Cyclic growth of dermal papilla and regeneration of follicular mesenchymal components during feather cycling

Ping Wu, Tingxin Jiang, Mingxing Lei, Chih-Kuan Chen, Shu-Man Hsieh Li, Randall Widelitz and Cheng-Ming Chuong

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MS TITLE: Regeneration of dermal papilla stem cells and mesenchymal components during feather cycling

AUTHORS: Ping Wu, Tingxin Jiang, Mingxing Lei, Chih-Kuan Chen, Shu-Man Hsieh Li, Randall Widelitz, and Cheng-Ming Chuong

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 also have several 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 is expected to 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 be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please 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 study Wu and co-authors characterize the proliferation and migration dynamics of mesenchymal dermal cells during physiological and injury-induced feather follicle regeneration. Using DNA incorporation of thymidine analogs combined with pulse-chase assays they demonstrate that transient amplified and label retaining cells within the dermal sheath, dermal papilla and the pulp display specific spatiotemporal patterns during the growth and resting phases of the feather regeneration. Furthermore, they show evidence for a flow of label retaining cells from the apical region of the dermal papilla to the newly formed pulp during the growth phase. In complementary experiments the authors perform gene expression analysis which reveals differentially expressed genes between the discrete dermal compartments.
Finally, the authors surgically ablate portions of the dermal papilla and show that the remaining dermal niche is capable to compensate and drive the regeneration of a new feather, albeit with notable defects. From these experiments the authors infer that the partial excision of the dermal papilla alters the interface between the epithelium and the mesenchyme illustrating the importance of this cross-talk for the fidelity of the regeneration process.

Overall this is an interesting study that sheds light into the cellular dynamics of the mesenchymal component of a regenerating epidermal appendage.
Given the importance of the dermal papilla for the regeneration of hair follicles in mammals this study provides critical information to highlight the common and diverging mechanisms involved in the regeneration of these organs. My major criticism is that the data in this study are largely descriptive and the functional significance of many of the observations is not tested further and, in many cases, not even sufficiently discussed.
Furthermore, I have some concerns for the rigor behind some of the statements and interpretations.

Comments for the author

Below are my specific comments that hopefully will help further improve the manuscript before publication.

1) Fig2G. I am not sure what the blue arrows are indicating in this figure.
I can see a streak of IdU labeled cells but the arrows don't seem to be properly aligned with them. Furthermore, it is not clear if this "streak" is actual labeling or a staining artifact.

2) Page 7. " In the pulp the TA cell zone expanded toward the cPP during the 24-hour labeling (Fig. 2G, second column), compared to the 2-hour labeling period (Fig. G, first column)". It is hard for the reviewer to come to this conclusion based on the images that are provided. The authors need to provide quantifications to substantiate this statement.

3) Fig. 3. "After 1-week labeling, about 90 percent of the pPP cells are BrdU positive (Fig. 3C, C', yellow arrows), but the DP and the DS are rarely positive. After a 2-week chase, the number of BrdU positive cells in the pPP decreased by 30 percent." The authors need to provide detailed quantification and statistical analysis to support these statements.

4) The gene expression analysis provided in Figs. 4 and 5 are useful but seem disconnected from the rest of the paper. I'd like to see at least some cohesive hypothesis discussed for how the differentially expressed genes are involved in the proliferation and migration dynamics that are presented in the rest of the manuscript.

5) Page 9. "Resting phase DP retains putative stem cells in the apical region (Fig. 3F)". I am not sure that this is sufficiently supported by the current data. I understand that many of the conventional lineage tracing tools are not yet available in the chicken model however calling the

apical DP cells, stem cells is premature without further analysis and based solely on the pulse-chase data.

6) Fig 6E. I am not sure why the authors chose a different pulse-chase timecourse for this experiment compared to the one in Fig. 3 but it is hard to compare the data. At the very least images right before plucking should be provided here to show where the LRCs are right before the induced regeneration.

7) Page 10. "…we detected numerous TA cells in the epidermis… we also detected some LRDCs in the new PP." These statements lack rigor. Like in other parts of the manuscript the authors need to provide the quantifications and the sample numbers and biological replicates used for the statistical analysis.

8) Fig. 6F "We also detected some LRDCs in the new PP". There seem to be many more LRCs that TAs. How do the authors explain the lack of label dilution given the massive growth in the new PP in the two days of growth after plucking?

8) Fig. 6G "We found that among IdU positive cells, 50% are also CldU positive." Need to see the quantifications, same as above.

9) Fig 6I-K. I am not intimately familiar with this assay, so I have to take the author's interpretations of the data at face value. I am wondering what negative controls would be appropriate for this and whether the authors should provide them to inspire confidence to the reader.

10) Page 11. "After 8 weeks of growth, the regenerated feathers lost feather branches on the right side, especially in the pennaceous regions (Fig. 7B) (N=10/10)." The authors provide the sample numbers but not the quantification or statistical analysis.

11) Page 11. "thinner in width than normal control feathers." How was this quantified?

12) Page 11. "LRDCs in the dermis coordinate with LRDCs in the epidermis"
This is a rather vague statement that I am not sure how strongly it is supported by the current data.

Reviewer 2

Advance summary and potential significance to field

Avian feather dermal papilla is an excellent model to study how dermal mesenchymal cells behave in vivo because of its large size. The authors take advantage of the feather model to address whether dermal components also display cyclic behaviors during natural molting and regeneration. This question is difficult to study in the hair dermal papilla due to its small size. Despite genetic tagging tools are not available in their system, they apply RNA in situ assays, BrdU/IdU/CIdU labeling, and DiI dye-mediated cell tracing to show that dermal cells have a distinct migration trail during the growth and resting phases. In addition, they also conducted transcriptome analyses to identify specific markers and signaling components that will facilitate further studies of distinct dermal components or compartments in feather follicles. Overall, it is a solid work. I find their figures and images are in high quality, interpretations are appropriate, and key conclusions are well-supported. I have no major issues to comment but offer some minor points to consider.

Comments for the author

Minor:
1. Page 7, paragraph 3. The authors state "…long-term label-retaining dermal cells (LRDC)- BrdU positive pPP cells are "about 90 percent…" and "… decreased by 30 percent". How do the authors come up with these numbers?
Please be specific about how the quantifications were conducted.

2. Page 9, paragraph 3. The in situ expression patterns of CRABP-1 and NCAM

(Fig. 5F and 5H) are not in consistent with transcriptome data shown in Fig. 5E. Any speculations?

3. Page 11, paragraph 2. "…we performed surgery to remove the right half of the DP midway along the left-right axis". In Fig. 7A and 7B, the drawings show the "left half" of the DP is removed. Please check.

4. Fig. 1S and 1T. It is confusing for readers to go over schematic drawings that assign different colors to the same components or compartments. Please be consistent especially when they are displayed in the same figure. If pink color is used to label DP in one drawing, the same pink color shall be used to label DP in another drawing.

5. Fig. 2I and 2J. Same issue. fe and DP shall be labeled with consistent color across different figures. Same for Fig. 3C'' and 3G; Fig.6I to L and Fig. 6H; Fig. 7E and 7G.

6. Fig. 2E. Mis-spelling of 'double'.

7. Fig.4D and 4E. The axis labels are difficult to read. Same for Fig. 5E.

8. Fig. 6B, 2nd row, middle column (K15 day 2). Please label the bDP domain.

Reviewer 3

Advance summary and potential significance to field

Using the feather as a model system, Wu et al document the proliferative behavior of dermal fibroblasts that comprise the feather mesenchyme across the regenerative growth and rest cycle. The authors provide a comparison of various markers and bulk sequencing previously associated with hair follicle dermal stem cells, dermal papilla and dermal sheath from mouse studies. They also complete bulk sequencing to provide transcriptional signatures of each compartment within the feather meseenchyme, which shows greatest similarity between DS and DP relative to cells in pulp. By performing short and long term pulse chase experiments they provide an approximate location of proliferative cells in DS and PP and putative "stem cells" based on longer term label retention. Although interesting, label retention alone cannot be used to identify a putative stem cell and so the conclusions need to be tempered. That said it is strengthened by the fact that despite the difference in appendage anatomy, there appears to be congruence in the location of dermal progenitors in mouse hair follicle and feather (dermal sheath and basal DP). This is very intriguing and further highlights the importance of these cells in appendage maintenance and regeneration across species. Overall this is an interesting study, in a novel model system, that further supports the existence of a stem/progenitor cells within mesenchyme of regenerative appendages (both feathers and hair follicles).

Comments for the author

Review Manuscript # DEV198671 Regeneration of dermal papilla stem cells and mesenchymal components during feather cycling

1. The title is misleading and inaccurate. The authors show themselves that the DP is almost entirely non-proliferative and without lineage tracing cannot claim the definitive origin or location of a stem cell within the DP and the claim that the stem cell pool is being repopulated can only be a guess, based on the data presented. The title needs to be revised.

2. The authors pose the question "We wonder how the homeostasis of dermal mesenchymal cells is controlled". Previous work has provided considerable insight about the existence and identity of hair follicle dermal stem cells and how the hair follicle mesenchyme is maintained (Rahmani et al 2014; Shin et al 2020; Chi et al 2013). The rationale for this study should be stated as determining whether similar mesenchymal progenitor location, markers, and dynamics exist in the feather.

3. I find it difficult to know where the compartment boundaries are in the images provided. It would be helpful to provide combined compartment specific immunostaining with the IdU/Edu/Brdu staining. This would allow the authors to more precisely verify the location/origin of the IdU/EdU/BrdU labeling (particularly for the LRC studies).

4. The identification of putative mesenchymal stem/progenitors in the DS compartment is interesting given that this parallels work done in the mouse hair follicle. It would greatly elevate the importance of the manuscript if the authors could co-localize those proliferative cells within the DS (suspected to represent putative feather dermal stem cells) with immunofluorescence staining and high magnification imaging with the candidate genes identified in the bulk RNAseq. This would help to better define the regulatory signature of these cells and what distinguishes them from other cells within the compartment.

5. I fail to see the significance or the question being tested in the dermal papilla amputation studies. The objective and approach are entirely unclear. The data is speculative and seems out of place within the rest of the manuscript. I recommend that this is removed or better explained.

6. The introduction and discussion sections lack clarity and there are a number of incorrect references provided throughout. The discussion on page 13 (De novo production of dermal papilla cells during the anagen phase of the hair cycle) is confusing and contains a number of incorrect statements and citations. For example, “Whether stem cells exist within hair DP is unknown. In hair cycling, dermal stem cells regenerate a new DS and supply cells to the DP (Jahoda, 2003; Rahmani et al., 2014). The adult DS harbors dermal stem cells, which regenerate a new DS and supply cells to the DP (Biernaskie, 2010).”
These statements are contradictory. Moreover, the existence of a dermal stem cell has been demonstrated in work by Rahmani et al 2014 using both lineage tracing and in vivo clonal analysis. Although speculated by Jahoda (2003) no direct evidence of a stem/progenitor in the hair follicle mesenchyme was provided and so this citation should be removed. This section should be rewritten and clarified.

7. Pg 12 Discussion Line 1 - The statement “In this study, we show a mesenchyme cycling model in which multiple dermal components in a discrete follicle unit undergo physiological regeneration of dermal stem cells.” This does not make sense. Nowhere in this study do you provide sufficient evidence for regeneration of a dermal stem cell. This needs to be clarified.

8. The images provided in Figure 6 are poor and make it difficult to determine the fate of the labeled cells. Location of progeny should be detrmined by compartment specific markers as the tissue sections appear broken and the anatomy of the mesenchyme is difficult to discern with confidence.

9. How does the dermal sheath differ from the basal DP ? My assumption would be that these two structures are contiguous and that the basal DP would be equivalent to the dermal cup in the mouse hair follicle. This should be discussed. As well, the findings regarding stem/progenitors in the aDP, seem to take priority over the progenitor pools in the dermal sheath and the basal DP. Further explanation of the results is warranted because it is still not clear the lineage relationship between these compartments.

10. The study is highly descriptive. It would be interesting to provide some quantification of LRCs within each compartment. How much heterogeneity is there? How many of the aDP and bDP and DS are label retaining? How many are LRCs?

Minor
Pg 8 - Further examination of gene expression levels among different developmental stages reveals that CDK1, SOSDTC1 – spelled incorrectly.
Figure 2E “double labeling” spelled wrong

First revision

Author response to reviewers' comments

Response to Reviewers

Reviewer 1. Advance summary and potential significance to field
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Reviewer 1 Comments for the author
Below are my specific comments that hopefully will help further improve the manuscript before publication.

1) Fig 2G. I am not sure what the blue arrows are indicating in this figure. I can see a streak of IdU labeled cells but the arrows don't seem to be properly aligned with them. Furthermore, it is not clear if this "streak" is actual labeling or a staining artifact.

Originally the blue arrows were meant to indicate the IdU labeled cells, and white arrow to point to the CldU labeled cells, so readers could compare the position of both. This was not clear to the reviewers. We have removed the arrows in Fig 2G and use new quantification in Fig. 2I, J to show the position shift (Please also see answer to point (2)).
We state: "We found that TA cell zones in both feather epidermis and pulp expanded with time (Fig. 2G)". Here we focus on the analyses of dermal components. The major point here is TA cells are produced in the peripheral pulp first, and then shifted toward the central pulp region.
The 'streak' is a blood vessel. We now clarify this in the figure legend.

2) Page 7. "In the pulp the TA cell zone expanded toward the cPP during the 24-hour labeling (Fig. 2G, second column), compared to the 2-hour labeling period (Fig. G, first column)". It is hard for the reviewer to come to this conclusion based on the images that are provided. The authors need to provide quantifications to substantiate this statement.

Thank you for the opportunity to clarify this point. First, we define peripheral and central pulp clearer with three criteria: "(i) differences in cell density (Fig. 1A'), (ii) functional demonstration that they control barb branching patterns (Li et al., 2017) and pigmentation patterns (Lin et al., 2013) and (iii) molecular expression patterns (summarized in Fig. 1S)."
Second, we quantify the percentage of CldU and IdU positive cells in pPP versus cPP. While there is no anatomical boundary between pPP and cPP, the cell density and arrangement are different (Fig. 1A', S1). Molecular expression further defines pPP (Fig. 1C-G, H-K). Based on these,

100 μm is estimated and used to mark the boundary between the pPP and cPP (Fig. 2I) for quantification.
In the result part, we now state: "We calculate the percentage of 2-hour labeled CldU cells versus 24-hour labeled IdU cells in pPP and cPP and show that the percent of TA cells increases more in the cPP than the pPP (Fig. 2I and J). This double TA cell labeling result demonstrates the migration and expansion of pulp cells and illustrates their possible migration route (Fig. 2K)".

3) Fig. 3. "After 1-week labeling, about 90 percent of the pPP cells are BrdU positive (Fig. 3C, C', yellow arrows), but the DP and the DS are rarely positive. After a 2-week chase, the number of BrdU positive cells in the pPP decreased by 30 percent." The authors need to provide detailed quantification and statistical analysis to support these statements.
Thank you for the comment. We quantified the BrdU positive cells in pPP, DP and DS after labeling with BrdU for 1 week as well as after chasing the label for 2, 4 and 7 weeks. In the 7-week chase period, feathers have entered the resting phase, there is no pulp anymore, so we calculated the BrdU positive cells in aDP instead of the pulp. We added a new panel in Fig. 3G.
Now we state in the results: "After 1-week labeling, 90.2 ± 4.1 percent of the pPP cells are BrdU positive (Fig. 3C, C', yellow arrows), but the DP and the DS are rarely positive. After a 2-week chase, the number of BrdU positive cells in the pPP decreased to 31.5 ± 5.1 percent. These BrdU long-term label-retaining dermal cells are concentrated in the pPP, adjacent to the epidermal LRC in the collar bulge, previously shown to be the site of feather epidermal stem cells (Fig. 3D, D', white arrows). Some LRDCs can also be detected in the DS (Fig. 3D, green arrows). Feathers collected after a 4-week chase period showed that the LRDCs moved downward surrounding the DP (Fig. 3E, E', white arrows) and some DS cells are BrdU positive (Fig. 3F, green arrows). Notably, downward movement of putative dermal stem cells accompanies the movement of the epidermal stem cell zone (yellow bracket line in Fig. 3D and E). After a 7-week chase period, the Resting phase feather follicle has LRDCs in the aDP (Fig. 3F, F', blue arrows) and some positive cells also are seen in the DS (Fig. 3F, F', green arrows). The schematic drawing in C" to F" shows the relative position of BrdU positive cells (blue dots, no chase; yellow dots, LRDCs in epidermis; red dots, LRDCs in dermis). The percentage of BrdU positive cells before and after 2-, 4- and 7-weeks chase periods are shown in Fig. 3G. These results demonstrate the accompanied downward shift of epidermal and dermal LRCs, during feather cycling (Fig. 3H). In Resting phase, LRDCs are present in the aDP but not in the bDP, suggesting that the aDP region may retain dermal progenitor cells for the next feather cycle. The bDP is more quiescent than the aDP."

4) The gene expression analysis provided in Figs. 4 and 5 are useful but seem disconnected from the rest of the paper. I'd like to see at least some cohesive hypothesis discussed for how the differentially expressed genes are involved in the proliferation and migration dynamics that are presented in the rest of the manuscript.
Thank you for the opportunity to clarify this point. We added this paragraph to the discussion to explain some molecules that may be involved in cell proliferation or adhesion / migration.
"Our bulk RNA-seq for pulp at different developing phases (Fig. 4) and in different dermal components (Fig. 5) suggest the possible molecular circuit regulating cell proliferation and migration in feather growth. For example, decreasing CDK1expression from Early growth PP to Resting phase aDP (Fig. 4D, F) is accompanied by the declining number of BrdU positive cells (Fig. S2). NCAM is another molecule which showed an intriguing pattern. NCAM transcripts are found at higher levels in the DP and DS (Fig. 5E), while RNAscope results reveal the heterogenous NCAM transcript distribution in the DP: the peripheral DP expresses higher NCAM levels than the central DP (Fig. 5H). This heterogeneity highlights the possible route for DS cell migration into the inward feather follicle (Fig. 7C). On the other hand, co-staining of LRDCs and NCAM by RNAscope (Fig. S5) reveals higher NCAM expression levels in the Near Resting phase PP may facilitate LRDC accumulation in the PP close to the DP. Future studies will further characterize the function of these molecular markers."

5) Page 9. "Resting phase DP retains putative stem cells in the apical region (Fig. 3F)". I am not sure that this is sufficiently supported by the current data. I understand that many of the conventional lineage tracing tools are not yet available in the chicken model however calling the apical DP cells, stem cells is premature without further analysis and based solely on the pulse-chase data.
We agree more lineage data will be required to name them stem cells. We modified the sentence to be: "Resting phase DP retains LRDCs in the apical region (Fig. 3F)."

6) Fig 6E. I am not sure why the authors chose a different pulse-chase time course for this experiment compared to the one in Fig. 3 but it is hard to compare the data. At the very least images right before plucking should be provided here to show where the LRCs are right before the induced regeneration.
For Fig. 6, we address regeneration after plucking induced injuries. The data show the principles are similar to physiological cycling data in Fig. 3. We performed 1-week IdU labeling on growth phase feathers in order to identify resting phase feathers which retain the LRCs in the apical DP. Two hours before collecting the regenerating feather follicle, we injected CldU to label the TA cells. This method allows us to visualize LRDCs and TA cells in the same follicle. We have added a panel (Fig. 6F) in which feather follicles were labeled with IdU and CldU to show the LRDCs in the aPP before plucking.

7) and 8) Page 10. "…we detected numerous TA cells in the epidermis… we also detected some LRDCs in the new PP." These statements lack rigor. Like in other parts of the manuscript the authors need to provide the quantifications and the sample numbers and biological replicates used for the statistical analysis.

Fig. 6F "We also detected some LRDCs in the new PP". There seem to be many more LRCs that TAs. How do the authors explain the lack of label dilution given the massive growth in the new PP in the two days of growth after plucking?

All cells are labeled by the 1-week IdU labeling period. The LRDCs are slow cycling cells that rarely divide. Most will retain the label through the 4-week chase period. Then the TA cells are labeled with CldU during the last 2 hours. This will label a subset of TA cells that are within S-phase of the cell cycle during this 2-hour period. Cells will only divide approximately once per 24 hours. Two days of regeneration only shows early stages of feather growth. Massive growth has not yet been accomplished over these two days. In the new Fig. 6F, we show LRCs in unplucked feather follicles. Then in panel G, we show feather follicles that have regenerated for 2 days. Quantification data is shown in panel I.

Fig. 6G "We found that among IdU positive cells, 50% are also CldU positive." Need to see the quantifications, same as above.

We added panel I to show a resting phase feather follicle before plucking in panel 6F. We also added quantification to count CldU positive (TA cells), IdU positive (LRDCs) and CldU/IdU (double positive) cells representing LRDCs that began to divide during the CldU labeling window in resting aDP versus new PP after 2 days of regeneration.

9) Fig 6I-K. I am not intimately familiar with this assay, so I have to take the author's interpretations of the data at face value. I am wondering what negative controls would be appropriate for this and whether the authors should provide them to inspire confidence to the reader.
DiI is a lipophilic dye that labels cell membranes and it is a traditional method to label and trace cells when genetic reporter tracing methods used in mice are not available. We micro-injected DiI to different regions of the feather follicle at different phases of the feather cycle. Cells retain the label while passing some to their daughter cells over a 5-day period. This enables us to see the expansion of the labeled cell population. We have many examples of this, but this data will not increase the understanding of the experiment and is not included here. We added images for DiI labeling at time 0 in the new Fig. 7. We did not show the 0 time point for Fig. 7D because GFP needs at least 10 hours to be expressed.

Some transgenic chicken lines with loxP reporters which can be cleaved by micro-injected Cre recombinant protein have recently become available in the Roslin Institute in the UK. We plan to use these chickens to carry out higher resolution experiments in the future when feasible. But the logistics will take some time to arrange.

10) Page 11. "After 8 weeks of growth, the regenerated feathers lost feather branches on the right side, especially in the pennaceous regions (Fig. 7B) (N=10/10)." The authors provide the sample numbers but not the quantification or statistical analysis.

We will do more analyses of these DP ablation assays in a different paper in the future. For this manuscript, we removed Fig 7A-F per R3's suggestion. Thanks for your opinion.

11) Page 11. "thinner in width than normal control feathers." How was this quantified?
We removed Fig 7A-F per R3's suggestion.

12) Page 11. "LRDCs in the dermis coordinate with LRDCs in the epidermis" This is a rather vague statement that I am not sure how strongly it is supported by the current data.
We removed Fig 7A-F per R3's suggestion and have eliminated this statement.

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Reviewer 2 Comments for the author

Minor:
1. Page 7, paragraph 3. The authors state "…long-term label-retaining dermal cells (LRDC)- BrdU positive pPP cells are "about 90 percent…" and "…decreased by 30 percent". How do the authors come up with these numbers?
Please be specific about how the quantifications were conducted.
Thank you for the opportunity to clarify this point. We quantified the BrdU positive cells of pPP after labeling with BrdU for 1 week as well as after chasing the label for 2, 4 and 7 weeks. These data are now shown in our new Fig. 3G.

2. Page 9, paragraph 3. The in situ expression patterns of CRABP-1 and NCAM (Fig. 5F and 5H) are not in consistent with transcriptome data shown in Fig.5E. Any speculations?
Thank you. We now use the RNAscope method to redo some expression data. We replaced Figure F-I with RNAscope data. This is a more sensitive method to detect mRNA expression and shows the distribution of RNAs in higher resolution. NCAM can be seen to be enriched in the DP, Fzd8 is enriched in the DS, TnC in the DP and part of DS.

3. Page 11, paragraph 2. "…we performed surgery to remove the right half of the DP midway along the left-right axis". In Fig. 7A and 7B, the drawings show the "left half" of the DP is removed. Please check.
Thank you. We decided to analyze the DP ablation data further in future studies. This paragraph has been removed.

4. Fig. 1S and 1T. It is confusing for readers to go over schematic drawings that assign different colors to the same components or compartments. Please be consistent especially when they are displayed in the same figure. If pink
color is used to label DP in one drawing, the same pink color shall be used to label DP in another drawing.
Thank you for your suggestion. In figure 1, we need more colors to illustrate more components, In Fig. 2-7. we have revised the colors in the diagram, so they are consistent.

5. Fig. 2I and 2J. Same issue. fe and DP shall be labeled with consistent color across different figures. Same for Fig. 3C'' and 3G; Fig.6I to L and Fig. 6H; Fig. 7E and 7G.
Revised as suggested. Thank you.

6. Fig. 2E. Mis-spelling of ‘double’.
Revised. Thank you.

7. Fig.4D and 4E. The axis labels are difficult to read. Same for Fig. 5E.
Revised. Thank you.

8. Fig. 6B, 2nd row, middle column (K15 day 2). Please label the bDP domain.
Revised. Thank you.

Reviewer 3 Advance summary and potential significance to field
Using the feather as a model system, Wu et al document the proliferative behavior of dermal fibroblasts that comprise the feather mesenchyme across the regenerative growth and rest cycle. The authors provide a comparison of various markers and bulk sequencing previously associated with hair follicle dermal stem cells, dermal papilla and dermal sheath from mouse studies. They also complete bulk sequencing to provide transcriptional signatures of each compartment within the feather meseenchyme, which shows greatest similarity between DS and DP relative to cells in pulp. By performing short and long term pulse chase experiments they provide an approximate location of proliferative cells in DS and PP and putative “stem cells” based on longer term label retention. Although interesting, label retention alone cannot be used to identify a putative stem cell and so the conclusions need to be tempered. That said it is strengthened by the fact that despite the difference in appendage anatomy, there appears to be congruence in the location of dermal progenitors in mouse hair follicle and feather (dermal sheath and basal DP). This is very intriguing and further highlights the importance of these cells in appendage maintenance and regeneration across species. Overall this is an interesting study, in a novel model system, that further supports the existence of a stem/progenitor cells within mesenchyme of regenerative appendages (both feathers and hair follicles).

Reviewer 3 Comments for the author

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Regeneration of dermal papilla stem cells and mesenchymal components
during feather cycling

1. The title is misleading and inaccurate. The authors show themselves that the DP is almost entirely non-proliferative and without lineage tracing cannot claim the definitive origin or location of a stem cell within the DP and the claim that the stem cell pool is being repopulated can only be a guess, based on the data presented. The title needs to be revised.
We agree more data will be required to call them stem cells. These apical DP are mainly responsible for the cyclic growth of pulp. We now revised the title to be “Cyclic growth of dermal papilla and regeneration of follicular mesenchymal components during feather cycling”.

2. The authors pose the question “We wonder how the homeostasis of dermal mesenchymal cells is controlled”. Previous work has provided considerable insight about the existence and identity of hair follicle dermal stem cells and how the hair follicle mesenchyme is maintained (Rahmani et al 2014; Shin et al 2020; Chi et al 2013). The rationale for this study should be stated as determining whether similar mesenchymal progenitor location, markers, and dynamics exist in the feather.
Hairs and feather are independently evolved and have been apart for approximately 200 million years. The motivation of this study is to compare the parallels and differences on how they manage cyclic regenerating follicles. To make this point clearer, we explained this better in the introduction with this paragraph:
“The topology of skin appendage follicles, allowing stem cells to be protected in the proximal follicle and distal differentiated appendage to be shed, is a successful strategy to organize the integuments (Lai and Chuong, 2016). Yet, the configurations of hair and feather follicles are achieved via convergent evolution, separated by approximately 200 million years. While the fundamental principles of epidermal stem cells – dermal niche are shared (Yue et al., 2005; Chu et al., 2014; Morgan, 2014; Fuchs 2018), the specific way to molt and regenerate the epidermal and dermal components during cycling are different. For example, the developing feather filament is a cylinder with pulp inside, while mouse and human hair filaments are concentric epithelial cords. To enter resting phase, feather follicles keep the follicular walls more or less intact, while hair follicles undergo catagen to destroy the lower follicles. Thus, it would be interesting to compare

how these two major skin appendages, for avian and mammalian classes, may use different strategies to manage their cycling regeneration. In this study, we will focus on dermal components. Recent work provided considerable insight about the existence and identity of hair follicle dermal stem cells and how the dermal sheath, destroyed in catagen, is regenerated and cells in the dermal papilla are replenished (Chi et al., 2013; Rahmani et al., 2014; Shin et al., 2020). On the other hand, in feather cycling, there is much less remodeling of the dermal sheath, but very large-scale pulp regeneration and degeneration within the follicle. We wonder whether, in parallel to the epidermal cells in hair and feather follicles, there are also label-retaining cells (LRC), TA cells, stem cell clusters, progenitor cell zones in the dermis of growing feather follicles? If so, where are they located and how do they behave or transit during feather cycling?"

We also modified the previous Table 1 which summarizes the differences and put it as the new Fig. 8B. The above work about hair DS stem cells are cited and compared with the findings from feather follicles.

3. I find it difficult to know where the compartment boundaries are in the images provided. It would be helpful to provide combined compartment specific immunostaining with the IdU/Edu/Brdu staining. This would allow the authors to more precisely verify the location/origin of the IdU/EdU/BrdU labeling (particularly for the LRC studies).
We co-stain Col1, Tenascin-C, Sox2 with LRDCs. We add a paragraph to describe the result.
"We co-stained the LRDCs and the marker genes shown in Fig. 1. Examples (Col1, Tenascin-C and Sox2) are shown in Fig. S3. The distribution of LRDCs in different developing stages are accompanied with the differential expression of marker genes. For example, when LRDCs move downward from Middle Growth to Late Growth and eventually present in the aDP in Resting phase (Fig. S3C, F, I), the expression of Sox2 antigen changed from the whole DP (Middle Growth and Late Growth) to the bDP (Resting phase)."

4. The identification of putative mesenchymal stem/progenitors in the DS compartment is interesting given that this parallels work done in the mouse hair follicle. It would greatly elevate the importance of the manuscript if the authors could co-localize those proliferative cells within the DS (suspected to represent putative feather dermal stem cells) with immunofluorescence staining and high magnification imaging with the candidate genes identified in the bulk RNAseq. This would help to better define the regulatory signature of these cells and what distinguishes them from other cells within the compartment.
Thank you for the constructive suggestion. We performed a new RNAscope experiment to locate Tenascin-C and NCAM in the late growth phase feather follicle with higher resolution, and co-stained LRCs in the same section (new Fig. S5). We found the accumulation of LRCs in pPP is accompanied by a higher level of NCAM expression. However, in the DS, fewer LRCs are detected (Fig. S4L) and both NCAM and Tenascin-C are expressed at a lower level. Our result is consistent with what has been found in the mouse hair follicle and we will need more data in the future to fully understand the mechanism.
We add a paragraph in the result section "Molecular profiling in different dermal components".
"Furthermore, we examined whether dermal LRDCs co-express the molecules enriched in dermal components. We used RNAscope to detect Tenascin-C and NCAM transcripts in the Near Resting phase feather follicle and co-stained the LRDCs in the same section (Fig. S5). We focused on the distribution of dermal LRDCs in pPP and DS. We found the accumulation of LRDCs in the pPP (Fig. S5G) is accompanied by higher NCAM expression levels (Fig. S5H). In the DS, fewer LRDCs are detected (Fig. S5L) and both NCAM and Tenascin-C are expressed at lower levels than in the pPP (Fig. S5M and N). Based on this data, we speculate that the higher expression levels of cell adhesion molecules, such as NCAM, in the Near Resting phase PP (Fig. 4E) may be involved for the accumulation of LRDCs required for feather cycling. More study will be required to characterize these LRDCs and the DS in the future. In this study, we focus more on the regeneration of feather pulp.".

5. I fail to see the significance or the question being tested in the dermal papilla amputation studies. The objective and approach are entirely unclear. The data is speculative and seems out of place within the rest of the manuscript. I recommend that this is removed or better explained.
We decided to analyze the data of DP ablation further in future studies. Thus we removed DP amputation studies.

6. The introduction and discussion sections lack clarity and there are a number of incorrect references provided throughout. The discussion on page 13 (De novo production of dermal papilla cells during the anagen phase of the hair cycle) is confusing and contains a number of incorrect statements and citations. For example, “Whether stem cells exist within hair DP is unknown. In hair cycling, dermal stem cells regenerate a new DS and supply cells to the DP (Jahoda, 2003; Rahmani et al., 2014). The adult DS harbors dermal stem cells, which regenerate a new DS and supply cells to the DP (Biernaskie, 2010).”
These statements are contradictory. Moreover, the existence of a dermal stem cell has been demonstrated in work by Rahmani et al 2014 using both lineage tracing and in vivo clonal analysis. Although speculated by Jahoda (2003) no direct evidence of a stem/progenitor in the hair follicle mesenchyme was provided and so this citation should be removed. This section should be rewritten and clarified.
Thank you. This is revised as advised. We now state “Using in vivo lineage tracing and in vitro clonal analysis, it is shown that the adult DS harbors dermal stem cells, which repopulate the DS and the DP with new cells (Rahmani et al., 2014). Hair follicle dermal stem cells can regenerate the DS and repopulate the DP (Chi et al., 2010; Rahmani et al., 2014). Platelet-derived growth factor (Pdgfra) signaling is important for the function of hair follicle dermal stem cells (González et al., 2017). The hair DS has a distinct precursor population which may act as a reservoir for regenerating DP cells in aging (Agabalyan et al., 2017). Injury of hair follicles was shown to recruit more dermal stem cell progeny to become DP cells (Abbasi and Biernaskie, 2019).”

7. Pg 12 Discussion Line 1 - The statement “In this study, we show a mesenchyme cycling model in which multiple dermal components in a discrete follicle unit undergo physiological regeneration of dermal stem cells.” This does not make sense. Nowhere in this study do you provide sufficient evidence for regeneration of a dermal stem cell. This needs to be clarified.
As stated, apical DP controls the cycling of pulp, but not all the dermal components of feather follicles. We now delete the phrase “of dermal stem cells.” The sentence now reads: “In this study, we show a mesenchyme cycling model in which multiple dermal components in a discrete follicle unit undergo cyclic physiological regeneration.”

8. The images provided in Figure 6 are poor and make it difficult to determine the fate of the labeled cells. Location of progeny should be determined by compartment specific markers as the tissue sections appear broken and the anatomy of the mesenchyme is difficult to discern with confidence.
The current DiI labeling method has limitations in its resolution, therefore we also used the pTol2-H2BGFP labeling method. The specimen here is obtained after feather plucking and the broken tissue is due to the damage caused by feather plucking.

9. How does the dermal sheath differ from the basal DP ? My assumption would be that these two structures are contiguous and that the basal DP would be equivalent to the dermal cup in the mouse hair follicle. This should be discussed. As well, the findings regarding stem/progenitors in the aDP, seem to take priority over the progenitor pools in the dermal sheath and the basal DP. Further explanation of the results is warranted because it is still not clear the lineage relationship between these compartments.
Thank you. Our data in feathers (Fig. 7C, D) also made us think that feather DS and basal DP may be a contiguous structure. Our study is more focused on the apical DP - pulp regeneration, and the DS - DP regeneration here is preliminary. Whether there is a parallel dermal cup-like equivalent remains to be studied in the future. We now state the comparison in feathers and hairs clearer in the discussion section.
“We compare the morphological/structural similarities and differences between chicken feathers and mouse hairs (Fig. 8B). In mouse hair cycling, the lower hair follicle is destroyed in catagen. In every anagen, DS is regenerated along with ORS and hair matrix. Thus, the regeneration of dermal components is mainly DS and some DP, with cells coming from the dermal cup, the location of dermal stem cells (Rahmani et al., 2014). In contrast, in feather cycling, the major degeneration events occur in the pulp and epidermal collar within the follicle. Thus, the major feather dermal components that require regeneration are the pulp and some DP, not DS. In feather follicles, in addition to the inductive function of DP (Chu et al., 2014), the cPP in the filament core provides nutrition continuously for feather growth. Furthermore, an additional pPP - epidermal interface is extended distally above the DP (discussed further in the next section). Feather DS, DP and PP are contiguous structures. When feathers enter the Resting phase, no pulp component remains and

LRDCs descend to reside in the aDP. Because the dermal components that require regeneration are differently positioned in hair and feather follicles, the topological arrangement of dermal LRCs and TA cells are also different. Whether there is a parallel dermal cup equivalent in the lower sheath remains to be studied using transgenic chickens in future investigations."

10. The study is highly descriptive. It would be interesting to provide some quantification of LRCs within each compartment. How much heterogeneity is there? How many of the aDP and bDP and DS are label retaining? How many are LRCs?

Indeed, the DP is heterogeneous in the feather. Also, this heterogeneity changes during feather cycling which can also be appreciated in the making of pennaceous and plumulaceous portions of contour feathers (Chang et al., 2019). The molecular heterogeneity can be appreciated by looking at the staining data in Fig. 1, showing growth and resting phase follicles.
For cell labeling, we now quantified the BrdU and IdU positive cells in pPP, DP and DS after labeling with BrdU for 1 week as well as after chasing the label for 2, 4 and 7 weeks. In the 7-week chase period, feathers have entered the resting phase, there is no pulp anymore, so we calculated the BrdU positive cells in aDP instead of pulp. These quantification data are shown in Figs 2I, 2J, 3G and 6I to help address this issue.
Now we state: "After 1-week labeling, 90.2 ± 4.1 percent of the pPP cells are BrdU positive (Fig. 3C, C', yellow arrows), but the DP and the DS are rarely positive. After a 2-week chase, the number of BrdU positive cells in the pPP decreased to 31.5 ± 5.1 percent. These BrdU long-term label-retaining dermal cells are concentrated in the pPP, adjacent to the epidermal LRC in the collar bulge, previously shown to be the site of feather epidermal stem cells (Fig. 3D, D', white arrows). Some LRDCs can also be detected in the DS (Fig. 3D, green arrows). Feathers collected after a 4-week chase period showed that the LRDCs moved downward surrounding the DP (Fig. 3E, E', white arrows) and some DS cells are BrdU positive (Fig. 3F, green arrows). Notably, downward movement of putative dermal stem cells accompanies the movement of the epidermal stem cell zone (yellow bracket line in Fig. 3D and E). After a 7-week chase period, the Resting phase feather follicle has LRDCs in the aDP (Fig. 3F, F', blue arrows) and some positive cells also are seen in the DS (Fig. 3F, F', green arrows). The schematic drawing in C" to F" shows the relative position of BrdU positive cells (blue dots, no chase; yellow dots, LRDCs in epidermis; red dots, LRDCs in dermis). The percentage of BrdU positive cells before and after 2-, 4- and 7-weeks chase periods are shown in Fig. 3G. These results demonstrate the accompanied downward shift of epidermal and dermal LRCs, during feather cycling (Fig. 3H). In Resting phase, LRDCs are present in the aDP but not in the bDP, suggesting that the aDP region may retain dermal progenitor cells for the next feather cycle. The bDP is more quiescent than the aDP."

Minor

Pg 8 - Further examination of gene expression levels among different developmental stages reveals that CDK1, SOSDTC1 – spelled incorrectly.
Revised, thank you.

Figure 2E "double labeling" spelled wrong
Changed. Thank you.

Second decision letter

MS ID#: DEVELOP/2020/198671

MS TITLE: Cyclic growth of dermal papilla and regeneration of follicular mesenchymal components during feather cycling

AUTHORS: Ping Wu, Tingxin Jiang, Mingxing Lei, Chih-Kuan Chen, Shu-Man Hsieh Li, Randall Widelitz, and Cheng-Ming Chuong

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 reviewers are positive and we would like to publish your manuscript in Development, provided that the referees' minor comments can be satisfactorily addressed. As you see, Reviewer #2 requests some additional information in a few of the figure legends. I ask that you include this information in a revised version that I do not expect to return to referees. 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

This study that sheds light into the cellular dynamics of the mesenchymal component of the regenerating feather appendage. Given the importance of the dermal papilla for the regeneration of hair follicles in mammals this study provides critical information to highlight the common and diverging mechanisms involved in the regeneration of these organs.

Comments for the author

The revised manuscript by Wu et al. is significantly improved. The changes that were incorporated improve the clarity and bolster confidence for the major conclusions. The authors have addressed most of my comments and based on the overall significance of the findings support the publication of this manuscript without further review. I do however recommend that the authors implement minor changes suggested below to improve on the reporting of their statistical analyses.

Major comments:
1. The authors response to my comment is satisfactory.
2. I am partially satisfied with the authors response to my comment. Sample number (n) and p values for this quantification need to be included in the figure legend. In the Materials and Methods section the authors indicate a “n = 5” for Fig. 2I, however it is not clear what the “n” refers to.
3. I am satisfied for the most part with the authors response to my comment. Sample number (n) and p values for the graph in Fig. 3G need to be included in the figure legend to indicate statistical significance.
4. The authors response to my comment is satisfactory.
5. The authors response to my comment is satisfactory.
6. The authors response to my comment is satisfactory.
7-8. I am satisfied for the most part with the authors response to my comment. Sample number (n) and p values for the graph in Fig. 6I need to be included in the figure legend to indicate statistical significance.
9. The authors response to my comment is satisfactory.
10. The authors response to my comment is satisfactory. I agree that this part needs further development, and it is wise to remove it from the current manuscript.
11. The authors response to my comment is satisfactory.
12. The authors response to my comment is satisfactory.

Reviewer 2

Advance summary and potential significance to field

The authors characterize cyclic growth of dermal papilla during feather molting and regeneration. They identify proliferation and migration dynamics of mesenchymal dermal cells. They also conducted transcriptome analyses on different mesenchymal compartments for specific markers and signaling components. Their findings are relevant to future studies of mesenchymal stem cells in regenerative appendages.

Comments for the author

The revised version looks good to me. I have no further questions.

Reviewer 3

Advance summary and potential significance to field

The authors employ the feather to examine dermal fibroblast dynamics during regeneration. Given the importance for the mesenchyme in skin appendage formation/regeneration this paper provides new insights into fibroblast functional heterogeneity and mechanisms by which these inductive structures are maintained to support continuous rejuvenation of these mini-organs. Using RNAseq and RNAscope the authors also identify some new markers and potential regulators within the different dermal subsets comprising the feather.

Comments for the author

The authors appear to have reasonably addressed my major concerns/suggestions in their revised draft and is now suitable for publication in Development.

Second revision

Author response to reviewers' comments

We have addressed Reviewer 1's points.

Changes in Figures:
-Fig. 2J, 3G and 6I, we added * to show statistics significance.

Changes in Figure legends:
-Fig. 2J, 3G and 6I, we added the sample size and p values.

Changes in Materials and Methods:
-We now make it clear that "n=5 feather follicles"

Third decision letter

MS ID#: DEVELOP/2020/198671

MS TITLE: Cyclic growth of dermal papilla and regeneration of follicular mesenchymal components during feather cycling

AUTHORS: Ping Wu, Tingxin Jiang, Mingxing Lei, Chih-Kuan Chen, Shu-Man Hsieh Li, Randall Widelitz, and Cheng-Ming Chuong

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.