FGF signaling regulates salivary gland branching morphogenesis by modulating cell adhesion

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Original submission

First decision letter

MS ID#: DEVELOP/2022/201293

MS TITLE: FGF signaling regulates salivary gland branching morphogenesis by modulating cell adhesion

AUTHORS: Ayan T Ray and Philippe Soriano

I sincerely apologize for the long delay before being able to come back to you with a decision, due to difficulties finding available reviewers. 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 considerable interest in your work, but have some significant criticisms and recommend a 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. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

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 well-written follow-up to the previous Ray et al. paper, the authors provide interesting additional evidence for a role in development of FGF receptors that is independent of canonical downstream signaling in branching morphogenesis.
As indicated in their manuscript title, they claim that FGF signaling “regulates” salivary gland morphogenesis “by modulating cell adhesion.” They provide various lines of evidence similar to those in the Ray et al. paper that focused instead on neural crest cells, plus other data, that support the importance of non-canonical FGF receptor signaling with interesting intersections with cadherin-based cell- reported cell adhesion and integrin-based cell-matrix adhesion. Since the details in the literature of why FGF receptors are so important in salivary gland branching morphogenesis appears incomplete, this linkage between FGF signaling and cell adhesion provides a useful new dimension to understanding the mechanisms of FGF signaling.
The high-quality genetic analyses with the integrin-activation and antibody-inhibition approaches applied to analyze the role of FGF receptors in salivary gland development are interesting and valuable for elucidating the frequently reported central roles of FGF receptor function in salivary gland development in mice and humans. The conclusion about non-canonical signaling independent of ERK is novel for this particular field of development (organogenesis), even though the broad concept was suggested in the previous Ray et al. paper from this laboratory.
If all the data had been convincing, this paper would have been a very good candidate for publication in Development.

Comments for the author

This manuscript is generally well-written and carefully documented including the use of super-plots, curvature quantification, and generally convincing confocal microscopy. Nevertheless, there are several major specific concerns involving an apparent internal contradiction and the absence of one set of seemingly obvious important results, plus other concerns make this otherwise solid manuscript currently regrettably but clearly not appropriate for publication without major revisions.

1. The authors emphasize the importance of FGF non-canonical signaling in regulating cell adhesion in developing salivary glands. They clearly distinguish between cell-cell and cell-matrix adhesion in the text. They show convincingly that there are likely disruptions in cell-cell interactions, most convincingly documented by the major loss of the key cell adhesion protein E-cadherin according to immunofluorescence analysis. Very puzzlingly, they fail to apply the same type of analysis to integrins. They show immunostaining for total and activated beta1 integrins in Supplementary Figure 6, yet they do not show these crucial types of analyses for the double conditional knockout, nor for the FCPG mutant that provides a partial rescue of phenotype. This omission does not make sense. The integrin activation experiment that they show to be negative in terms of a rescue of the double knockout could have been merely due to reduced levels of one or more critical integrins, for which further activation would be ineffective. Even if the levels of integrins turn out not to change, that is critical information concerning cell-matrix adhesion parallel to the type of analysis used for cell-cell adhesion. Otherwise, this paper is clearly incomplete.

2. There is an apparent major contradiction in the text when comparing basement membrane (laminin) staining in the double knockout. Near the bottom of page 9, the text states: “…in Fgfr1cKO/cKO; Fgfr2cKO/cKO mutants, the BM appeared disintegrated in certain domain, with large gaps, variable thickness, and minimal interaction with GFP+ cells” and yet at the bottom of page 10, the text states: “Fgfr1cKO/cKO; Fgfr2cKO/cKO compound mutants did not show overt defects in the BM in the untreated condition” and experimental integrin inhibition was required to show defects. It will be important to have more definitive, consistent, and convincing analyses of the claimed role of non-canonical FGF receptor signaling in cell-matrix adhesion. Although it is not essential, it is puzzling that the authors did not perform a similar type of analysis to that reported in their original Ray et al. manuscript, which showed effects defects in adhesion by another cell type concerning the formation of focal adhesions. Although salivary epithelial cells might not show that same type of defect, especially if confronted with a more appropriate basement membrane or basement membrane extract-coated substrate, such evidence would be much more direct concerning claims about adhesion than the current dependence on morphological changes. However, if the authors cannot perform such experimentation, they should at a minimum characterize in detail the nature of the interactions of cells with the basement membrane e.g., comparing close-up views of cells interacting with the basement membrane in the various

conceptually important mutant mice with the essential integrin immunofluorescence analyses. Otherwise, the evidence for the claim in Figure 7 panel D seems to be based only on inference.

3. The use of the CA Braf mutant to activate the ERK1/2 pathway appears to be useful conceptually, but have the authors shown that this mutant can induce the same levels and pattern of ERK1/2 signaling in salivary glands cells as the FGF receptor itself?
Conversely, what is the actual level of EKR1/2 signaling with the FCPG mutant that partially rescues the phenotype?

4. The statistical analyses for virtually all graphs appear to be incorrect. The authors state that they used unpaired two-tailed Students t-tests to obtain P values. Unfortunately, as indicated in statistics textbooks and current Internet information comparing the use of t-tests to ANOVA make the point strongly that the t-test is meant to be used only for a comparison of a pair of conditions. To be able to use a t-test for more than two conditions, the Bonferroni correction is essential to avoid over-estimating statistical significances. However, most labs use ANOVA with an appropriate post--hoc statistical test. Without such corrections for the number of conditions being compared, the current reported levels of statistical significance are too optimistic.

Minor points:

5. Anatomical texts and medical dictionaries use “submandibular” and “sublingual” rather than “Sub-mandibular” and “Sub-lingual” on page 3.
6. At the end of the Introduction, the authors refer to receptor functions “impinging on cell matrix interactions” even though the evidence for this claim is weak, and the evidence for effects on cell-cell interactions is strong.

7. Statistical analysis is missing for the graphs on the bottom of Supplementary Figure 7.

Reviewer 2

Advance summary and potential significance to field

This is an interesting and important study that explores the role of FGF signaling through two of its receptors, Fgfr1 and Fgfr2, in the branching/budding morphogenesis of the mouse salivary gland (submucosal gland, SMG). The authors make creative use of their suite of conditional mutant genes that allow them to compare the role in the epithelial cells of straightforward gene deletions versus expression of mutant Fgf receptors. Significantly, the mutant alleles encode receptors that have been disabled for canonical downstream signaling through ERK1/2, PI3K/Akt, PLCγ and STATs but still retain intact extracellular domains that the authors surmise can interact with cell surface proteins involved in cell matrix interactions.
This study builds strongly on recent elegant work from the Yamada lab on the dynamic behaviors of surface epithelial cells in the SMG during terminal bud growth, expansion and clefting, which is essential for “branching morphogenesis” in this organ. Importantly, the Yamada study revealed very dynamic behaviors of the surface epithelial cells. These express high levels of cell-matrix adhesion but low levels of cell-cell adhesion. Importantly for the Yamada model, surface cells which move internally to divide retain low cell-cell adhesion as measured by cadherin expression. This low level is critical for the subsequent forced extrusion of cells back into the surface layer, the expansion of which drives clefting. The internal cells are therefore heterogeneous, with some having low cell-cell adhesion and some having high. The current work, elegantly uses mutant alleles to test the role of FGF in SMG development. The results are important but there are some limitations which need to be addressed to increase confidence in their interpretation.

Comments for the author

Specific comments
1. An important component of the authors’ model is that Fgfr2 protein is expressed at higher levels on the surface layer than internally. This conclusion is reached by quantifying the level of fluorescence from H2B-GFP and H2B-mCherry alleles which are translated from gene transcripts. However, they do not discuss for the reader whether there are differences between the stability/turnover of reporter proteins in the cell nucleus and wild type proteins at the cell surface.

According to their model, it would seem that surface cells have to downregulate the level of Fgf receptor quickly to be able to move inside. Indeed, Figure 1D appears to show considerable heterogeneity in H2B GFP and H2B mCherry levels in the surface epithelium. Ideally, the authors need to do live imaging of surface cells and show that changes in mCherry precede or accompany cells moving into the interior of the bud. If this is not possible in a reasonable timeframe then adequate discussion of the issues is essential.
2. A key feature of the Yamada model is that surface cells that move into the interior retain low cell-cell adhesion compared with the bulk of interior cells. These interior cells then drive the surface cells back out again by competitive adhesion. It is hard to see how Fgf receptor extracellular domains can be modulating both the function of integrins for matrix adhesion and cadherins for cell-cell adhesion. Again, it would seem that there has to be dynamic heterogeneity in Fgf protein expression in a minority of interior cells, versus the bulk, but that the bulk interior cells still have lower levels than bulk surface cells. But protein levels are not measured directly vis-a-vis reporter expression

Minor points
1. Relevant to point (1) above, the arrows in Figure IC do not always point to cells. There is an E14.5 lost in the schematic of D. We are not told whether differences in intensity levels in D are statistically significant.
2. In the discussion the authors state " and inward folding of surface cells with weaker cell adhesion promotes budding and accompanying clefting". This is a bit ambiguous since all surface cells have lower adhesion not just the ones which are folding in.

Reviewer 3

Advance summary and potential significance to field

FGF signaling is critical for the development of the salivary glands and other organs; however, the exact mechanisms through which FGF signaling functions are not known. In the current study, the authors use a knockout/knockin strategy with specific FGFR1 and FGFR2 point mutations to examine the contribution of canonical and non-canonoical FGFR-driving signaling pathways to salivary gland braching morphogenesis. First, they use FGFR1 and FGFR2 reporter strains to confirm previous reports that FGFR1 is widely expressed in the murine embryonic salivary gland mesenchyme and is also expressed in the epithelium, whereas FGFR2 is very restricted to the embryonic salivary gland epithelium. Using transgenic reporters for FGFR1 and FGFR2, they further show that in the epithelium both receptors are enriched in the outer bud cells contacting the basement membrane.
Using epithelial conditional mutants, they confirm that FGFR1 and FGFR2 contribute to branching morphogenesis, with FGFR2 having a greater role relative to FGFR1 in the epithelium. The primary new and significant data involve the use of their multiple knock-in point mutation alleles that prevent binding of signaling effectors to the receptors and prevent canonical downstream signaling cascades. Previous data from this group using these mutants demonstrated that the kinase active, signaling cascade defective mutants exhibit cell matrix and cell adhesion regulation in cells in culture. In developing salivary glands of mice, both FGFR1-FCPG and FGFR2-FCPG knock in mutations, which restore canonical FGFR signaling, can partially rescue the defects in branching morphogenesis observed in the knockout mutations, with FGFR2-FCGP having greater activity, as predicted based on the more significant function of FGFR2 in branching morphogenesis. They further demonstrate that the FGFR knockout mutants exhibit defects in basement membrane integrity and preferential localization of knockout-reporter cells to the outer bud periphery adjacent to the basement membrane, which was exacerbated by blocking integrin function. Further, they demonstrate that non-canonical FGFR signaling interacts with integrin activation during branching morphogenesis. The data presented support the authors' conclusions that non-canonical FGFR functions drive salivary gland branching morphogenesis through regulation of previously unknown regulation of cell-cell adhesion and basement membrane interactions. The study makes a significant contribution to our understanding of the contributions of FGF signaling to salivary gland branching morphogenesis.

Comments for the author

The authors have thoroughly described the study. Only minor revisions are needed to clarify minor points.

Minor points:
1. It's not clear if any of the mice are commercially available. If so, that should be noted in the methods.
2. What was the duration of the sucrose incubations during tissue fixation?
It should be noted in the methods.
3. The duration of the secondary antibody incubation should be noted.
4. It is indicated that unpaired t-tests were used for statistical comparisons. This is acceptable for paired comparisons but not for comparisons of multiple samples. ANOVA with post-hoc tests should be used instead.
5. The n for each strain should be indicated for each experiment in the methods or figure legend. Likewise, the number of tissue sections used for quantification should also be indicated.

First revision

Author response to reviewers' comments

Response to reviewer comments (changes in the manuscript are in red):

Reviewer 1
This manuscript is generally well-written and carefully documented including the use of super-plots, curvature quantification, and generally convincing confocal microscopy. Nevertheless, there are several major specific concerns involving an apparent internal contradiction and the absence of one set of seemingly obvious important results, plus other concerns make this otherwise solid manuscript currently regrettably but clearly not appropriate for publication without major revisions.

1. The authors emphasize the importance of FGF non-canonical signaling in regulating cell adhesion in developing salivary glands. They clearly distinguish between cell-cell and cell-matrix adhesion in the text. They show convincingly that there are likely disruptions in cell-cell interactions, most convincingly documented by the major loss of the key cell adhesion protein E-cadherin according to immunofluorescence analysis. Very puzzlingly, they fail to apply the same type of analysis to integrins. They show immunostaining for total and activated beta1 integrins in Supplementary Figure 6, yet they do not show these crucial types of analyses for the double conditional knockout, nor for the FCPG mutant that provides a partial rescue of phenotype. This omission does not make sense. The integrin activation experiment that they show to be negative in terms of a rescue of the double knockout could have been merely due to reduced levels of one or more critical integrins, for which further activation would be ineffective. Even if the levels of integrins turn out not to change, that is critical information concerning cell-matrix adhesion parallel to the type of analysis used for cell-cell adhesion. Otherwise, this paper is clearly incomplete.

We thank the reviewer for having brought up this very important issue, and agree that this analysis was lacking in our manuscript! We decided to analyze integrin expression by immunohistochemistry as this would allow us to identify the integrins in the mutant cells of conditional knock-outs. We analyzed the expression of Integrins $\alpha 1$, $\alpha 5$, and $\beta 1$, and found no difference in null mutants compared to controls or Fgfr1$^{FCPG/-}$ or Fgfr2$^{FCPG/FCPG}$ mutants. We have now included these results in a new Supplementary Figure 6B and discuss these findings in the results section on page 10, lines 262-265.

2. There is an apparent major contradiction in the text when comparing basement membrane (laminin) staining in the double knockout. Near the bottom of page 9, the text states: "…in Fgfr1cKO/cKO; Fgfr2cKO/cKO mutants, the BM appeared disintegrated in certain domain, with large gaps, variable thickness, and minimal interaction with GFP+ cells" and yet at the bottom of

page 10, the text states: “Fgfr1cKO/cKO; Fgfr2cKO/cKO compound mutants did not show overt defects in the BM in the untreated condition” and experimental integrin inhibition was required to show defects. It will be important to have more definitive, consistent, and convincing analyses of the claimed role of non-canonical FGF receptor signaling in cell-matrix adhesion. Although it is not essential, it is puzzling that the authors did not perform a similar type of analysis to that reported in their original Ray et al. manuscript, which showed effects defects in adhesion by another cell type concerning the formation of focal adhesions. Although salivary epithelial cells might not show that same type of defect, especially if confronted with a more appropriate basement membrane or basement membrane extract-coated substrate, such evidence would be much more direct concerning claims about adhesion than the current dependence on morphological changes. However, if the authors cannot perform such experimentation, they should at a minimum characterize in detail the nature of the interactions of cells with the basement membrane, e.g., comparing close-up views of cells interacting with the basement membrane in the various conceptually important mutant mice with the essential integrin immunofluorescence analyses. Otherwise, the evidence for the claim in Figure 7 panel D seems to be based only on inference.

We thank the reviewer for bringing up these issues. There is a major difference in the experimental conditions used on page 9 and page 10. The experiments discussed on page 9 refer to the analysis of salivary glands in vivo, in the mouse embryo. Those on page 10 refer to experiments where the salivary glands are put in culture. These conditions fail to recapitulate all aspects of normal development, including the rupture of the BM, and we have now highlighted this limitation in the text on page 11, lines 288-289. Regarding the reviewer’s second comment, there is a long history of studying focal adhesions, but almost all studies are done in cells in culture. Analyzing focal adhesions directly in the embryo has been shown to be more complex. In our previous study (Ray et al., 2020), we were able to analyze cells of neural crest origin in culture. However, despite numerous attempts, we failed to find conditions allowing salivary gland epithelial cells to grow in culture. For this revision, we have attempted again to characterize focal adhesions in vivo by vinculin or paxillin staining but have failed to obtain meaningful results. An extensive study of focal adhesions of salivary gland epithelial cells seems to be beyond the scope of the present paper. Nonetheless the data that we show on cell-cell adhesion and on modulating integrin activity support the model set forth in Figure 7D.

3. The use of the CA Braf mutant to activate the ERK1/2 pathway appears to be useful conceptually, but have the authors shown that this mutant can induce the same levels and pattern of ERK1/2 signaling in salivary glands cells as the FGF receptor itself? Conversely, what is the actual level of EKR1/2 signaling with the FCPG mutant that partially rescues the phenotype?

Braf works in a linear cascade that regulates MEK and thus ERK. This necessarily works outside of the FGF pathway and is not expected to induce the same level of ERK. BRAF, MEK, and ERK are detected broadly and should encompass the FGF signaling expression domain. The FCPG mutants abrogate all ERK1/2 activation, as shown in our previous study (Ray et al., 2020).

4. The statistical analyses for virtually all graphs appear to be incorrect. The authors state that they used unpaired two-tailed Students t-tests to obtain P values. Unfortunately, as indicated in statistics textbooks and current Internet information comparing the use of t-tests to ANOVA make the point strongly that the t-test is meant to be used only for a comparison of a pair of conditions. To be able to use a t-test for more than two conditions, the Bonferroni correction is essential to avoid over-estimating statistical significances. However, most labs use ANOVA with an appropriate post--hoc statistical test. Without such corrections for the number of conditions being compared, the current reported levels of statistical significance are too optimistic.

We thank the reviewer for having brought up this really important issue! We have now reanalyzed all of the results that were not pairwise comparison using one way ANOVA with a Bonferroni multiple comparisons test, and corrected all of the diagrams. We have modified our description in the methods section accordingly. Our general conclusions have not changed, but some values did: Fig. 2D lower; Fig. 6A upper (controls only); Fig. S6D; Fig. S7A.

Minor points:

5. Anatomical texts and medical dictionaries use “submandibular” and “sublingual” rather than “Sub-mandibular” and “Sub-lingual” on page 3.

Thank you! We have made changes throughout the text accordingly.

6. At the end of the Introduction, the authors refer to receptor functions “impinging on cell matrix interactions” even though the evidence for this claim is weak, and the evidence for effects on cell-cell interactions is strong.

We thank the reviewer for bringing up several issues relating to the integrin expression that have strengthened the manuscript.

7. Statistical analysis is missing for the graphs on the bottom of Supplementary Figure 7.

Thank you! We have now made this correction.

Reviewer 2

Specific comments

1. An important component of the authors’ model is that Fgfr2 protein is expressed at higher levels on the surface layer than internally. This conclusion is reached by quantifying the level of fluorescence from H2B-GFP and H2B-mCherry alleles which are translated from gene transcripts. However, they do not discuss for the reader whether there are differences between the stability/turnover of reporter proteins in the cell nucleus and wild type proteins at the cell surface. According to their model, it would seem that surface cells have to downregulate the level of Fgf receptor quickly to be able to move inside. Indeed, Figure 1D appears to show considerable heterogeneity in H2B GFP and H2B mCherry levels in the surface epithelium. Ideally, the authors need to do live imaging of surface cells and show that changes in mCherry precede or accompany cells moving into the interior of the bud. If this is not possible in a reasonable timeframe then adequate discussion of the issues is essential.

We thank the reviewer for bringing up this interesting issue. We failed to clarify in the text that the GFP and mCherry are expressed off the endogenous Fgfr1 and Fgfr2 promoters through a T2A self-cleaving peptide and now clarify this on page 5, lines 103-106. We have found the existing FGFR1 and FGFR2 antibodies unsuitable for subcellular localization. We never generated alleles that might express a protein (including a Myc or Flag tag for instance) from the null and that would have been ideal to examine the questions the reviewer brings up. We are therefore unable to perform the experiment that the reviewer suggests. However, we now discuss the possibilities brought up by the reviewer on page 13, lines 350-357.

2. A key feature of the Yamada model is that surface cells that move into the interior retain low cell-cell adhesion compared with the bulk of interior cells. These interior cells then drive the surface cells back out again by competitive adhesion. It is hard to see how Fgf receptor extracellular domains can be modulating both the function of integrins for matrix adhesion and cadherins for cell-cell adhesion. Again, it would seem that there has to be dynamic heterogeneity in Fgf protein expression in a minority of interior cells, versus the bulk, but that the bulk interior cells still have lower levels than bulk surface cells. But protein levels are not measured directly vis-a-vis reporter expression.

The FGFR extracellular domain can interact with both integrins and cadherins. We have never made mutations in the extracellular domain interaction sites in combination with the intracellular domain mutations as we had indicated on page 12, lines 349-351. As explained in the answer to point 1, we are unable to analyze FGFR expression levels in interior cells vs. the bulk, but now discuss the possibilities brought up by the reviewer on page 13, lines 351-358.

Minor points

1. Relevant to point (1) above, the arrows in Figure IC do not always point to cells. There is an E14.5 lost in the schematic of D. We are not told whether differences in intensity levels in D are statistically significant.

Thank you for catching these errors, they have now been rectified in the revised manuscript.

2. In the discussion the authors state “and inward folding of surface cells with weaker cell adhesion promotes budding and accompanying clefting”. This is a bit ambiguous since all surface cells have lower adhesion not just the ones which are folding in.

We have modified the sentence and removed “with weaker cell adhesion”.

Reviewer 3
The authors have thoroughly described the study. Only minor revisions are needed to clarify minor points.

Minor points:
1. It’s not clear if any of the mice are commercially available. If so, that should be noted in the methods.

Thank you very much for catching this omission! All of the mice are available from mutant repositories and we have indicated sources in the methods sections.

2. What was the duration of the sucrose incubations during tissue fixation? It should be noted in the methods.

This has been corrected, thank you.

3. The duration of the secondary antibody incubation should be noted.

This has been corrected, thank you.

4. It is indicated that unpaired t-tests were used for statistical comparisons. This is acceptable for paired comparisons but not for comparisons of multiple samples. ANOVA with post-hoc tests should be used instead.

Thank you for catching this error. We have now reanalyzed all of the results that were not pairwise comparison using one way ANOVA with a Bonferroni multiple comparisons test, and corrected all of the diagrams. We have modified our description in the methods section accordingly. Our general conclusions have not changed, but some values did: Fig. 2D lower; Fig. 6A upper (controls only); Fig. S6D; Fig. S7A.
5. The n for each strain should be indicated for each experiment in the methods or figure legend. Likewise, the number of tissue sections used for quantification should also be indicated.

The number of mice or slides used has been updated.

Second decision letter

MS ID#: DEVELOP/2022/201293

MS TITLE: FGF signaling regulates salivary gland branching morphogenesis by modulating cell adhesion

AUTHORS: Ayan T Ray and Philippe Soriano
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.

Reviewer 1

Advance summary and potential significance to field

See previous review

Comments for the author

This resubmitted manuscript has resolved and clarified all issues raised in the original reviewing process. The authors have been conscientious in their revisions, and acceptance of the manuscript for publication can now be recommended with enthusiasm.

Reviewer 2

Advance summary and potential significance to field

See comments for the original submission

Comments for the author

The authors have addressed my concerns in the discussion.