Diverse epithelial cell populations contribute to the regeneration of secretory units in injured salivary glands

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DOI: 10.1242/dev.192807

Editor: Thomas Lecuit

Review timeline
Original submission: 11 May 2020
Editorial decision: 2 July 2020
First revision received: 17 July 2020
Accepted: 11 September 2020

Original submission

First decision letter

MS ID#: DEVELOP/2020/192807

MS TITLE: Diverse Epithelial Cell Populations Contribute to Regeneration of Secretory Units in Injured Salivary Glands

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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 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.

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 a combined use of lineage tracing and an induced injury model, the authors have provided evidence for a high degree of cellular plasticity within salivary gland cells. The results show that cells of several different terminally differentiated types can contribute to the rapid regeneration of secretory acinar cells following an injury of short duration. Plasticity has only recently been recognized in the salivary glands, and the implications for this finding are significant. The ability to adopt an alternate fate could be harnessed therapeutically to generate damaged or lost cells for regenerative medicine.
However, whether the conclusions provide a significant and novel contribution to the understanding of developmental mechanisms is open to question. The cellular responses to acute injury are novel and informative, but because the injury model involves damage to the nerve, presumably resulting in the absence of important trophic signals, they may not be physiologically relevant to developmental mechanisms. A comment included in the discussion speaks to this issue. It was previously reported that, using a less disruptive ligation procedure, non-acinar cells do not generate acini, and the authors mention that they confirmed those results. It is only with the more disruptive ligation procedure that the extensive cellular plasticity is observed. In other words, the cellular response has been changed by the degree of perturbation.

Comments for the author

1. The results support the conclusions, but there are technical issues that should be considered in the interpretation of the results. The first is the validation of the model used to examine regeneration. The use of a more disruptive ligation, including periductal tissue with the excretory duct, should insure that extensive acinar cell death occurs. However, controls should include proof of extensive apoptosis, as well as proof of acinar cell loss. It has been shown by others that AQP5 is rapidly down-regulated after injury, but the loss of AQP5 does not necessarily indicate that acinar cells are completely gone. If there are acinar cells remaining one could interpret Fig.1e as showing ectopic activation of K14 in acinar cells that have down-regulated Aqp5.
In fact, in an early publication, using the same K14Tre:tetO-H2BGFP system, H2BGFP was activated in Aqp5+ acinar cells (Kwak et al. 2016; Fig. 2B).
2. To confirm the interpretation that it is proliferation of K14 duct cells that produce regenerated acini, it is also important to show co-labeling of K14+ cells with Ki67 proliferation marker. It would also be informative to compare the K14-YFP staining at L3dL10 with K14 antibody staining (Fig. 1e). It is hard to draw conclusions about the cellular contributions after the 5-week recovery period, when acinar cells have significantly expanded.
3. Finally, additional characterization of the regeneration model could include staining of the tubular structures with Sma and cKit at L3dL10, to determine whether the Cre recombinases driven by these promoters are activated in the presumptive cells driving regeneration.
4. The second technical concern is the potential for leakiness in the genetic systems used. The Dox -induced Cre system may require more than 4 days prior to injury to be effectively silenced. The inclusion of controls showing injury in the absence of Dox would partially address this, although it would be interesting to compare the observed labeling pattern with that obtained if Dox is added at the time of ligation. Similarly, tamoxifen administration via chow is dependent on the eating behavior of the animals, and the speed of Cre induction can therefore vary significantly. The length of time necessary for Cre activation, as well as for tamoxifen clearance should be addressed in the controls.
5. The text states that 24.4% of regenerated acini were derived from MECs, whereas 32.5% or (if labeling efficiency is considered) 65% of regenerated acini were derived from K14-expressing cells. It is not clear why the authors maintain that MECs make the dominant contribution to acini.
6. The suggestion that MECs and cKit cells dedifferentiate to a common bipotent progenitor is speculative and has not been demonstrated. Perhaps co-staining with Sox10 could address this and provide evidence for de-differentiation?
7. It may be that the response of salivary glands to each type of injury is different, so the assertion that “plasticity of committed parenchymal cells is the predominant mechanism of acinar regeneration in response to a severe injury” should be limited to this particular injury model.

Reviewer 2

Advance summary and potential significance to field

The manuscript describes lineage contribution to regeneration of acinar cells following severe injury in salivary glands. The study combines gland injury by ductal, blood vessels and nerve ligation with several genetic models to ask which cell-types regenerate acinar cells after severe damage. This is an important unanswered question as lineage relationships during acinar regeneration are not well understood. The results clearly show that several cell populations are able to regenerate the secretory cells in a surprising way. The study is novel and an important advance for the field to understand the regenerative potential in salivary glands. The study has broad implications for many other organ systems, in terms of understanding how in vivo models and in vitro models compare, and how the extent/severity of damage influences the type of cells that respond and regenerate.

Comments for the author

The study is novel and an important advance for the field to understand the regenerative potential in salivary glands. Some aspects of the study need to be clarified.

Major comments:
1) The authors use a severe damage model of reversible ductal ligation, where the ligature also surrounds the nerve and blood vessel. Previous literature shows that after duct ligation if the nerve is permanently damaged the gland will not regenerate. This point needs to be explicitly stated in the introduction to avoid reader confusion about the model. In this model when nerve and blood vessels are constricted, but not permanently damaged, it is not shown which cell types survive the damage. This is important, because the proportion of cells that survive in this severe damage model, may influence their ability to regenerate. For example, if MECs have a higher survival rate compared to ductal K14 cells, that could result in more lineage tracing from one population over the other. This important point could be addressed by showing staining patterns of SMA, Axin2 and cKit in damaged glands at an early time-point (L3dL0) or very shortly after the de-ligation, since all lineages studied show the potential to regenerate.

2) The authors conclude that both MECs and cKit cells go through a “bi-potent progenitor” as an intermediate population to regenerate acinar cells. This is based on analysis of acini/ID junctions labeling patterns. Although they report that most of the acini are continuously labeled with ID markers, they also find acini that are lineage traced without labeled IDs. They also report that cKit cells are lineage traced heterogeneously. Together, these findings do not exclude direct trans-differentiation without a “bi-potent progenitor cell”. In addition, using the SMA-cre, the authors do not report lineage traced cKit ID cells without an acinar label. Therefore, the possibility that cKit cells only lineage trace from MECs through proacinar/acinar cells cannot be excluded. Please revise the model or discus these possibilities. If there is a “bi-potent progenitor” what are its defining markers, or is it simply a transitional state depending on what cell is being used as a progenitor?

3) The authors use two well-established methods to compare in vitro growth from K14+ cells and MECs. Their experiments indicate that in vitro results do not directly correlate to in vivo predictions of potential. The authors conclude that the cells are therefore not stem cells. This is at odds with their manuscript given the extensive in vivo evidence for conditional proliferative potential. Their findings suggest that there is not a “one size fits all” in vitro stem cell assay and highlights the limitations of in vitro assays. The in vitro models do not recreate the complex multicellular and matrix-rich stem cell niche that occurs in vivo. This should be discussed.

4) In the discussion, the authors refer to parallel studies obstructing salivary duct with and without periductal tissue with very different outcomes, but do not include discussion of these other results. Discussion of these results would help explain to readers their key finding, about salivary gland cell plasticity and how different damage models result in different regenerative scenarios.

Minor comments:
1) The authors pose two scenarios “The broad expression of K14 in regenerative glands could be either due to expansion of the surviving K14+ cells or upregulation of K14 in other cell types”. But do not include the third option that the increase is relative to loss of other cell types (point 1 above). The results show the lineage contribution from pre-existing K14 cells, but they do not directly address/discuss the other scenarios in light of their results
2) They state that traced MECs formed very few organoids despite comparable TdT-labeling efficiency in vivo in the two transgenic lines. However, the ~50% labeling efficiency of K14 cells includes both duct and MECs, while ~50% labeling efficiency of SMA+ cells is only MECs. Thus, this is not an accurate comparison as the actual number of labeled cells in the SMA-Cre could be much lower compared to the K14-Cre. This can in turn lead to a lower number of labeled cells in the spheres after 7 days. If the goal is to compare proliferative potential, it is more accurate to look for the percentage increase from day 0.
3) The authors state on page 9, line14: “…suggesting a different mechanism for regeneration of MECs than that used for acinar and ductal cells.” This is confusing as the model is showing that MECs are surviving and some regenerating other cell types. What is meant by MEC regeneration? Please clarify.
4) The authors switch between using the terms differentiated, committed and more committed cells when referring to MECs and duct cells, e.g, in the abstract MECs and duct cells are differentiated while in the discussion they are committed and fully differentiated. They also refer to K14 duct cells as either stem/progenitor cells, or ductal stem cells, or K14 stem cells. This is confusing, please use terms consistently.
5) In Figure 1: If “non-injured” is the same as “control” please revise figure for consistency. If it is not the same, please specify why there was an additional control.
6) Page 4, line 22: The gland weight loss results are not referred to in any figure. Please include them.
7) Page 8, line 4: “…which is likely due to migratory nature of MECs..” please include a reference to this migratory phenomenon.
8) Page 13, line 11: In reference to ductal stem cells, “…however, their contribution to acinar regeneration is not significant as MECs, possibly due to their scarcity and physical distance from acini.” This is confusing as the authors show that K14 increases after ligation while acini are lost. How scarce are ductal stem cells after damage and how would their distance from acini matter once the acinar cells are lost? Or do they regenerate in a different location?
9) Page 11, line2: “However, whether MECs display similar characteristics have not been determined.”
Sentence should include the word “salivary MECs”. If not, this is confusing as the previous sentence refers to mammary MECs.
10) The authors should refer to this damage model as severe duct ligation as they show that ligating with or without the periductal vascular and nerve tissue are basically two different damage models. Also, page 15 line 22: Please clarify that this is severe ligation injury since severe injury is often used to describe irradiation damage.

Reviewer 3

Advance summary and potential significance to field

This study seeks to investigate the plasticity of non-acinar cells in the salivary gland by examining their contribution to the formation of secretory units after severe injury. To induce severe damage, the authors used a modified method of the submandibular gland ductal ligated model where, alongside the WhartonÂ’s duct, periductal tissues were also ligated. This method was proposed to induce a more severe injury in terms of acinar cell loss than the obstruction of the WhartonÂ’s duct alone, allowing cells to increase their plasticity and expand their lineage commitment to acinar secretory fates. Although their data from lineage tracing studies support their proposed observation, I would suggest that further experiments are required before conclusions can be safely drawn. This includes further validation of their main finding, that myoepithelial cells transdifferentiate into a common ckit+ progenitor to regenerate the secretory units. With this revision, that their study would greatly increase our understanding of the plasticity of myoepithelial cells in salivary gland regeneration.

Comments for the author

Specific concerns:
1. Given the importance of the severity of injury on cellular plasticity, it would be very informative to present a more detailed analysis of the degree of damage that the ductal vs the ductal-periductal ligation cause to the tissue. In particular, it would be interesting to quantify the proportion of remaining acinar cells after the 3 day ligation (L3D0) period (Fig. S1B). For the quantification, acinar cells could be characterised by using, in addition to Aqp5, other acinar-specific markers, since Aqp5 expression is reduced after ligation (Cotroneo E et al., 2008. Cell Tissue Res). How consistent is the ligation procedure in terms of the amount of injury caused? In the discussion, the authors mention that they performed a parallel lineage tracing study where a milder injury was induced by obstructing only the duct and not the periductal tissue and they did not observe any acinar contribution. It would be very informative to include this data in the supplementary material.

2. During the regeneration phase the authors found a 4 to 5-fold expansion of K14 positive cells by comparing the proportion of K14+ cells in the control and regenerating gland (Fig. 1E). However, this expansion might be overestimated given that the acinar/ductal ratio, and therefore the K14 negative/K14 positive ratio, is reduced in the regenerating gland. K14 positive cells could be normalised by the total number of myoepithelial and ductal cells instead of the total number of cells as a better indication. It would be also interesting to identify the cell type (myoepithelial or ductal) expressing K14 for each condition.

3. Using the K14TRE:tetOH2GFP mice, the author show that regenerating acinar cells are produced by K14+ cells. Although this is an important observation, it is unclear what is the overall contribution of K14+ regenerating cells producing acinar cells. Therefore, it would be instructive to have a quantification of Aqp5+ GFP+ cells (Fig. 1H). In addition, although it is known that, in homeostasis, acinar cells do not express K14, a control image showing no K14 expression in the regenerating acinar cells would also be important to include. What is the number of GFP+ myoepithelial cells during regeneration? Is GFP found only in the tubular structures?

4. To identify the population of cells with regenerative potential, lineage tracing experiments were performed on multiple cell types using the K14CreTRE:R26RYFP (K14-YFP), αSMACreERT2;R26R9tdTomato (SMA-TdT), Axin2CreERT2;R26RTdT (Axin2-TdT) and cKitCreERT2/+; R26RTdT (cKit-TdT) mouse lines. For the K14-YFP and Axin2-TdT mice, induction was performed for 4-5 days through diet, and then mice were returned to normal diet for 4 days before they underwent injury. However, for the SMA-TdT and cKit-TdT, induction was performed for 4-5 days, and then mice were returned to normal diet for 4 weeks before they underwent injury. Was the induction in the latter case performed during the postnatal stage? A more thorough clarification is required on why the authors decided to increase the tracing period to 4 weeks for the SMA-TdT and ckit-TdT mice before performing the ligation. In addition, given the importance of lineage tracing studies in defining the cellular plasticity after severe injury, it is important to ensure that there are no minor leakages of the transgenes that would be further expanded during the regeneration phase due to the high proliferation. Therefore, an additional control is required particularly for the SMA-TdT line, where non-induced mice would undergo the same regime of surgical procedures as the induced mice.

5. Using the K14-YFP mice, the authors demonstrated the remarkable plasticity of the K14+ ductal and/or myoepithelial cells to produce acinar cells when exposed to injury (Fig. 2). To identify the K14+ cells that contribute to the formation of acini (ductal vs myoepithelial), the authors looked at the distribution of the labelling across the acinar/ductal axis. Based on this strategy the authors suggested that acinar cells were regenerated mainly from K14+ myoepithelial cells. Although this strategy might be informative if it is performed in a homeostatic tissue and at a clonal level, it is hard to interpret when the labelling efficiency is 50% and when the tissue morphology is perturbed after ligation (including the intercalated-granular duct junction). It would therefore be more informative to describe the distribution and the cell types produced by K14+YFP+ cells at the time of ligation (L3D0) and during regeneration (L3D10) on salivary glands induced at a clonal level on day -7 (Fig. 2A). Given the difficulty in interpreting these results from the lineage tracing experiment, it is hard to exclude the possibility that K14+ ducal cells had also highly contributed to the acinar cell regeneration apart from the K14+ myoepithelial cells.

6. By tracing the myoepithelial cells before injury, the authors found that these cells can contribute to the formation of acinar and intercalated ductal cells after severe injury (Fig. 3). Given the novelty of this finding, it is important to describe how the labelled myoepithelial cells transition from myoepithelial to a progenitor state. Similarly to the point above, it would therefore be very informative to describe the distribution and the cell types produced by the SMA+TdT+ cells at the time of ligation (L3D0) and during regeneration (L3D10) on salivary glands labelled at a clonal level on day -7.

7. To understand this transition more deeply, the authors isolated SMA+TdT+ and found a significantly low efficiency of cells to form clones and organoids in vitro compared to the K14+YFP+ cells (Fig. 4). Although they concluded that this further supports their idea of myoepithelial cell transdifferentiation, this argument is not completely clear. Organoid formation assays promote regeneration, and therefore it would be more likely to provide the required conditions for the SMA+TdT+ to transdifferentiate into a progenitor state and produce acinar and intercalated ductal cells. Interestingly some isolated SMA+TdT+ cells where able to produce organoids. However, there is no information on the cell types produced (eg. Aqp+, ckit+, K14+). Further characterisation of the in vitro progeny of the SMA+TdT+ cells is required.

8. By tracing the pattern of labelling in the regenerated cKit-TdT glands induced before injury, the authors found that this pattern resembled that seen in the SMA+TdT+ glands (Fig. 6). They therefore suggested that SMA+ cells and cKit+ cells transition through the same progenitor. However further validation is required. It would be interesting to examine whether SMA+TdT+ and cKit+-TdT+ cells express the same progenitor markers in the regenerating glands (L3D10 or before).

First revision

Author response to reviewers' comments

Date: 7/17/2020

Dear Reviewers,

Please accept our thanks for the time and consideration given this manuscript and for clarifying the ambiguities in our initial submission. Below please find a point-by-point response to your comments.

Reviewer 1:

1. The results support the conclusions, but there are technical issues…….

A) should ensure that extensive acinar cell death occurs. However, controls should include proof of extensive apoptosis, as well as proof of acinar cell loss. It has been shown by others that AQP5 is rapidly down-regulated after injury, but the loss of AQP5 does not necessarily indicate that acinar cells are completely gone.

As indicated in the introduction, the model of injury used here has been described previously and shown that the inclusion of the nerve and blood vessels in the ligature induce acinar cell death rather than atrophy (Walker and Gobe, 1987) (for review please see Denny, 1997). The histopathology of SMG at L3dL0 (Fig. 1B and S1A and S1E) is consistent with a necrotic tissue and severe damage to acinar and granular duct cells. We revised the text to clarify this (page 4 line 17). Aqp5 staining is consistent with the histological findings. Regardless, to address the issue raised with the down regulation of Aqp5, we have included images of injured gland stained for Mist1(a transcription factor expressed in acinar cells) in Fig. S1C to confirm acinar cell loss (Page 4, line 23).

B) If there are acinar cells remaining, one could interpret Fig.1e as showing ectopic activation of K14 in acinar cells that have down-regulated Aqp5. In fact, in an early publication, using the same K14Tre:tetO-H2BGFP system, H2BGFP was activated in Aqp5+ acinar cells (Kwak et al. 2016; Fig. 2B).

We acknowledged this issue in the original manuscript Page 6 line 21 stating that "either due to expansion of the surviving $K14^{+}$ cells or upregulation of K14 expression in other cell types" and addressed this directly by inducible lineage tracing of K14+ cells (Figure 2). Since $K14^{+}$ cells are labeled prior to the injury, lineage traced cells derive from the initially labeled K14+ cells not from those expressing K14 after deligation. As for the comment in our earlier publication, there is no leakage of K14 promoter activity in acinar cells. The GFP-labeled cell in Fig. 2B in Kwak 2016 is not an acinar cell but a myoepithelial cell that is located in between acinar cells (for example please see Figure 3D and 3E). Please note that as indicted in Fig. 1A, K14 is known to be expressed in both ductal stem cells and MECs. We have revised the application to reiterate this in the text as well (Page 6 line 9).

2) To confirm the interpretation that it is proliferation of K14 duct cells that produce regenerated acini, it is also important to show co-labeling of K14+ cells with Ki67 proliferation marker. It would also be informative to compare the K14-YFP staining at L3dL10 with K14 antibody staining (Fig. 1e). It is hard to draw conclusions about the cellular contributions after the 5-week recovery period, when acinar cells have significantly expanded.

Immunohistochemical staining for Ki67 staining was used to assess overall proliferative response after injury and as stated in the text (page 5, line 9) "is consistent with the rapid expansion of tubular and acini-like structures". We have further clarified that K14 was used as a marker of SG embryonic and adult progenitor cells (Page 5, line 15). We used $K14^{TRE}$:tetO- Histone2B-GFP system to show more specifically that K14+ cells actively cycle, expand and give rise to $K14^{neg}$ cells that generate acinar cells as depicted in the Fig1G-H and the revised Fig. S2. Furthermore, the final conclusion for cellular contribution is not drawn from this short- term pulse-chase experiment but from the subsequent lineage tracing studies using K14- Cre:RosaYFP mice which provides a strong evidence in support of contribution of the initially labeled K14+ cells to regenerated acinar cells. Please note that even though newly formed acinar cell have divided exponentially to repopulate acini, they originated from the initially labeled $K14^{+}YFP^{+}$ cells (Fig. 2).

3. Finally, additional characterization of the regeneration model could include staining of the tubular structures with Sma and cKit at L3dL10, to determine whether the Cre recombinases driven by these promoters are activated in the presumptive cells driving regeneration.

Clearly, as shown by our data, not all cells in the tubular structures are K14+ (Fig. 1E) and lineage tracing of SMA and cKit+ cells indicates that they survive and contribute to acinar regeneration (Fig. 3 and 6). However, we have revised the manuscript and included images of control and L3dL10 stained for SMA and cKit in the revised Fig. S2 (please also see page 6, lines 9 and 16). These stainings were done on K14-H2BGFP to distinguish between K14+ ductal cells and MECs (which also express K14) and K14+ cells and cKit+ cells. Regardless, please note that Cre-recombinase is not activated in the presumptive ducts of the regenerative gland, but prior to injury in the normal gland.

4. (A)The second technical concern is the potential for leakiness in the genetic systems used. The Dox –induced Cre system may require more than 4 days prior to injury to be effectively silenced. The inclusion of controls showing injury in the absence of Dox would partially address this.

All transgenic lines used in our studies presented here have been used previously either by us or others to trace cell lineages in the SMG during development or homeostasis as cited in the manuscript. Therefore, leakiness of these systems has been addressed previously. However, even if the system is not leaky, the promoters deriving Cre are not specific to the target population that is being traced. For example, cKit promoter is not only expressed in the ID cells, but in Tuft cells and in hematopoietic cells; SMA is expressed in MECs as well as smooth muscle cells surrounding the blood vessels and, K14 is expressed in MECs and ductal stem cells. The best way these

problems could be addressed is to use multiple transgenic lines for lineage tracing which we have done here. For example, to distinguish between contribution from MECs and smooth muscle cells when the tissue is severely damaged and lost its architecture, we rely on the results of K14-Cre mice (K14 is not expressed in smooth muscle cells). The similar pattern of lineage-labeled cells in the acini and their contiguous ID cells in SMA-Cre and K14-Cre mice (Fig. 2 and 3) indicate that smooth muscle cells did not contribute to acini. The same is applied to K14 Stem cells and the use of K14Cre and Axin 2Cre (Fig. 2 and Fig. 5). As for cKit, we rely on the ability to trace the labeled cells from acini to the associated ID but not to the Tuft cells that reside in the GD. In general, the problem often is not leakiness in the absence of tamoxifen, rather leakiness in some mice when treated with tamoxifen. We have dealt with this potential problem by using non-injured gland for each mouse as a control to check for labeling efficiency and potential leakage before analyzing the injured gland. Therefore, our approach has been as rigorous as it could be for lineage tracing studies.

As for the period of the induction Cre when using either K14-Cre or Axin-Cre, we have compared administrating the last dose of Dox or Tam either 4 days or 2 weeks before the injury and did not see any difference in the outcome. The reason we prefer to use a short time is that K14 stem cells are actively dividing cells that give rise to K19+ ductal cells and in this case it would be difficult to convince a reviewer that K19+ ductal cells did not contribute to acinar cell regeneration. For this reason, we induced injury before K14+ ductal stem cells have a chance to expand and generate K19 ductal cells. We have revised the text in page 7 line 8 to clarify this.

4. (B) Although it would be interesting to compare the observed labeling pattern with that obtained if Dox is added at the time of ligation.

These studies are not within the scope of the current manuscript and will be published in the future.

4. (C). Similarly, tamoxifen administration via chow is dependent on the eating behavior of the animals, and the speed of Cre induction can therefore vary significantly. The length of time necessary for Cre activation, as well as for tamoxifen clearance should be addressed in the controls.

As per manufacturer a 250 mg/Kg tam-containing diet used here is equivalent to 40 mg/Kg tam given by gavage and is based on uptake of 3.2 g chow/day. Based on this information and before switching to tam-diet, we compared administrating tamoxifen through gavage and diet in our mice and found that diet gave us a more reproducible labeling efficiency. Furthermore, as mentioned in the manuscript, for every mouse used in our studies, we collect both injured and contralateral non-injured gland and use the latter to determine the labeling efficiency in each mouse and have used the same base line (50% labeling efficiency) for analysis of the injured glands presented in this manuscript.

5. The text states that 24.4% of regenerated acini were derived from MECs, whereas 32.5% or (if labeling efficiency is considered) 65% of regenerated acini were derived from K14-expressing cells. It is not clear why the authors maintain that MECs make the dominant contribution to acini.

K14 is expressed by both ductal stem cells and MECs, therefore, both K14+ ductal cells and myoepithelial cells were initially labeled before injury in the K14-Cre:Rosa YFP system. Therefore, the lineage labeled acinar cells in the regenerated gland could have originated from both K14+ ducal cells and MECs. To distinguish between these possibilities, we used SMACre:RosaTdT (Fig 3) to specifically target MECs and used Axin2-Cre to specifically target ductal stem cells. Given that TdT-labeled MECs contributed to 24% of acinar and YFP-labeled K14 conribute to 32.5% indicates that the majority of lineage-labeled cells in the latter derived from MECs. This conclusion was further verified in Axin2Cre/TdT mice.

6. The suggestion that MECs and cKit cells dedifferentiate to a common bipotent progenitor is speculative, and has not been demonstrated. Perhaps co-staining with Sox10 could address this and provide evidence for de-differentiation?

The identical pattern of lineage labeled cells in SMA-TdT and Kit-TdT mice in acini and associated ID indicating a bi-lineage contribution and is consistent with reversion of MECs and cKit+ cells to a bipotent progenitor population. That is why we have stated that these data suggest reversion to a bipotent progenitor-like state.
We did co-stain the injured glands with Sox10 and phenotypic markers but to find out that Sox 10 is broadly expressed in MECS, acinar cells and their associated IDs in the adult SMG in the absence of any injury as described previously (Ohtomo et al., 2013), Therefore, we cannot use Sox10 as a marker of cell plasticity or adult progenitor cells. Although this information was already in the literature, we now include Sox 10 staining in the revised Fig. S8. Clearly the molecular mechanism of lineage reversion in the adult SG needs to be further investigated as indicated in the discussion.

7. It may be that the response of salivary glands to each type of injury is different, so the assertion that "plasticity of committed parenchymal cells is the predominant mechanism of acinar regeneration in response to a severe injury" should be limited to this particular injury model.

Thank you for your comment; we revised this sentence in the discussion Page 16-lines 3-5.

Reviewer 2
Major comments:
1) The authors use a severe damage model of reversible ductal ligation, where the ligature also surrounds the nerve and blood vessel. Previous literature shows that after duct ligation if the nerve is permanently damaged the gland will not regenerate. This point needs to be explicitly stated in the introduction to avoid reader confusion about the model. In this model when nerve and blood vessels are constricted, but not permanently damaged, it is not shown which cell types survive the damage.

We have revised the introduction (page 3 line 20-22) to address the concern about the severity of nerve damage and have made distinctions between the mild and severe duct ligation injury (Page 4 lines 2-4).

1) This is important, because the proportion of cells that survive in this severe damage model, may influence their ability to regenerate. For example, if MECs have a higher survival rate compared to ductal K14 cells, that could result in more lineage tracing from one population over the other. This important point could be addressed by showing staining patterns of SMA, Axin2 and cKit in damaged glands at an early time-point (L3dL0) or very shortly after the de-ligation, since all lineages studied show the potential to regenerate.

The point about the possibility of differential survival of ductal stem cells and myoepithelial cells is not supported by our data. Lineage tracing analysis of the regenerated glands in K14Cre-YFP system shows extensive contribution of YFP-labeled cells to both acini and GDs. Unlike acini that can be regenerated by induced plasticity of diverse cell types, regeneration of GD is strictly dependent on contribution from ductal SC. If K14+ ductal stem cells survival was disproportionately lower than MECs, we would have observed a significantly lower proportion of YFP-labeled GDs which is not shown by our data. Moreover, as indicated by the histopathology of SMG at L3dL0 (Fig. 1B and S1A), the ligated gland is necrotic with a high degree of cell damage. The loss of tissue architecture and a low signal to noise ratio makes identification and quantification of these relatively rare cells technically challenging and unreliable. To avoid misinterpretation of such results, we have relied on the initial labeling of MECs and ductal SC and the extent of their contribution to lineage-traced acini and GDs.

2) The authors conclude that both MECs and cKit cells go through a "bi-potent progenitor" as an intermediate population to regenerate acinar cells. This is based on analysis of acini/ID junctions labeling patterns. Although they report that most of the acini are continuously labeled with ID markers, they also find acini that are lineage traced without labeled IDs. They also report that cKit cells are lineage traced heterogeneously. Together, these findings do not exclude direct trans-differentiation without a "bi-potent progenitor cell". In addition, using the SMA-cre, the authors do not report lineage traced cKit ID cells without an acinar label.

Therefore, the possibility that cKit cells only lineage trace from MECs through proacinar/acinar cells cannot be excluded. Please revise the model or discus these possibilities. If there is a "bi-potent progenitor" what are its defining markers, or is it simply a transitional state depending on what cell is being used as a progenitor?

The possibility that MECs give rise to cKit$^+$ ID cells through proacinar/acinar cells is not supported by our data as a small number (3% of acini-ID junctions) of MEC-derived lineage- traced ID cells connected to non-labeled acini are detected in the SMA-TdT model. This data is now included in the revised Figure 3I and S4G. We also revised the text (page 10 lines 20-23) and Figure 3J to better clarify the frequency of different labeling pattern at the acini/ID junction. Clearly further investigation is needed to understand the molecular mechanism of the lineage plasticity and reversion of MECs as indicated in the discussion.

3) The authors use two well-established methods to compare in vitro growth from K14+ cells and MECs. Their experiments indicate that in vitro results do not directly correlate to in vivo predictions of potential. The authors conclude that the cells are therefore not stem cells. This is at odds with their manuscript given the extensive in vivo evidence for conditional proliferative potential. Their findings suggest that there is not a "one size fits all" in vitro stem cell assay and highlights the limitations of in vitro assays. The in vitro models do not recreate the complex multicellular and matrix-rich stem cell niche that occurs in vivo. This should be discussed.

It is not clear to us how the reviewer has come to this conclusion from Figure 4. The data actually show a clear and direct correlation between *in vitro* proliferation capacity and *in vivo* stemness. These culture methods are used to assess proliferative potential of stem cells in normal tissue not the injured tissue. The purpose of our experiment was to determine if MECs in normal SMG behave as quiescent stem cells as has been described for mammary MECs. Accordingly, we have used normal SMG to assess the proliferative potential of MECs in culture either by clonogenicity or by organoid formation. As mentioned correctly by the reviewer culture condition does not represent or simulate the complexities of the wound environment, but it promotes proliferation of tissue stem cells. In many tissues including skin which I have vast experience with, differentiated cells do not grow in culture, however, they undergo lineage plasticity and regenerate multi-lineage tissue when transplanted into a skin wound (Please see Mannik et al, 2010). We have revised the text to better clarify the conclusion (Page 12 line 9- 10). We also have revised the discussion to clarify this (Page 17 lines 21-23)

4) In the discussion, the authors refer to parallel studies obstructing salivary duct with and without periductal tissue with very different outcomes, but do not include discussion of these other results. Discussion of these results would help explain to readers their key finding, about salivary gland cell plasticity and how different damage models result in different regenerative scenarios.

We have included data showing the extent of acinar cells loss after the mild and severe ligature-induced injury and the contribution of cKit cells in these two injury models in the supplementary Figure 7 and revised the discussion accordingly (Page 16 line 23 and Fig. S7). Previous studies by Dr. Ovitt's laboratory cited in the manuscript have shown that regeneration of acinar cells after ligation of Wharton's duct is achieved by self-duplication and not from K5+ cells that include both ductal stem cells and MECs.

Minor comments:

1) The authors pose two scenarios "The broad expression of K14 in regenerative glands could be either due to expansion of the surviving K14+ cells or upregulation of K14 in other cell types". But do not include the third option that the increase is relative to loss of other cell types (point 1 above). The results show the lineage contribution from pre-existing K14 cells, but they do not directly address/discuss the other scenarios in light of their results

We revised the text in page 6 line 22-23 accordingly "The broad expression of K14 in regenerative glands could be either due to disproportionate survival of K14$^+$ cells and their subsequent expansion, or upregulation of K14 expression in other cell types.

2) They state that traced MECs formed very few organoids despite comparable TdT- labeling efficiency in vivo in the two transgenic lines. However, the ~50% labeling efficiency of K14 cells includes both duct and MECs, while ~50% labeling efficiency of SMA+ cells is only MECs. Thus, this is not an accurate comparison as the actual number of labeled cells in the SMA-Cre could be much lower compared to the K14-Cre. This can in turn lead to a lower number of labeled cells in the spheres after 7 days. If the goal is to compare proliferative potential, it is more accurate to look for the percentage increase from day 0.

The objective of data shown in Fig. 4 was to assess if MECs contain quiescent stem cells. In Fig. 4A, ductal stem cells and MECs were purified and their colony forming efficiency was directly compared. The organoid formation assay was used as a second approach to confirm that. Regardless, the number of ductal stem cells is significantly lower than MECs in the adult SMG (2.5% vs. 8% of total parenchymal cells) (Kwak, et. al 2016) (also please see the flow chart in Fig. 4A), therefore even in K14Cre:TdT mice, the majority of initially labeled cells are MECs.

3) The authors state on page 9, line14: "…suggesting a different mechanism for regeneration of MECs than that used for acinar and ductal cells." This is confusing as the model is showing that MECs are surviving and some regenerating other cell types. What is meant by MEC regeneration? Please clarify.

We meant repopulation of MECs, we have revised the text (page 9-line 18-19) "suggesting that repopulation of MECs is not driven by the same progenitor that replenishes acinar and ID cells".

4) The authors switch between using the terms differentiated, committed and more committed cells when referring to MECs and duct cells, e.g, in the abstract MECs and duct cells are differentiated while in the discussion they are committed and fully differentiated. They also refer to K14 duct cells as either stem/progenitor cells, or ductal stem cells, or K14 stem cells. This is confusing, please use terms consistently.

in the original manuscript we referred to cKit as committed cells. However, we agree with the reviewer and for consistency we have revised the manuscript and refer to MEC and ID cells as differentiated cells and K14 ductal cells as ductal stem cells.

5) In Figure 1: If "non-injured" is the same as "control" please revise figure for consistency. If it is not the same, please specify why there was an additional control.
It is the same and figure revised accordingly.

6) Page 4, line 22: The gland weight loss results are not referred to in any figure. Please include them.
This data and an image of the control and ligated glands are now included in the Fig. S1D

7) Page 8, line 4: "…which is likely due to migratory nature of MECs.." please include a reference to this migratory phenomenon.
We revised the sentence to clarify that migration of MECs during regeneration and cited accordingly.

8) Page 13, line 11: In reference to ductal stem cells, "…however, their contribution to acinar regeneration is not significant as MECs, possibly due to their scarcity and physical distance from acini." This is confusing as the authors show that K14 increases after ligation while acini are lost. How scarce are ductal stem cells after damage and how would their distance from acini matter once the acinar cells are lost? Or do they regenerate in a different location?

The reviewer is correct that due to loss of architecture in the injured gland, the location of stem cell would not matter. We have revised the sentence to clarify this on page 13 lines 12-13.

9) Page 11, line2: "However, whether MECs display similar characteristics have not been determined." Sentence should include the word "salivary MECs". If not, this is confusing as

the previous sentence refers to mammary MECs.
Thank you, we have revised the text accordingly.

10) The authors should refer to this damage model as severe duct ligation as they show that ligating with or without the periductal vascular and nerve tissue are basically two different damage models. Also, page 15, line 22: Please clarify that this is severe ligation injury since severe injury is often used to describe irradiation damage.

We have revised the entire manuscript to distinguish between the two models of injury. The indicated sentence was revised to "Therefore, at least in the model of severe glandular injury used in our studies, plasticity of differentiated parenchymal cells is the predominant mechanism of acini regeneration."

Reviewer 3 Comments for the Author:

Specific concerns:

1. Given the importance of the severity of injury on cellular plasticity, it would be very informative to present a more detailed analysis of the degree of damage that the ductal vs the ductal-periductal ligation cause to the tissue. In particular, it would be interesting to quantify the proportion of remaining acinar cells after the 3 day ligation (L3D0) period (Fig. S1B). For the quantification, acinar cells could be characterised by using, in addition to Aqp5, other acinar-specific markers, since Aqp5 expression is reduced after ligation (Cotroneo E et al., 2008. Cell Tissue Res). How consistent is the ligation procedure in terms of the amount of injury caused? In the discussion, the authors mention that they performed a parallel lineage tracing study where a milder injury was induced by obstructing only the duct and not the periductal tissue and they did not observe any acinar contribution. It would be very informative to include this data in the supplementary material.
We have revised the SI and have included MIST1 staining of the injured gland in Fig. S1C. In addition, we included a comparative analysis of duct ligation +/- periductal and quantification of remaining acini is now included in Figure S7.

2. During the regeneration phase the authors found a 4 to 5-fold expansion of K14 positive cells by comparing the proportion of K14+ cells in the control and regenerating gland (Fig. 1E). However, this expansion might be overestimated given that the acinar/ductal ratio, and therefore the K14 negative/K14 positive ratio, is reduced in the regenerating gland. K14 positive cells could be normalised by the total number of myoepithelial and ductal cells instead of the total number of cells as a better indication. It would be also interesting to identify the cell type (myoepithelial or ductal) expressing K14 for each condition.

K14+ cells include MECs and K14+ ductal stem cells, and in the regenerative gland the majority of epithelial cells are ductal cells. We refer the reviewer to Fig. S2A in which an image of normal and regenerative gland labeled with K14-H2BGFP included to see a significant increase in the number of K14+ cells upon injury. Clearly, disproportionate survival of K14+ cells could have attributed to this which we have clarified in the revised manuscript Page 6-line 22-23.

2. Using the K14TRE:tetOH2GFP mice, the author show that regenerating acinar cells are produced by K14+ cells. Although this is an important observation, it is unclear what is the overall contribution of K14+ regenerating cells producing acinar cells. Therefore, it would be instructive to have a quantification of Aqp5+ GFP+ cells (Fig. 1H). In addition, although it is known that, in homeostasis, acinar cells do not express K14, a control image showing no K14 expression in the regenerating acinar cells would also be important to include. What is the number of GFP+ myoepithelial cells during regeneration? Is GFP found only in the tubular structures?

We have added a new Figure S2 in the revised manuscript to address all the points raised here.

4. To identify the population of cells with regenerative potential, lineage tracing experiments were performed on multiple cell types using the K14CreTRE:R26RYFP (K14- YFP),

αSMACreERT2;R26R9tdTomato (SMA-TdT), Axin2CreERT2;R26RTdT (Axin2-TdT) and cKitCreERT2/+; R26RTdT (cKit-TdT) mouse lines. For the K14-YFP and Axin2-TdT mice, induction was performed for 4-5 days through diet, and then mice were returned to normal diet for 4 days before they underwent injury. However, for the SMA-TdT and cKit- TdT, induction was performed for 4-5 days, and then mice were returned to normal diet for 4 weeks before they underwent injury. Was the induction in the latter case performed during the postnatal stage? A more thorough clarification is required on why the authors decided to increase the tracing period to 4 weeks for the SMA-TdT and ckit-TdT mice before performing the ligation. In addition, given the importance of lineage tracing studies in defining the cellular plasticity after severe injury, it is important to ensure that there are no minor leakages of the transgenes that would be further expanded during the regeneration phase due to the high proliferation. Therefore, an additional control is required particularly for the SMA-TdT line, where non-induced mice would undergo the same regime of surgical procedures as the induced mice.

This is addressed in detail in response to reviewer I comment 4A.

5. Using the K14-YFP mice, the authors demonstrated the remarkable plasticity of the K14+ ductal and/or myoepithelial cells to produce acinar cells when exposed to injury (Fig. 2). To identify the K14+ cells that contribute to the formation of acini (ductal vs myoepithelial), the authors looked at the distribution of the labelling across the acinar/ductal axis. Based on this strategy the authors suggested that acinar cells were regenerated mainly from K14+ myoepithelial cells. Although this strategy might be informative if it is performed in a homeostatic tissue and at a clonal level, it is hard to interpret when the labelling efficiency is 50% and when the tissue morphology is perturbed after ligation (including the intercalated-granular duct junction). It would therefore be more informative to describe the distribution and the cell types produced by K14+YFP+ cells at the time of ligation (L3D0) and during regeneration (L3D10) on salivary glands induced at a clonal level on day -7 (Fig. 2A). Given the difficulty in interpreting these results from the lineage tracing experiment, it is hard to exclude the possibility that K14+ ducal cells had also highly contributed to the acinar cell regeneration apart from the K14+ myoepithelial cells.

We have clearly addressed the issue of contribution of K14+ ductal stem cells and MECs by using Axin2-Cre and SMA-Cre drivers in the subsequent experiments.

6. By tracing the myoepithelial cells before injury, the authors found that these cells can contribute to the formation of acinar and intercalated ductal cells after severe injury (Fig. 3). Given the novelty of this finding, it is important to describe how the labelled myoepithelial cells transition from myoepithelial to a progenitor state. Similarly to the point above, it would therefore be very informative to describe the distribution and the cell types produced by the SMA+TdT+ cells at the time of ligation (L3D0) and during regeneration (L3D10) on salivary glands labelled at a clonal level on day -7.

As indicated in the discussion, the molecular mechanism regulating lineage reversion of MECs is subject of future studies and beyond the scope of this manuscript.

7. To understand this transition more deeply, the authors isolated SMA+TdT+ and found a significantly low efficiency of cells to form clones and organoids in vitro compared to the K14+YFP+ cells (Fig. 4). Although they concluded that this further supports their idea of myoepithelial cell transdifferentiation, this argument is not completely clear. Organoid formation assays promote regeneration, and therefore it would be more likely to provide the required conditions for the SMA+TdT+ to transdifferentiate into a progenitor state and produce acinar and intercalated ductal cells. Interestingly some isolated SMA+TdT+ cells where able to produce organoids. However, there is no information on the cell types produced (eg. Aqp+, ckit+, K14+). Further characterisation of the in vitro progeny of the SMA+TdT+ cells is required.

The purpose of the studies presented in Fig 4 was to make sure that MECs do not function as quiescent stem cells, and we believe our date clearly shows that. We disagree with the reviewer

on the statement that culture conditions simulate the wound environment as using skin as a model, we have previously shown that differentiated cells that do not grow in culture, when placed in the wound environment revert to multipotent stem cell (Mannik, et. al. 2010).

The types of cells generated in SG organoids has been characterized previously and we did not feel that it has relevance to the conclusion of this manuscript.

8. By tracing the pattern of labelling in the regenerated cKit-TdT glands induced before injury, the authors found that this pattern resembled that seen in the SMA+TdT+ glands (Fig. 6). They therefore suggested that SMA+ cells and cKit+ cells transition through the same progenitor. However further validation is required. It would be interesting to examine whether SMA+TdT+ and cKit+-TdT+ cells express the same progenitor markers in the regenerating glands (L3D10 or before).

The model we suggested is based on the pattern of lineage traced cells in SMA-TdT and Kit- TdT mice. Clearly further investigation is needed to gain clear mechanistic insights into cell plasticity in this model of injury. We discuss this issue on page 18 line 20-22.

Second decision letter

MS ID#: DEVELOP/2020/192807

MS TITLE: Diverse Epithelial Cell Populations Contribute to Regeneration of Secretory Units in Injured Salivary Glands

AUTHORS: Ninche Ninche, Mingyu Kwak, and Soosan Ghazizadeh
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

In a combined use of lineage tracing and an induced injury model, the authors have provided evidence for a high degree of cellular plasticity within salivary gland cells. The results show that cells of several different terminally differentiated types can contribute to the rapid regeneration of secretory acinar cells following an injury of short duration. Plasticity has only recently been recognized in the salivary glands, and the implications for this finding are significant. The ability to adopt an alternate fate could be harnessed therapeutically to generate damaged or lost cells for regenerative medicine.
However, whether the conclusions provide a significant and novel contribution to the understanding of developmental mechanisms is open to question. The cellular responses to acute injury are novel and informative, but because the injury model involves damage to the nerve, presumably resulting in the absence of important trophic signals, they may not be physiologically relevant to developmental mechanisms.

Comments for the author

While the authors argue that the events they are seeing involve cellular plasticity, they do not take into account that plasticity involves changes in cell phenotype and, most likely, gene expression. The results obtained after the severe ligation injury do clearly suggest cell plasticity, but the conclusions derived from the results are still open to questions. Some of these questions, including

potential leakiness of Cre or the use of cell proximity to argue for progenitor-progeny relationships, are concerns posed by at least 2 reviewers.
However, the authors have not adequately addressed these concerns in the resubmission.
Additional concerns regarding the timeline used and what cellular events/behavior is missed during the intervening 4 weeks have not been satisfactorily addressed. The 3-day ligation clearly induces rapid responses which would be important and intriguing to characterize and understand, given the authors' observations that MECs and cKit duct cells appear to lineage trace to regenerated acinar cells. The novelty of these findings would be significant if the caveats pointed out by the reviewers can be rigorously ruled out.

Reviewer 2

Advance summary and potential significance to field

The authors have made a significant and surprising advance in identifying several cell types that respond to severe damage in the salivary glands and regenerate the acinar cells. The study is novel and an important advance for the field to understand the regenerative potential in salivary glands.

Comments for the author

The authors have addressed our concerns. We look forward to seeing the manuscript published.

Reviewer 3

Advance summary and potential significance to field

As detailed in the first round of review.

Comments for the author

The revised version has improved on the clarity of the in vivo lineage tracing studies. Overall, the reviewers have addressed almost all of the comments in a satisfactory manner. However, the argument of myoepithelial stem cell transdifferentiation is still not very well supported. The reduced colony forming and organoid formation efficiency of myoepithelial cells might be related to their differential requirements for growth conditions and it does not necessarily indicate myoepithelial cell transidifferentiation. The authors may consider revising their conclusion of myoepithelial cell transdifferentiation.