Retinoic acid synthesis and autoregulation mediate zonal patterning of vestibular organs and inner ear morphogenesis

Kazuya Ono, Lisa L. Sandell, Paul A. Trainor and Doris K. Wu
DOI: 10.1242/dev.192070

Editor: Patrick Tam

Review timeline
Original submission: 27 April 2020
Editorial decision: 28 May 2020
First revision received: 9 June 2020
Accepted: 1 July 2020

Original submission

First decision letter

MS ID#: DEVELOP/2020/192070

MS TITLE: Retinol dehydrogenase 10 is required for regional patterning of the vestibular organs and morphogenesis of the inner ear

AUTHORS: Kazuya Ono, Lisa Sandell, Paul Trainor, and Doris Wu

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

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

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.

Reviewer 1

Advance summary and potential significance to field

This is a very interesting and well-executed study that expands our understanding of the retinoic acid signaling responsible for zonal patterning of the vestibular sensory regions of the inner ear. The authors first identify the specific dehydrogenase for the first step in the synthetic pathway (RDH10) by showing its gene expression overlaps with the previously studied second step in the pathway (ALDH1A3) and that Rdh10 conditional mutant mice have vestibular phenotypes similar to

those Aldh1a3 mutants, which are opposite to those of mutants for the RA-degrading enzyme (CYP26B1). Importantly, the authors go on to elucidate some of the mechanism by which RA directs zonal patterning of the vestibular sensory organs. By using an in vitro culture system, they show that RA induces expression of the Cyp26b1 gene, and by examining proliferation in RA gain-and-loss of function mutants they that show that the level of RA signaling determines the timing of cell cycle exit in the vestibular sensory epithelia, which has implications for thinking about using this signaling system to promote repair following vestibular sensory cell loss. The data are convincing and the story will be appreciated by the inner ear field, and more generally by those interested in RA signaling in the patterning of organs.

Comments for the author

All of my concerns are minor:

1. It would be nice if the title could reflect the findings on RA patterning in the second part of the paper.
2. Defining the brackets used in Figure 2 inside the main text (not just in the legend) would help the explanation of the expression patterns. Also, some of the panels could use additional annotation to point out structures mentioned in the text (e.g. extrastriolar region of utricle and saccule and the peripheral zone of the canal cristae)
3. Figure 3 could be improved if the authors can provide a two-injection paintfill of the Rdh10 control and a one-injection paintfill of the Aldh1a3 control.
4. The authors mention that the Aldh1a3 mutants lack otoconia and are poor swimmers.
The Rdh10 otoconial phenotype is shown in Figure 4, but there is no mention of swim tests.
There is no need to do this test for publication, but if it can't be done because the cKO is lethal after birth, perhaps this could be mentioned somewhere (like in Materials and Methods).
5. It seems as if there is a word or phrase missing from the sentence on line 167.
6. I would like to see the data in Supplementary Figure 1 included with Figure 5.
7. The sample numbers for Figure 6 need to be included. Also, it would be helpful to place B' as a full panel with an indication of its time in culture (it currently says "extended").
8. The sentence in lines 222-225 starting "These results suggest that…" seems convoluted. Maybe there is a simpler way to state the conclusion.
9. Sample numbers are needed for Figure 8.

Reviewer 2

Advance summary and potential significance to field

Previous work defined the enzymes that synthesize and degrade retinoic acid (RA) in vestibular organs, their spatial distribution and their crucial importance for patterning the striolar region. Here this new work identifies the vestibular enzyme that produces retinaldehyde, a precursor/earlier step in RA synthesis. RDH10 was previously associated to early vestibular development, but not in later epithelial patterning. The authors show that Rdh10 loss-of-function essentially recapitulates Aldh1a3 inactivation, which is expected since both models prevent RA production. In addition, cross-regulation in the RA pathway is addressed, with ectopic RA treatment promoting expression of the RA degrading enzyme Cyp26b1, and inactivating Aldha13 conversely reducing Cyp26b1. The results in the study form a natural follow-up to the genetics and anatomical results in Ono et al., Nat Communication 2020. The work is very well-done, the manuscript is very well written and the conclusions are solid. Some results interestingly expand on previous conclusions. For example, Rdh10 affects zoning in cristae, unlike Aldh1a3, revealing that there must be an alternate crista-specific aldehyde dehydrogenase(s). I have no major concern, only two comments.

Comments for the author

1) This is a relatively short study that appears well suited for a Report format. Results feel stretched in 9 figures, with Fig. 1 only having a schematic and several figures including a single experiment/set of panels. Figures 1 to 4 could be compiled into a new Fig. 1 to show Rdh10

expression and high level phenotypes (fused utricle-saccule, lack of otoconia). Fig. 5 could be a new Fig. 2 (zonal phenotype). Fig. 6-8 could be a new Fig. 4 (cross-regulation). Fig 9 could be a new Fig. 4 (proliferation).
2) Line 282: the author discuss the observation that OCM expansion in Rdh10 mutant occurs only medial to the LPR in the utricle, but on both sides of the LPR in the saccule. This is probably simply because the striolar region itself is normally medial to the LPR in the mouse utricle but bisected by the LPR in the saccule (Li/ Peterson 2008, the authors' Jiang et al 2017). "The reason for this regional difference is not clear" (line 282) applies in my mind to the normal, distinct organization of the utricle vs saccule, and not to the way OCM expands, which is rather expected. I find this part of the discussion a little confusing. Why not point out more simply that striolar expansion respects the polarity boundary?

Reviewer 3

Advance summary and potential significance to field

The primary contribution this paper makes is to contribute to unravel how vestibular organs develop in mammals. Specifically, we know very little about the functions of the different specialized zones of the vestibular sensory epithelium; ditto for the signals that regulate formation of these regions. This paper introduces novel and exciting findings about how RA controls this process and opens doors for manipulative studies to test functions of the different specialized zones. These findings are of fundamental importance to sensory biology. The authors expand our knowledge of how RA controls regional development of other vestibular sensory epithelia (saccular macula and two cristae), and they elucidate some of the molecular mechanisms by which RA exerts control over inner ear development. One of the strengths of the study is they show, using gain of function and loss of function assays, that RA regulates Cyp26b1 expression. Although this has been shown for other sensory systems, the authors demonstrate this for the first time in the inner ear.

Comments for the author

This is a clearly written paper that follows up on a couple of prior studies demonstrating that RA controls 1) gross development of the membranous segments of the inner ear and 2) regional development of the utricular macula. Here, the authors expand our knowledge by showing that RA also controls regional development of other vestibular sensory epithelia (saccular macula and two cristae), and they elucidate some of the molecular mechanisms by which RA exerts control over inner ear development. One of the strengths of the study is they show, using gain of function and loss of function assays, that RA regulates Cyp26b1 expression. Although this has been shown for other sensory systems, the authors demonstrate this for the first time in the inner ear.

Overall, this is a concise paper with excellent descriptions of rationales for each experiment, helpful and beautiful images and quantitative figures of the data that support the interpretations, and strong discussion of the findings. My review describes edits I believe could strengthen the paper.

Essential revisions:

The anatomical nomenclature used by the authors is confusing at times. Throughout the paper, the terms crista and utricle or saccule are used together, but technically the crista = sensory epithelium, while the utricle/saccule = organ. I suggest using the sensory epithelial names (crista and macula) in sections that address the regional differences in the sensory epithelium and defining the specific terms early on in the paper.

Lines 247-259. This section on EdU labeling is confusing. As the authors explain, the findings in Figure 9C,C' could be explained by 1) cell cycle exit before E14.5 or 2) high rates of cell division between E14.5 and E18 that diluted the EdU. But, the findings could also be explained by migration of cells born on E14.5 from the striolar region, which was not considered. By giving EdU at an earlier time (E11.5) and labeling for a HC marker, the authors tested 1) if earlier cell division occurred in that region and 2) if new HCs were generated from those divisions. However, it is not clear how this second experiment distinguished between early cell cycle exit and high rates of

division. This could have been addressed by EdU pulse/fix at E14.5 for which labeling would have supported a higher rate of cell division. Furthermore, it is not clear why the higher number of labeled HCs in the mutant supports the interpretation that cell cycle exit was premature. Why would the rate of HC production be higher earlier on, if mitosis were to halt earlier than normal? Also, this finding could also indicate that the mutant cells experience a fate-switch from SC to HC. Finally, this is picky but HCs do not exit the cell cycle; they never divide.

Figure 3C,D. Relative to A and B, it's difficult to see the paint fill. Is it possible to increase signal in C,D?

Figure 4. Panels B and C look very different from A. It is very hard to compare and to see difference or where otoconia are/are not. Can this figure be altered to make this easier for the reader?
¬
Figure 9. The utricle in panel F has an odd shape, so it is difficult to see whether EdU labeling is in the striolar region. Can this be improved? Also, if the authors can clarify the significance of EdU-labeled HCs, could they add a high magnification image of these cells (e.g., between panels F and G)?

Figure 6B. The images are low magnification, so it is difficult to distinguish where the hybridization signal is localized. How tissue-specific was Cyp26b1 upregulation? It looks like the signal extends beyond sensory epithelium. If so, this should be described. One limitation of this analysis is it is non-quantitative and although the authors explain that they carefully controlled the timing of the hybridization reaction, they did so only in the legends. Concerns about the qualitative aspects of this experiment would be better allayed if the timing component were explained in the Results text.

Figures 5, 7 and Supplementary Figure 1. It is not clear what is meant by % as a measure (% of control, % increase of control, % of a given region or cell type, or other)? This should be clearly described in the Results text and figure legends. E.g., for Figure 7: Does % = % of utricular macula or % of striolar region?

Supplementary Fig 1. The ß spectrin labeling, as shown at low magnification, is not very useful. Perhaps add an inset so the readers can see where the kinocilium exits each cuticular plate – i.e., the cell planar cell polarity? The authors need to describe the white line (LPR) somewhere. For this figure, when analyzing the regional changes, did they cut it into anatomic sections? If so, they should explain this in the Methods and "box" or otherwise indicate the specific regions they analyzed.

Discussion: One interesting finding – that otoconia fail to form – is not discussed. Is this a novel finding? Does it suggest that striolar differentiation required for otoconial differentiation? How do otoconia form - central to peripheral? Also, the authors should explain why they rule out migration of post-mitotic cells as an explanation for large areas lacking EdU-positive cells in the Aldh1a3 as knockout. This might be discounted for instance, if numbers of EdU-positive cells did not increase in extrastriolar regions.

Since the paper is describing a mechanistic interaction, it would be helpful to add a schematic model for how RA signaling works to define the size of regions in the vestibular organs. One thing that struck me is the authors do not seem to know which cells types - sensory progenitors, supporting cells, or hair cells - express Cyp26b1, Rdh10, and Aldh1a3 in the macula at different stages. At the least, can they hypothesize which cells types express it from the ISH?

Minor criticisms:

Line 81. I recommend adding "reviewed in" before Eatock and Songer 2011.

Line 148. The wording "..suggest that both RDH10 and ALD1a3…are required" is confusing because double knockouts were not performed. Perhaps reword to "suggest that RDH10 and ALD1a3…are EACH required"?
Ditto for line 159.

Line 156. The wording is confusing here. Perhaps add “we found that otoconia, which were clearly seen in controls,…were absent”.

Line 232. The Sans and Chat paper should be cited here.

Line 237. EdU incorporation indicates that cells divided; it does not, in and of itself, demonstrate that cells underwent terminal division.

Line 248. It would be helpful to add in this sentence that EdU labeling was analyzed at E18.

Lines 576 and 594. The sensory epithelium, not the organ, has a striola or central zone. I suggest rewording to (line 576) “Each vestibular sensory epithelium consists of….” and (line 594) “Bracket represents part of the striola of the utricular and saccular sensory epithelium”.

Line 667: I believe this should be “macular area” not “utricular area”.

First revision

Author response to reviewers' comments

We are delighted to receive all the positive comments on our manuscript! We have now revised the manuscript according to reviewers' suggestions. Our main changes are: 1) combining Suppl figure 1 into Figure 5 as suggested by Reviewer #1, 2) combining the two in vitro results of Figures 6 and 7 into one figure as suggested by Reviewer #2, and 3) add a summary diagram as suggested by Reviewer #3. All the pertinent changes are highlighted in red and our point-by-point responses to the reviewers are listed below:

Reviewer 1
1. lt would be nice if the title could reflect the findings on RA patterning in the second part of the paper.
The title as well as the summary statement have been revised to include the second part of the paper.

2. Defining the brackets used in Figure 2 inside the main text (not just in the legend) would help the explanation of the expression patterns. Also, some of the panels could use additional annotation to point out structures mentioned in the text (e.g. extrastriolar region of utricle and saccule and the peripheral zone of the canal cristae)
Done.

3. Figure 3 could be improved if the authors can provide a two-injection paintfill of the Rdh10 control and a one-injection paintfill of the Aldh1a3 control.
We think the combination of one control with a single injection and one control with double injections is sufficient and an efficient use of space to illustrate our results. Since the NIH is still currently shut down due to COVID-19 pandemic, waiting on re-entry for more injections will delay our resubmission indefinitely.

4. The authors mention that the Aldh1a3 mutants lack otoconia and are poor swimmers. The Rdh10 otoconial phenotype is shown in Figure 4, but there is no mention of swim tests. There is no need to do this test for publication, but if it can't be done because the cKO is lethal after birth, perhaps this could be mentioned somewhere (like in Materials and Methods).
The cKO mutants die shortly after birth. This point has now been added to the Materials and Methods.

5. lt seems as if there is a word or phrase missing from the sentence on line 167. The sentence has been modified.

6. I would like to see the data in Supplementary Figure 1 ihcluded with Figure 5. Suppl Figure 1 has now been incorporated into Fig. 5.

7. The sample numbers for Figure 6 need to be included. Also, it would be helpful to place B' as a full panel with an indication of its time in culture (it currently says "extended").
Sample number has been added to the legend and panel B' has been enlarged to similar size as other panels. However, this is not a sample that was incubated for an extended time in culture but it is the same sample in B that has been incubated for a longer time during the color reaction step of the in situ hybridization. This has now been further clarified in the legend.

8. The sentence in lines 222-225 starting "These results suggest that..." seems convoluted. Maybe there is a simpler way to state the conclusion.
The sentence has been revised.

9. Sample numbers are needed for Figure 8.
Sample number has been added to legends.

Reviewer2
1) This is a relatively short study that appears well suited for a Report format. Results feel stretched in 9 figures, with Fig. 1 only having a schematic and several figures including a single experiment/set of panels. Figures 1 to 4 could be compiled into a new Fig. 1 to show Rdh10 expression and high level phenotypes (fused utricle-saccule, lack of otoconia). Fig. 5 could be a new Fig. 2 (zonal phenotype). Fig. 6-8 could be a new Fig. 4 (cross-regulation). Fig 9 could be a new Fig. 4 (proliferation).
We agree with the reviewer that the current manuscript is between the length of a report and an article. However, we feel Figure 1 will be too dense if we were to combine current Figures 1 to 4 as suggested. Reviewer #3 also suggested that we add a summary figure, which we did. Additionally, given the differences among the experimental paradigms used, we feel the clarity of our manuscript will be compromised if we have to reduce the text by at least 2500 words. Therefore, we prefer to keep our manuscript as an article format. However, we did incorporate the Reviewer's suggestion of combining the two in vitro results of Figures 6 and 7 into one.

2)Line 282: the author discuss the observation that OCM expansion in Rdh10 mutant occurs only medial to the LPR in the utricle, but on both sides of the LPR in the saccule. This is probably simply because the striolar region itself is normally medial to the LPR in the mouse utricle but bisected by the LPR in the saccule (Li/ Peterson 2008, the authors' Jiang et al 2017). "The reason for this regional difference is not clear" (line 282) applies in my mind to the normal, distinct organization of the utricle vs saccule, and not to the way OCM expands, which is rather expected. I find this part of the discussion a little confusing. Why not point out more simply that striolar expansion respects the polarity boundary?
It would be incorrect to say striolar expansion respects the polarity boundary since this only applies to the utricle and not the saccule. One could argue that the difference in 0cm expansion between the utricle and saccule is due to the distinct organization of these two sensory organs, which brings back to the point that we raised about regional differences in patterning. We have modified that part of the Discussion to be more inclusive.

Reviewer 3 Comments for the Author:
Essential revisions:
The anatomical nomenclature used by the authors is confusing at times. Throughout the paper, the terms crista and utricle or saccule are used together, but technically the crista = sensory epithelium, while the utricle/saccule = organ. I suggest using the sensory epithelial names (crista and macula) in sections that address the regional differences in the sensory epithelium and defining the specific terms early on in the paper.
We totally agreed with the reviewer. We were advised to reduce ear terminologies in our previous publication in *Nature Communications* and we did not make a distinction between the sensory tissue (macula) and its organ (utricle and saccule). We have now made the distinction in this revision.

Lines 247-259. This section on EdU labeling is confusing. As the authors explain, the findings in Figure 9C,C' could be explained by 1) cell cycle exit before E14.5 or 2) high rates of cell division between E14.5 and E18 that diluted the EdU. But, the findings could also be explained by migration of cells born on E14.5 from the striolar region, which was not considered.
Extensive cell migration in the developing utricle has not been described in the literature as far as we know. If cells migrate a lot in the developing utricle, we (Jiang et al, eLife 2017) and others (Yang et al, Sci. Rep. 2017) would not have observed the regional differences in hair cell birthdates. Our results in current Fig 8A also supported the previous findings. As for the mutants, we have shown that the total number of Ocm+ cells increased in the *Aldh1a3* KO mutants (Ono et al, 2020) and it is also shown in current Fig. 8G. Cell migration could not have accounted for the increase in Ocm+ HCs.

By giving EdU at an earlier time (E11.5) and labeling for a HC marker, the authors tested 1) if earlier cell division occurred in that region and 2) if new HCs were generated from those divisions. However, it is not clear how this second experiment distinguished between early cell cycle exit and high rates of division. This could have been addressed by EdU pulse/fix at E14.5 for which labeling would have supported a higher rate of cell division.
An EdU pulse/fix experiment at E14.5 would tell us if precursor cells are still dividing in the striola. However, we think a pulse/chase experiment at E14.5 will yield a negative result with little EdU positive cells because previous results (Jiang et al.; Yang et al) indicated that cells in the striolar region start to exit from the cell cycle at E11.5 and cell cycle exit is largely completed by E14.5, which is why the striolar region is devoid of EdU in the control (Fig. 8A). Based on the hypothesis that the lack of RA signaling causes an expansion of the striolar domain, we interpreted that the expanded EdU-negative domain observed in the center of the *Aldh1a3* KO utricle (Fig. 8C) is a gain of striolar fate, i.e. an earlier cell cycle exit than the rest of the utricle. But it could also mean that the larger blank zone in the mutants (Fig. 8C) than controls (Fig. 8A) was caused by cells that are still dividing at E14.5, picked up EdU, but the EdU label was diluted by additional cell division between E14.5 to E18. If this is correct, we would expect earlier EdU injections at E11.5 will show similar EdU labeling as controls. However, we observed many more heavily labeled cells medial to the striola by E18.0 that are also 0cm positive, indicating that these are cells in the extrastriolar region that exited from the cell cycle shortly after EdU administration and turned on striolar characteristics, consistent with our previous results (Ono et al, 2020). We have added words in the Results section to make this clearer.

Furthermore, it is not clear why the higher
number of labeled HCs in the mutant supports the interpretation that cell cycle exit was premature. Why would the rate of HC production be higher earlier on, if mitosis were to halt earlier than normal? Also, this finding could also indicate that the mutant cells experience a fate-switch from SC to HC.
Cells in the striola, either hair cells or supporting cells, exit from cell cycle earlier than the rest of the utricle starting at E11.5. The expanded striolar region in *Aldh1a3* ko mutants led us to hypothesize that more HCs will be undergoing terminal mitosis at that age and our hypothesis panned out, more strongly labeled EdU-positive cells found at E18 when EdU was administered at E11.5.

Finally, this is picky,
but HCs do not exit the cell cycle; they never divide.
Thank you for pointing out the oversight.

Figure 3C,D. Relative to A and B, it's difficult to see the paint fill. Is it possible to increase signal in C,D?
We have increased the contrast of the paint fill shown in C and D. It should be clearer.

Figure 4. Panels B and C look very different from A. It is very hard to compare and to see difference or where otoconia are/are not. Can this figure be altered to make this easier for the reader?
Short of conducting a scanning EM experiment, it is very difficult to illustrate the otoconia phenotype across the entire macula. We have now outlined the surface of the utricular macula, where it is not obscured by tissues from the roof of the utricle. We hope it will be easier to see the difference between the opaqueness of the otoconia in controls and the lack of it in mutants.

Figure 9. The utricle in panel F has an odd shape, so it is difficult to see whether EdU labeling is in the striolar region. Can this be improved? Also, if the authors can clarify the significance of EdU-labeled HCs, could they add a high magnification image of these cells (e.g., between panels F and G)?
To make it easier to see Ocm+ striolar region, separate panels as well as high magnification images were added to demonstrate EdU and 0cm co-labeled HCs in the mutant medial to the striola that are not present in the controls. (current Fig. 8E-E''', F-F''').

Figure 6B. The images are low magnification, so it is difficult to distinguish where the hybridization signal is localized. How tissue-specific was Cyp26b1 upregulation? It looks like the signal extends beyond sensory epithelium. If so, this should be described. One limitation of this analysis is it is non-quantitative and although the authors explain that they carefully controlled the timing of the hybridization reaction, they did so only in the legends. Concerns about the qualitative aspects of this experiment would be better allayed if the timing component were explained in the Results text.

We have expanded panel B' in Fig. 6 into the same size as the rest of the panels in the figure and explained our results in the text.

Figures 5, 7 and Supplementary Figure 1. It is not clear what is meant by % as a measure (% of control, % increase of control, % of a given region or cell type, or other)? This should be clearly described in the Results text and figure legends. E.g., for Figure 7: Does%=% ofutricular macula or% of striolar region?
The % are total number of Ocm+ per all the myosin 7a+ HCs in the entire utricle, saccule or lateral crista. The y-axis has been modified to convey this point better.

Supplementary Fig 1. The β spectrin labeling, as shown at low magnification, is not very useful. Perhaps add an inset so the readers can see where the kinocilium exits each cuticular plate - i.e., the cell planar cell polarity? The authors need to describe the white line (LPR) somewhere. For this figure, when analyzing the regional changes, did they cut it into anatomic sections? If so, they should explain this in the Methods and "box" or otherwise indicate the specific regions they analyzed.
The Suppl Fig.1 has been merged into Fig.5 as requested by Reviewer #1. Nevertheless, we have added a high magnification panel of spectrin staining to show how the line of polarity reversal (LPR) was determined.

Discussion: One interesting finding - that otoconia fail to form - is not discussed. Is this a novel finding? Does it suggest that striolar differentiation required for otoconial differentiation? How do otoconia form - central to peripheral?
The otoconia formation is dependent on the sensory epithelium of the utricle. In the Aldh1a3 knockout, the otoconial phenotype was attributed to the severe reduction of gene expression of otopetrin1, which is mutated in the tilted mice showing similar otoconial phenotype as Aldh1a3 and Rdh10 mutants. We have added this information in the Discussion.

Also, the authors should explain why they rule out migration of post-mitotic cells as an explanation for large areas lacking EdU-positive cells in the Aldh1a3 as knockout. This might be discounted, for instance, if numbers of EdU-positive cells did not increase in extrastriolar regions.
See our explanation about cell migration above. Briefly, the increase in total number of EdU-positive cells detected at E18 when EdU was administered at E11.5 indicate that cell migration alone could not have accounted for the increase in cell number. Secondly, many of these EdU positive cells are also Ocm- positive, indicating they have striolar-like properties.

Since the paper is describing a mechanistic interaction, it would be helpful to add a schematic model for how RA signaling works to define the size of regions in the vestibular organs.
We have now added a schematic model (Fig. 9) to describe how RA signaling works in patterning the vestibular organs.

One thing that struck me is the authors do not seem to know which cells types - sensory progenitors, supporting cells, or hair cells - express Cyp26b1, Rdh10, and Aldh1a3 in the macula at different stages. At the least, can they hypothesize which cells types express it from the ISH?

In our previous paper (Ono et al. 2020), we showed that Aldh1a3 immunoreactivities are located in the supporting cells and are also in the striolar hair cells at birth. Our gene expression results suggest that *Cyp26b1* and *Rdh10* are expressed in the supporting cell layer as well but short of good antibodies, we cannot confirm this notion currently.

Minor criticisms:

Line 81. I recommend adding "reviewed in" before Eatock and Songer 2011.
Wording "Reviewed in" was added.

Line 148. The wording "..suggest that both RDH10 and ALD1a3...are required" is confusing because double knockouts were not performed. Perhaps reword to "suggest that RDH10 and ALD1a3... are EACH required"? Ditto for line 159.
Done.

Line 156. The wording is confusing here. Perhaps add "we found that otoconia, which were clearly seen in controls,...were absent".
Corrected.

Line 232. The Sans and Chat paper should be cited here.
Added.

Line 237. EdU incorporation indicates that cells divided; it does not, in and of itself, demonstrate that cells underwent terminal division.
Done.

Line 248. It would be helpful to add in this sentence that EdU labeling was analyzed at E18.
Done.

Lines 576 and 594. The sensory epithelium, not the organ, has a striola or central zone. I suggest rewording to (line 576) "Each vestibular sensory epithelium consists of...." and (line 594) "Bracket represents part of the striola of the utricular and saccular sensory epithelium".
Done

Line 667: I believe this should be "macular area" not "utricular area".
Done.

We hope you will find our revised manuscript in good order and acceptable for publication. We look forward to hearing from you.

Second decision letter

MS ID#: DEVELOP/2020/192070

MS TITLE: Retinoic acid synthesis and autoregulation mediate zonal patterning of vestibular organs and inner ear morphogenesis

AUTHORS: Kazuya Ono, Lisa Sandell, Paul Trainor, and Doris K. Wu
ARTICLE TYPE: Research Article

I am satisfied with your response to Reviewers 1 and 2 and the revision of the manuscript. Your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 3

Advance summary and potential significance to field

This was stated in my original application.

Comments for the author

The authors did an excellent job responding to my suggestions. I have no further recommendations.