another two-dimensional plot, which barely contributes to clearer visualization. Thus, we concluded that our data require slightly complicated visualizations, as long as we avoid overly simplifying the complexity innate to our experiments.

Reviewer 2 Comments for the Author:
Major Comments:
The current state of the manuscript makes it challenging for the reader to understand what aspects of this work are novel. There are many reports on the role of the actomyosin machinery in epithelial cleft formation and branching morphogenesis in the submandibular gland (Joo, E. and Yamada, K., 2014; Kadoya, Y. and Yamashina, S., 2010; Daley, W. P. et al., 2009; Hsu et al., 2013; Harunaga, J.

S. et al., 2014). There are also reports on the role of YAP/TAZ regulating SMG branching morphogenesis(Enger et al. 2013) and specifying cell fate in the developing SMG epithelium (Szymaniak et al., 2017). The authors highlight where their data either agrees or conflicts with many of these findings, but don't make it clear how their findings add to the field.

First of all, we gratefully appreciate the invaluable comments of the reviewer, which is very helpful in terms of improving our manuscript. As the reviewer pointed out, actomyosin contractility plays a critical role in the branching morphogenesis of epithelial organs, including salivary glands. However, it is not clearly identified how the forces generated from actomyosin contractility sculpture epithelial morphology during the developmental process. In this study, we suggested the buckling mechanism of epithelial layers in the process of cleft formation, which is a previously unidentified mechanism during salivary gland-branching morphogenesis. Through multidisciplinary experimental approaches, we dissected the physical process in which the spatial difference of forces in epithelial buds is transmitted to the triggering force of cleft formation.

Moreover, we found that the physical effect of the actomyosin machinery regulates the expression of key genes of progenitor cell-patterning via the mechanotransduction process. In this process, YAP/TAZ were revealed as a key mediator of genetic regulation in acinar/ductal pattering. Previous articles (Enger et al. 2013; Szymaniak et al. 2017) have also described the role of YAP/TAZ in the differentiation process. The major differentiation point (rather than conflict) of our study is the upstream signals and operational mechanism of YAP/TAZ, which is the mechanotransduction process independent of the canonical Hippo pathway. Enger et al. emphasized the role of the TAZ protein during E-cadherin-mediated ductal differentiation, which is different in the context of our concepts. Szymaniak et al. reported the change in K14 expression is mediated by epiregulin-EGFR signaling, and the Hippo pathway acts as an upstream signal of this downregulation. This point is also distinct from our Hippo pathway-independent mechanotransduction process of YAP. Notably, the core finding of this study is that KIT, the crucial mediator of acinus-duct differentiation, is regulated by the mechanotransduction process.

In the Discussion section of the revised manuscript, we described key findings that can benefit the field and various points to previous research (p11-12).

The most interesting finding is that mesenchyme-free epiSMG cultures undergo buckling when myosin is inhibited. This suggests that when epithelial cell contractility is reduced, the tissue folds due to mechanical buckling. This buckling phenomenon is found in many epithelial organs (Nelson, C. M., 2016; Varner, V. D. et al. 2015; Shyer, A. E. et al. 2013), and to my knowledge has not been shown in the SMG prior to this work. The work would have more impact if the authors reframe their results in this context. Their finding of more buckling when actomyosin contractility is inhibited could add to our understanding of how compression leads to epithelial buckling by showing that tension caused by actomyosin contractility decreases buckling.

Thank you for your suggestion. As the reviewer commented, this study first introduces a buckling mechanism in the context of branching morphogenesis of the salivary glands. The core mechanism is actomyosin contractility of the outer epithelial cells efficiently transmit forces from the epithelial layers via an inward buckling force (Figure 3H). When actomyosin contractility was perturbed, striking morphological irregularity of the epithelial buds was observed (Fig. 1C and 1E). Based on the reviewer's interpretation of this phenomenon, we can anticipate the underlying processes in terms of the morphological distortion of epithelial buds as follows: first, decreased actomyosin contractility allows epithelial outgrowth with disrupted integrity of the epithelial layers. Moreover, perturbated contractility decreases epithelial tension, then lateral compressive stress, the triggering factor of the buckling mechanism, and it might be passively increased in epithelial layers. In addition to compressive stress, decreased stiffness of epithelial cells by blebbistatin treatment can alter buckling patterns more sporadically and transiently (Nelson 2016; Martens and Radmacher 2008). We suggest that aforementioned processes collectively elicit the morphological changes of epithelial buds.

We have newly added the content surrounding the buckling mechanism in the Abstract and Discussion sections of the revised manuscript to emphasize the novelty of the buckling mechanism in this study (p.2 and 11).

The authors switch between whole SMG explants and mesenchyme-free epiSMG frequently throughout the manuscript without rationalizing which model is used for a given experiment/analysis. This made interpreting the findings quite challenging. The authors need to more clearly state their questions and hypotheses for each set of data.

Throughout this study, we tried to primarily use epithelial isolation culture of SMGs (epiSMG) because the model can efficiently exclude the cellular effect in the mesenchyme, especially avoiding the unwanted impact of blebbistatin on mesenchymal cells. Moreover, optical visualization and perturbation of signaling activities is feasible in an epiSMG model. Whole SMG cultures (including the epithelium and mesenchyme) were used as supportive data to confirm the result of epiSMG cultures and conduct experiments that are difficult with epiSMG cultures (e.g., paraffin-embedding section, ductal differentiation staining and other epithelial organ experiments). Notably, there is little or no discrepancy in the results between the two models (epiSMG versus whole SMG culture) in this study.

The authors try to extend their findings regarding the role of cell contractility in the SMG epithelium to include other branching organs. These data were confusing and not entirely conclusive. It would be best to focus the findings on the SMG and move all other organs to the supplement. The universal role of epithelial cell contractility in branching epithelial organs should also be de-emphasized in the text, as there is not much data to support these claims.
As the reviewer commented, we moved all data for other organs to supplementary fields, and corrected the manuscript to avoid the overestimation of our findings.

The authors do not address how fibronectin (FN) may be altered in blebbistatin treatments. Considering this is an important part of cleft formation in the SMG and could be produced by the epithelial explants, it is surprising that any analysis on FN staining/distribution was omitted.
As the reviewer commented, we investigated the pattern of FN through immunostaining. In the SMG culture, overall FN signaling intensity around epithelial buds and clefts did not change upon blebbistatin treatment (Figure S3A and S3B). Although blebbistatin-treated SMGs displayed the relaxed pattern of FN, the concentrated signals in the cleft region were not modified (Figure S3A, lower panels). In the epiSMG culture, there was no clear FN pattern around the outer epithelial layers in either group (Figure S3C). These data indicate that morphological changes of epithelial buds with actomyosin perturbation is mainly mediated by the alteration of internal stress in epithelial buds.

Minor Comments:
Throughout the manuscript, all stages of each tissue should be listed in the respective figure panel (or at least in the figure legend) and the methods section.
As the reviewer commented, we listed all stages of each tissue in the respective figure panel, legend, and Methods sections.

In Figure 1A-C, the authors claim that the staining intensity of filamentous actin varies in certain regions of the tissues but the quantification appears to just show cells listed from dimmest to brightest instead of showing where in the organ the brighter or dimmer cells are. The authors also need to be more clear about where the staining was quantified within each cell: at the cortex or throughout the cytoplasm?
The quantified data shows the distribution (proportion) of F-actin intensity of each epithelial cell. To provide spatial information, we presented the arrowheads to indicate concentrated F-actin signals (Figure S1A). To analyze F-actin intensity, we quantified the cortical actin region of single cells, and the actin signals were manually detected using ImageJ software. We included this content in the Methods section.

Additionally, the presence of actomyosin complexes does not necessarily mean there is active force. Throughout the manuscript the authors need to weaken their claims about force within epithelia since this was not directly measured and it is not clear whether the cell shape changes are the result of externally applied forces or active shape change of the cells.

As reviewer commented, we added the description about the limitation of this study regarding the force patterns within epithelial cells in revised manuscript (p. 7).

Many of the staining patterns throughout the manuscript seem to have a lot of epithelial markers in mesenchymal tissue. For example, excessive mesenchymal staining of ZO-1 in Figure 2G and keratin staining in Figure 4I. Why does this happen? Several of the images throughout the paper are highly saturated (4I, 1A, 2G) adding to this difficulty of interpretation.
We are sorry for the saturated images that interfere with the exact interpretation of the data. We

have strictly chosen antibodies proven in previous articles to have a strong relationship with this project (Nedvetsky et al. 2014; Lombaert et al. 2013). Nevertheless, there are several unavoidable methodological limitations to show the exact signals of target proteins. The following is a point-to-point response for each issue.
Figure 1A (moved to Figure S1A in the revised figure): the epithelial buds of the tooth germ intrinsically exhibit very low signal intensity for F-actin (Figure S1A, arrowheads) compared to mesenchymal cells. We set the image window focusing on the epithelial actin signals so that mesenchymal signals were saturated inevitably.
Figure 2G (moved to Figure 4A in the revised figure): the excessive ZO-1 intensity in the mesenchymal part is actually the patterns of developing endothelial cells. To present ZO-1 midline condensation during tubulogenesis, we enhanced the contrast of images to focus on the signals in the ductal region, and consequently endothelial ZO-1 signals were saturated (Response Fig. 2A). To address this point, we presented the colocalization patterns of ZO-1 and CD31 (vascular marker) in the mesenchymal part (Response Fig. 2B).
Figure 4I (moved to Figure 4F in the revised figure): we estimate that K14 signals in the mesenchymal part might be based on non-specific binding of primary or secondary antibodies. However, K14 signals in the acinar part clearly show characteristic distal patterns in line with previous results (Lombaert et al. 2013). Moreover, unavoidable accumulated signals at the explant-membrane-water interface sometimes appeared, and were caused by the long incubation of primary and secondary antibodies (over 48 hrs for each antibody) for whole-explant immunostaining procedures.

We have removed unpublished data provided for the referees in confidence.

Response Fig. 2. **Removed**

From Figure 2E and F, the authors conclude that cells expressing MRLC-AA change cell shape, similar to blebbistatin treatment. It is clear and obvious that peripheral epithelial cells change shape in blebbistatin treated explants. However, the images in Figure 2D indicate that the most peripheral epithelial cells maintain an elongated cell shape, despite expressing MRLC-AA. To compare similarities between these two experiments, it would be useful to quantify epithelial cell shape in the most peripheral layer versus interior epithelial cells in both conditions. Further, do the authors expect a single cell without myosin contractility within a sheet of normal cells to have similar shapes to cells in a blebbistatin treated explant? It is not clear that this should be the case.
As the reviewer suggested, we compared the aspect ratio of peripheral and central cells in two experimental designs: control (-BB) versus blebbistatin treatment (+BB) and MLC-AA- versus MLC-AA+ cells. In the control groups (-BB and MLC-AA-), peripheral cells presented a significantly higher aspect ratio (AR) than central cells whereas the discrepancies in AR were clearly reduced in actomyosin-perturbated groups (+BB and MLC-AA+) (Figure S2D). Moreover, MLC-AA-expressing (MLC-AA+) cells and blebbistatin-treated cells showed similar morphological changes compared to each control group – the significant decrease in AR occurred in peripheral cells, but not in central cells (Figure S2E). Notably, the AR values in MLC-AA+ cells were less reduced compared to blebbistatin-treated group (Figure S2E). Given the mosaic expression patterns, the reason seemed that the morphology of sporadically located MLC-AA-expressing cells could not be fully altered by the restriction of adjacent MLC-AA non-expressing cells that maintain their original columnar morphology.

Representative images used to quantify pMyo staining intensity in Figure 2J should be shown.
We presented the representative images of pMyo staining in Figure 4C in the revised manuscript.

The changes in ZO-1 distribution in Figure 2G and I should be more clearly annotated in the images. We annotated the condensed ZO-1 patterns by presenting arrowheads in the images.

The authors state the stiffness of synthetic ECM as defined by the manufacturer (Figure 2I). The authors should measure the elastic modulus of these gels if they want to list these values, as the stiffness of a gel can vary depending on how it is exactly prepared. Further, the authors do not address the fact that changing the matrigel concentration also changes the ligand density.
As the reviewer commented, the stiffness of Matrigel varies depending on diverse environmental conditions. Moreover, the stiffness is different among each sample and manufactured lot number.

These facts make Matrigel difficult (almost impossible) to maintain in terms of exactly the same stiffness states. Therefore, we concluded that the elastic modulus values from the manufacturer should be withdrawn in the revised manuscript. To overcome these limitations, all experiments were conducted using the same Matrigel sample from a single tube, and all data were compared relatively.
Another methodological limitation is in the regulation of gel stiffness without influencing ECM protein concentration. We designed this experiment based on the previous methods that assessed the biological effects of matrix stiffness through changes in gel concentrations (Aragona et al., 2013; Paszek et al., 2005). A method for decreasing stiffness without changing gel concentration, to the best of our knowledge, has not yet been established. ECM-free hydrogel (or polyacrylamide gel) could be an effective alternative, but the viability of SMG cultures is compromised in these models. We used growth factor-free Matrigel to minimize modifications in the amount other ECM proteins, and supplied the proteins from culture media. In addition, the results from this experiment are confined to supporting data with limited interpretation.

In Figure 3, the authors discuss force in their traction measurements. While bead displacement is related to force imparted on the matrix, force cannot be inferred without also measuring a zero-stress state. The authors should be more explicit in what the bead displacement shows.
While our traction-force assay was inspired by traditional single-cell traction-force microscopy, there exists an important conceptual and practical discrepancy. In single-cell experiments, it is possible to reconstruct the quantitative force distribution via zero-stress measurement, as the reviewer pointed out, and making several assumptions on the elasticity of the matrix. At the scale of tissues, however, those assumptions are mostly infeasible and thus it is extremely challenging to quantitatively calculate the force distribution around the tissues. With these difficulties in mind, we sought to measure the microbead displacement field as a rough proxy of the traction force rather than accurately deriving the absolute force values.

Also, according to the displacement fields shown in Fig 3E, bead displacement slows as clefts form. If force is driving cleft formation, and bead tracking is a measurement of force, then why does traction slow as clefts are forming?
As summarized in Fig. 3H, the key point of our suggested model is that cleft formation is induced by the de-stressing of the concentrated force in the epithelial layer. This is consistent with our observation of decreased traction as clefts are forming.

Why are there no clefts forming in the blebbistatin examples in Figure 3F? This result seems inconsistent with data described in Figure 1F and 3B. If there are clefts, then the authors should annotate them in 3F as they did in 3E.
As shown in Figs. 1C and 3B, a large number of shallow clefts are formed with blebbistatin treatment. We intended to highlight the diminished traction and shallow clefts in Fig. 3F as the formation of the clefts was clear from Figs. 1C and 3B. Unlike the snapshot images in Fig. 1C and 1E, it was not straightforward whether we could annotate the dynamically evolving shallow clefts through the time- lapse data.

The authors show only one or two salivary glands in Figure 3 and the results appear variable from cleft to cleft. They need to quantify multiple clefts and summarize the results in a clearer graphical display to show their results are consistent across multiple explants, particularly for panels E, F, and G.
We regret that the current circumstances significantly limited our experimental capability in terms of collecting extensive new data. Yet, we note that there were consistent differences between the control and blebbistatin conditions without significant cleft-to-cleft variability (see the colormap), strongly supporting our main point herein.

In Figure 3G the text implies the vectors are the tangential component of the bead displacements just outside the epithelium, however, the vectors are not tangential to the epithelium. It needs to be more clear what this panel adds, because panel D also shows that the vectors point in towards the clefts.
As specified in the caption for Fig. 3G, the visualized arrows are full displacement vectors but not its tangential components. The text describes its tangential components converging into cleft locations, which are indicated by the black and gray arrowheads in Fig. 3G. We intended to visualize the converging force directions to highlight the concentrated force in our model, which is not shown

in Fig. 3D.

In Figure 4, the authors discuss the importance of Kit, however the volume plot where they identify Kit as a differentially expressed gene was listed in the supplement. This key finding would be better suited in the figures in the main text.
As suggested, we moved the volume plot data from the supplemental information to the main manuscript (Figure 5B).

In Figure 4I, it is difficult to interpret K14/K19 staining patterns in a merged image. These channels need to be split and the staining intensity at the cellular level should be quantified. Similarly, it should be made clear how Kit staining intensities were quantified.
We presented single channel images of K14 and K19 separately (Figure 4G) and conducted line-scan analysis to show the signaling patterns (Figure 4H). The analytical method for the KIT signals was included in the Methods and Materials section.

Having 4 panels of figure 4 showing gene ontology or pathway analysis does not add much to the story and the motivation for RNA-sequencing was not clear in the text. This figure might be improved by starting with the change in K14 and K19 distributions and using RNA-sequencing to understand the mechanism. Displaying changes in the RNA-seq data for K14, K19, and YAP/TAZ and target genes would then be very informative in this figure.
According to the suggestion, we reconstructed the whole structure of the manuscript, and the revised version consequently consists of two distinct parts. The first part describes the mechanical effect on cleft formation and the second part surrounds the genetic impact on duct formation. To this end, we conducted the following tasks.
- Moved the data regarding immature duct formation by actomyosin perturbation (Fig. 2G-K) to Fig. 4A-4E.
- Re-located the data surrounding the K14-K19 spatial patterns ahead of the RNA-seq results (Fig. 4F-4H).
- Added the KIT inhibitor (ISCK03) experiment results (Fig. 5H).

Based on the aforementioned changes, we sought to strengthen the relevance and motivation behind the RNA-seq analysis for the mechanistic study of K14-19 distribution changes.

The authors claim that YAP is not phosphorylated in SMG peripheral epithelium. The representative images do not reflect this. Quantification of pYAP and LATS staining at the cellular level would aid in the reader's interpretation of these data.
We quantified the phosphorylated YAP signal intensity in each bud and added the results (Figure S9A, right panel). In the case of phosphorylated LATS, we calculated the cell density of pLATS+cells in each single bud (Figure S9B, right panel).

The authors need to explain why different pathways might be acting upstream of YAP in their study compared to the study they mention which reported a decrease in K14 expression in Yap-cnull mice.
As described in the Discussion section, the authors of the previous report (Szymaniak et al. 2017) demonstrated that the Hippo pathway is a key upstream signal for YAP-mediated epiregulin expression, and the paracrine effect of epiregulin regulates K14 expression by the EGFR signaling pathway (YAP-Epiregulin-EGFR-K14 cascade). In this study, we suggested the different mechanism of K14 regulation mediated by the YAP-KIT-K14 cascade. In terms of the regulatory mechanism of YAP activity, previous studies have suggested the canonical Hippo pathway, while our study suggested the Hippo pathway-independent mechanotransduction process as an upstream signal. Based on the background, we anticipate that differential upstream signaling input has a multimodal effect on YAP activity that leads to different result with respect to K14 expression.
The corrected version of this content is included in the revised manuscript (p12).

In the discussion, the authors comment on the structure of the actin network in their tissues, specifically stating that the epiSMG explants have a stress fiber-free circumferential cortex, however this was not quantified. The authors should clearly quantify the structure of the actin network to make this claim.
Under the condition of high magnification with a high master gain value (high detector sensitivity), we found few stress fiber-like actin patterns in the epithelial cells (Response Fig. 3, arrows in right panel). However, the patterns are not clearly distinguished with cortical actin with high cell-to-cell

variability (Response Fig. 3). These characteristics make the quantification process difficult. Instead of a quantification result, we replaced the comments of “stress fiber-free circumferential cortex” to “circumferential cortex-dominant actin patterns”, a more accurate description depicting the actin patterns of epithelial buds.

We have removed unpublished data provided for the referees in confidence.

Response Fig. 3. **Removed**

It is not clear what information is gained from the epiSMG ECM experiments. It seems that low ECM concentration corresponds to decreased pMyosin, which is plausible. Then the authors show an inverse relationship between YAP/TAZ activation and ECM stiffness. This is a surprising finding and it is not clear how to interpret this finding. How is YAP activated in a microenvironment with low stiffness while cells are also exhibiting features of low contractility? The authors’ results suggest that if actomyosin machinery is inhibited, then the epiSMG explants buckle in culture. If this hypothesis is correct, culturing epiSMG explants within a stiff ECM in the presence of blebbistatin would enhance buckling. This experiment would be critical for understanding their data.
Although there are some exceptions, it is generally accepted that cell contractility and YAP/TAZ activity has a positive relationship. Such a regulatory mode of YAP has been demonstrated under the following experimental conditions: sparse cell density, 2D culture, and high ECM stiffness (usually plastic bottom). Under these conditions, many cell types present well-established actin stress fibers with high basal YAP/TAZ activity, and this activity is efficiently de-activated by the perturbation of actomyosin structure or contractility. Moreover, the activity of YAP/TAZ can be mediated by diverse external factors, such as cell density, cell adhesion, and ECM stiffness (Aragona et al. 2013; Poitelon et al. 2016; Totaro et al. 2018; Dupont et al. 2011). External factors also regulate YAP/TAZ activity by transmitting their signals to actomyosin contractility through actin stress fibers.
In our experimental setup of epithelial buds, the peripheral epithelial cells are subjected to extremely high cell density and are three-dimensionally clustered, surrounded by substrates with low stiffness (Matrigel or mesenchymal tissue). In addition, totally different actin patterns, well-organized cortical actin with weak stress fiber formation, were observed in the epithelial cells. These conditions result in low basal activity of YAP in the epithelial buds (Figure 6A) in contrast to the previous 2D culture models. We speculate that such differences in experimental conditions could be the major reason for the different results, specifically increased YAP activity with low actomyosin contractility.
Notably, a recent paper also introduced a distinct mode of YAP/TAZ regulation (Furukawa et al. 2017). The authors presented highly organized cortical actin (referred to as a “circumferential actin belt”) and low basal activity of YAP/TAZ in a Madin-Darby canine kidney (MDCK) monolayer with high density (Response Fig. 4). Blebbistatin treatment of the monolayer induced YAP/TAZ activation, which is consistent with our results (Response Fig. 4B and 4C). This result supports our findings, and the authors suggest that the structure of the actin pattern is a critical determinant in this phenomenon. Taken together, we can conclude that YAP/TAZ can be differentially regulated based on the biological context, especially with respect to characteristic actin structures. We expect that our findings will contribute to further understanding the mechanotransduction process in 3D organ models. This information and the relevant experimental data were included in the revised manuscript (p.12-13).

We have removed unpublished data provided for the referees in confidence.

Response Fig. 4. **Removed**

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Second decision letter

MS ID#: DEVELOP/2020/190785

MS TITLE: Mechanogenetic role of actomyosin complex in branching morphogenesis of epithelial organs

AUTHORS: Jin Man Kim, YoungJu Jo, Ju Won Jung, and Kyungpyo Park

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is clearly positive and we would like to publish a revised manuscript in Development. Following the reveiwers suggestions, this involves clarifications and text edits. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors have provided additional data and explanations, which satisfactorily answer several of my questions (fibronectin and presentational issues). However, I am still unsure i understand some of the physical reasoning (my previous two questions on buckling and their summary schematic in Fig. 3H). I thus have three comments left related to this (the first one being most important, and the other two being more presentational/rewriting issues i think):

Comments for the author

- on my question on buckling vs other forces/alternative hypotheses: the authors still very much equate myosin contractility with compressive/buckling stresses. While this is valid in some settings, many events of tissue bending depend not on absolute levels of myosin, but on its differential localisation (classical studies in Drosophila gastrulation or vertebrate neural tube formation for instance). The authors do mention for instance "The typical columnar morphology of the peripheral cells is formed by an apico-basal contraction force and intercellular junctional integrity," + "highly polarized morphology" and have added aspect ratio quantification in p6 and p7. But did the authors quantified the possibility of differential apico-basal regulation of myosin, that creates spontaneous curvature (which wouldn't really manifest mainly in aspect ratio, but mostly in apical vs basal differences in area), in addition or alternatively to compressive in-plane stresses/bending? This would be helpful to discuss this together with the bead experiments and discussion, to consider alternative/complementary possibilities.

- On the "continuous cell proliferation" statement in Fig. 3H, which appears as a key part of the mechanism: i thank the authors for the response figure, and the bibliographic detail, but am unsure why they don't include any of it in the main text? this is key background for the buckling argument to make sense in my opinion and should be clarified in intro and discussion.

- in abstract "determined the force flow that is essential for buckling mechanism to promote the branching process" is not very clear (i don't think "force flow" is a standard term in physics or biophysics). i think the authors want to say "force balance" or "spatial distribution/nature of forces" here? in general, this sentence is quite vague, and does not really indicate what the authors found, i would suggest significantly re-writing the abstract for clarity.

Reviewer 2

Advance summary and potential significance to field

The major restructuring of the manuscript has made the overall story much more focused and clear. The narrowed claims, specific to the salivary gland, are now quite compelling and the additional context on recent findings in the field of YAP/TAZ signaling in three dimensional tissues better highlights the importance of the results. There remain, however, some points for the authors to address

Comments for the author

Major points:
One of the main concerns in the initial review was about novelty. In the authors' response, they emphasized that their proposed role for Yap was downstream of mechanical signals and not Hippo signaling (as shown in previous studies (Szymaniak et al., 2017)). While this may be the case, the manuscript in its current form does not communicate effectively that Hippo signaling is not involved. To strengthen this point, the authors could move the data that supports this claim (pYap and pLATS immunostaining) from supplementary figure 9 to main figure 6.

The flow of the manuscript in terms of transitioning from a physical mechanism of buckling/branching to an investigation of the impacts on differentiation and patterning of

progenitors is much better. However, using the formation of ducts (assessed by ZO-1 staining) as a readout of how changes to actomyosin contractility and the resultant alterations to branching affect acinar/ductal differentiation may be slightly problematic. The mechanisms by which ducts form may require actomyosin activity, and therefore blebbistatin treatment could be interfering with this process directly (and not indirectly by affecting progenitor populations). Further, the authors show similar effects in the lacrimal gland, which does not have similar branching patterns to the submandibular gland, further supporting the idea that blebbistatin treatment is affecting duct formation more directly. This concern can be addressed by careful rewriting of certain sections of the results.

Finally, the initial review suggested that the authors culture epiSMG explants within a stiff ECM in the presence of blebbistatin to see if this affects buckling. While this may not be required, it would still add to the manuscript and should be included if possible. Also, one method for altering gel stiffness without diluting Matrigel is to include different concentrations of methylcellulose (perhaps this could be useful in future work to differentiate between the effects of stiffness and cell-ECM adhesion on buckling).

Minor points:
The abstract includes both “actin-myosin” and “actomyosin” - one should be chosen for consistency.

It would be helpful to state in the abstract (and perhaps keywords) that the epithelial organ focused on in the manuscript is the submandibular gland.

In figure 2C, the diagram depicting a square is placed next to an aspect ratio of 0, which is incorrect. The y-axis for this plot could instead just start at 1.

Figure 3 is still a bit confusing, although this is understandable given the complexity and dimensionality of the data being communicated. The figure legends could be more informative, and include information about how the surface plot relates to the kymographs (e.g. do the kymographs show the data from one slice or from an average of slices?)

The authors continue to use the word “traction” in multiple panels of figure 3. As discussed in the response letter, these data do not show forces and therefore the word traction should be replaced, perhaps with “bead velocity” to match the units provided.

In figure 3E the “Time [hour]” label is very close to the color bars indicating the traction rate and protrusion, making it look like the time label is meant for the color bar. It would be helpful to move this color bar and label to the top of the image to avoid confusion.

The authors state in their response that the vectors in 3G are full displacement vectors but the description of this panel in the text states “Decomposing the measured displacement vectors identified converging tangential traction alongside the epithelial layer at the cleft locations.” How were the vectors decomposed? The authors should clarify these statements, which seem to conflict.

In the results section on lines 247-248, the authors say that Figure 4A contains blebbistatin-treated epiSMG cultures. Is this correct, or are they just SMG cultures?

The line plots in figure 4H are helpful for understanding the data, but it would be beneficial, if possible, to quantify these data over multiple replicates.

In figure 5h, please label the grayscale panels with the stain they represent to avoid confusion.

The description of figure 5 in the text states that “actomyosin contractility acts as a major upstream mediator of KIT expression through transcriptional regulation” seems slightly overstated. The data support a slightly weaker statement more like “actomyosin contractility is required for normal kit expression”

The authors have replaced most references to matrigel stiffnesses with concentrations because stiffness was not directly measured in this work. However, figure 6d still contains Young's modulus values. Please remove these values.

In the discussion, remove the citation to Daley 2017 as this paper was recently retracted.

Second revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
The authors have provided additional data and explanations, which satisfactorily answer several of my questions (fibronectin and presentational issues). However, I am still unsure i understand some of the physical reasoning (my previous two questions on buckling and their summary schematic in Fig. 3H). I thus have three comments left related to this (the first one being most important, and the other two being more presentational/rewriting issues i think):

Reviewer 1 Comments for the Author:

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For the curvature formation via purse-string mechanism, the apically concentrated actomyosin contractility is required in peripheral epithelial layers of SMGs. Of course, we totally agree the possibility that the actomyosin contractility on apical part of the cells can elicit the spontaneous bending forces together with buckling process. Unfortunately, in our experimental conditions, we could not observe and demonstrate clear apico-basal difference in actin and p-myosin signals in peripheral epithelial cells (Fig. S1G and S2B). Therefore, we unavoidably suggested buckling mechanism triggered by internal stress instead of focusing spontaneous curvature formation by the concentrated forces at a subcellular level.

- On the "continuous cell proliferation" statement in Fig. 3H, which appears as a key part of the mechanism: i thank the authors for the response figure, and the bibliographic detail, but am unsure why they don't include any of it in the main text? this is key background for the buckling argument to make sense in my opinion and should be clarified in intro and discussion.
As reviewer suggested, we added the comment regarding continuous cell proliferation in the revised manuscript (lines: 229-231).

- in abstract "determined the force flow that is essential for buckling mechanism to promote the branching process" is not very clear (i don't think "force flow" is a standard term in physics or biophysics). i think the authors want to say "force balance" or "spatial distribution/nature of forces" here? in general, this sentence is quite vague, and does not really indicate what the authors found, i would suggest significantly re-writing the abstract for clarity.
As suggested, we corrected the terminology to clarify the meaning of the sentence.

Reviewer 2 Advance Summary and Potential Significance to Field:
The major restructuring of the manuscript has made the overall story much more focused and

clear. The narrowed claims, specific to the salivary gland, are now quite compelling and the additional context on recent findings in the field of YAP/TAZ signaling in three dimensional tissues better highlights the importance of the results. There remain, however, some points for the authors to address

Reviewer 2 Comments for the Author:
Major points:
One of the main concerns in the initial review was about novelty. In the authors' response, they emphasized that their proposed role for Yap was downstream of mechanical signals and not Hippo signaling (as shown in previous studies (Szymaniak et al., 2017)). While this may be the case, the manuscript in its current form does not communicate effectively that Hippo signaling is not involved. To strengthen this point, the authors could move the data that supports this claim (pYap and pLATS immunostaining) from supplementary figure 9 to main figure 6.
Thank you for the suggestion. We moved the figures to main figure 6F and 6G.

The flow of the manuscript in terms of transitioning from a physical mechanism of buckling/branching to an investigation of the impacts on differentiation and patterning of progenitors is much better. However, using the formation of ducts (assessed by ZO-1 staining) as a readout of how changes to actomyosin contractility and the resultant alterations to branching affect acinar/ductal differentiation may be slightly problematic. The mechanisms by which ducts form may require actomyosin activity, and therefore blebbistatin treatment could be interfering with this process directly (and not indirectly by affecting progenitor populations). Further, the authors show similar effects in the lacrimal gland, which does not have similar branching patterns to the submandibular gland, further supporting the idea that blebbistatin treatment is affecting duct formation more directly. This concern can be addressed by careful rewriting of certain sections of the results.
Thank you for the suggestion. First of all, we presented the data of sublingual glands (SLG), not lacrimal glands, and SLGs show similar branching patterns to SMGs. Of course, we agree that the possibility of direct effect of blebbistatin on acinar/ductal patterning. To avoid the overstatement, we added the comment about direct effect of blebbistatin in Result section of revised manuscript (lines: 259-262).

Finally, the initial review suggested that the authors culture epiSMG explants within a stiff ECM in the presence of blebbistatin to see if this affects buckling. While this may not be required, it would still add to the manuscript and should be included if possible. Also, one method for altering gel stiffness without diluting Matrigel is to include different concentrations of methylcellulose (perhaps this could be useful in future work to differentiate between the effects of stiffness and cell-ECM adhesion on buckling).
Thank you for the information about the method for the alteration of gel stiffness. We added the data about epiSMG explants within a stiff ECM (100% Matrigel concentration) in the presence of blebbistatin (Fig. S9).

Minor points:
The abstract includes both "actin-myosin" and "actomyosin" - one should be chosen for consistency.
We corrected the terms of actin-myosin to actomyosin.

It would be helpful to state in the abstract (and perhaps keywords) that the epithelial organ focused on in the manuscript is the submandibular gland.
We added the comment of SMGs both in the abstract and keywords sections.

In figure 2C, the diagram depicting a square is placed next to an aspect ratio of 0, which is incorrect. The y-axis for this plot could instead just start at 1.
We corrected the labels of y-axis of all aspect ratio graphs.

Figure 3 is still a bit confusing, although this is understandable given the complexity and dimensionality of the data being communicated. The figure legends could be more informative, and include information about how the surface plot relates to the kymographs (e.g. do the kymographs show the data from one slice or from an average of slices?) The authors continue to use the word "traction" in multiple panels of figure 3. As discussed in the response letter, these

data do not show forces and therefore the word traction should be replaced, perhaps with “bead velocity” to match the units provided.
We thank the Reviewer for acknowledging the complexity of our data. As suggested, we revised the legends for Figure 3 in order to (i) describe the kymograph quantification more precisely, (ii) emphasize that the visualized traction vectors do not show the absolute forces, and (iii) clarify the decomposition of the vectors (see below).

In figure 3E the “Time [hour]” label is very close to the color bars indicating the traction rate and protrusion, making it look like the time label is meant for the color bar. It would be helpful to move this color bar and label to the top of the image to avoid confusion.
We moved the labels as suggested.

The authors state in their response that the vectors in 3G are full displacement vectors but the description of this panel in the text states “Decomposing the measured displacement vectors identified converging tangential traction alongside the epithelial layer at the cleft locations.” How were the vectors decomposed? The authors should clarify these statements, which seem to conflict.

The vectors were decomposed into tangential and normal components with respect to the surface of the epithelia rudiments segmented via Otsu’s method. We revised the corresponding section in Materials and Methods as well as the legends for Figure 3 in order to clarify this decomposition.

In the results section on lines 247-248, the authors say that Figure 4A contains blebbistatin-treated epiSMG cultures. Is this correct, or are they just SMG cultures?
We corrected the contents as suggested.

The line plots in figure 4H are helpful for understanding the data, but it would be beneficial, if possible, to quantify these data over multiple replicates.
We corrected the graphs with multiple replicates (n=9 line scans per each group).

In figure 5h, please label the grayscale panels with the stain they represent to avoid confusion.
We labeled the grayscale panels of ZO-1 signals.

The description of figure 5 in the text states that “actomyosin contractility acts as a major upstream mediator of KIT expression through transcriptional regulation” seems slightly overstated. The data support a slightly weaker statement more like “actomyosin contractility is required for normal kit expression”
We corrected the contents as suggested.

The authors have replaced most references to matrigel stiffnesses with concentrations because stiffness was not directly measured in this work. However, figure 6d still contains Young’s modulus values. Please remove these values.
We corrected the contents as suggested.

In the discussion, remove the citation to Daley 2017 as this paper was recently retracted.
We removed the reference.
Thank you for great suggestions and comments that clearly improve our manuscript.

Third decision letter

MS ID#: DEVELOP/2020/190785

MS TITLE: Mechanogenetic role of actomyosin complex in branching morphogenesis of epithelial organs

AUTHORS: Jin Man Kim, YoungJu Jo, Ju Won Jung, and Kyungpyo Park

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is very positive and we would like to publish your manuscript in Development. Before we can proceed with this please attend to thhe few remaining reviewers' comments which require some text changes.

Reviewer 1

Advance summary and potential significance to field

I'm happy with the responses and changes of the author, which have made t hepapers clearer. One exception is the sentence in the answer latter:
"Unfortunately, in our experimental conditions, we could not observe and demonstrate clear apico-basal difference in actin and p-myosin signals in peripheral epithelial cells (Fig. S1G and S2B)"

Comments for the author

I think this is a result by itself (and albeit negative, actually quite important as this justifies the assumption of buckling vs some other sources of tissue bending).

So i would encourage the author to quantify this (i.e. measure apical vs basal levels of actomyosin in peripheral cells, to actually show that the impression they mention in their answer is correct), mention it quickly in main text and have it in supplement figures - next to S1G for instance.

Reviewer 2

Advance summary and potential significance to field

The authors show a role for actomyosin contractility in buckling of salivary epithelium.

Comments for the author

Our comments have been satisfactorily addressed with the exception of the comment that resulted in the new Fig. S9. Firstly, either the labels or the legend for Fig. S9 is incorrect (is it 10% and 50%, or 50% and 100%). On that note, it doesn't seem like the authors indicate anywhere what % Matrigel was used for Fig. 1E-F. Is it 50%?

Secondly, the way Fig. S9 is discussed in the text is quite vague (lines 310-312) and currently disrupts the flow of the results section discussing Fig. 6. The result of Fig. S9 seems to be that, although blebbistatin changes the amount of buckling in rudiments cultured in 50% and 100% Matrigel, there is no difference in the amount of buckling between 50% and 100% Matrigel, and that perhaps this stiffness range (unlike 10% to 50%, Fig. 4C) is irrelevant to the branching rudiments (which agrees with the very slight difference in Yap intensity depicted in Fig. 6D). If this is true, then perhaps another helpful experiment would have been to compare Blebbistatin treatment at 10% and 50%, although this is probably unnecessary at this stage of the review process. As is, Fig. S9 seems to support the authors' claims in Fig 1E and 4C that cleft depth and bud number are primarily determined by a buckling mechanism largely controlled by cell contractility rather than directly by the stiffness of the substratum. Fig. S9 therefore could either be omitted or moved to the section of the results discussing Fig. 1E or 4C.

Overall, the authors' improvements, in particular the changes to the descriptions and labels of Figure 3 and the inclusion of Figure 6F-G in the main text, have greatly improved the clarity of the manuscript. Addressing the minor comment above would make this article suitable for publication.

Third revision

Author response to reviewers' comments

Reviewer 1 Advance summary and potential significance to field
I'm happy with the responses and changes of the author, which have made the papers clearer. One exception is the sentence in the answer latter:
"Unfortunately, in our experimental conditions, we could not observe and demonstrate clear apico-basal difference in actin and p-myosin signals in peripheral epithelial cells (Fig. S1G and S2B)"

Reviewer 1 Comments for the author

I think this is a result by itself (and albeit negative, actually quite important as this justifies the assumption of buckling vs some other sources of tissue bending).

So i would encourage the author to quantify this (i.e. measure apical vs basal levels of actomyosin in peripheral cells, to actually show that the impression they mention in their answer is correct), mention it quickly in main text and have it in supplement figures - next to S1G for instance.

Thank you for the suggestion. We added the quantification data in Fig. S1H

Reviewer 2 Advance summary and potential significance to field
The authors show a role for actomyosin contractility in buckling of salivary epithelium.

Reviewer 2 Comments for the author
Our comments have been satisfactorily addressed with the exception of the comment that resulted in the new Fig. S9. Firstly, either the labels or the legend for Fig. S9 is incorrect (is it 10% and 50%, or 50% and 100%). On that note, it doesn't seem like the authors indicate anywhere what % Matrigel was used for Fig. 1E-F. Is it 50%?

Yes, the concentration of Matrigel used in this study is 50%. We used growth factor-reduced Matrigel diluted with DMEM/F12 with a 1:1 ratio (line 442-443).
Secondly, the way Fig. S9 is discussed in the text is quite vague (lines 310-312) and currently disrupts the flow of the results section discussing Fig. 6. The result of Fig. S9 seems to be that, although blebbistatin changes the amount of buckling in rudiments cultured in 50% and 100% Matrigel, there is no difference in the amount of buckling between 50% and 100% Matrigel, and that perhaps this stiffness range (unlike 10% to 50%, Fig. 4C) is irrelevant to the branching rudiments (which agrees with the very slight difference in Yap intensity depicted in Fig. 6D). If this is true, then perhaps another helpful experiment would have been to compare Blebbistatin treatment at 10% and 50%, although this is probably unnecessary at this stage of the review process. As is, Fig. S9 seems to support the authors' claims in Fig 1E and 4C that cleft depth and bud number are primarily determined by a buckling mechanism largely controlled by cell contractility rather than directly by the stiffness of the substratum. Fig. S9 therefore could either be omitted or moved to the section of the results discussing Fig. 1E or 4C.

Overall, the authors' improvements, in particular the changes to the descriptions and labels of Figure 3 and the inclusion of Figure 6F-G in the main text, have greatly improved the clarity of the manuscript. Addressing the minor comment above would make this article suitable for publication.

Thank you for the great comments. As the reviewer suggested, we decided to omit Fig. S9.

Fourth decision letter

MS ID#: DEVELOP/2020/190785

MS TITLE: Mechanogenetic role of actomyosin complex in branching morphogenesis of epithelial organs

AUTHORS: Jin Man Kim, YoungJu Jo, Ju Won Jung, and Kyungpyo Park
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.