Fgf8 genetic labeling reveals the early specification of vestibular hair cell type in mouse utricle

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MS TITLE: Fgf8 Lineage Tracing Reveals the Early Specification of Vestibular Hair Cell Type in Mouse Utricle

AUTHORS: Evan M Ratzan, Anne M Moon, and Michael R Deans

I have now received the reports of three referees on your manuscript and I 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, all the referees express great interest in your work, but they also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, they comment on the lack of quantification of the data. Referee 2 also requests that you re-analyse cells labelled by FGF8-mcm at an early stage and that you examine their fate at a postnatal stage when type I hair cells can be unambiguously identified. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their 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

Yes, nice paper suitable for publication in Development. Need some revision and quantifications of results though. See below.

Comments for the author

This manuscript describes the generation of an Fgf8-cre mouse (Fgf8-mcm) and this mouse strain was used to conduct an Fgf8-lineage study in the inner ear. The authors showed that Fgf8 lineage cells give rise to Type I hair cells (HCs) in the mouse utricle after terminal mitosis, suggesting that Type I and Type II HCs of the vestibular utricle are of separate lineages and refuted previous hypothesis that the two types of hair cells share a common lineage.

Overall, this is a nice study and the finding that Type I HCs are only found in the Fgf8 lineage is an important result. However, one does not get a good sense of percentages of Type I HCs or lack of the Type II HCs in the Fgf8-lineage as the manuscript currently stands. Using neck to base width ratio to analyze HC morphology at a developing stage is not clear enough. Adding quantifications to the staining results in the mature/postnatal stages would be useful. For example adding quantification and showing tdTomato and Ocm signals as separate panels in Figure 5C would give a better idea of how many striolar Type I HCs are tdTomato-positive. Quantification of the number of osteopontin (extrastriolar Type I marker) and Anxa4 (Type II hair cell marker) that overlap with tdTomato signal to support results in Fig. 6 would also be helpful.

In order to claim that Fgf8 lineage starts after HCs exited from the cell cycle based on the results shown in Fig 3 (Edu administration at E11.5, tamoxifen at E12.5, and analysis at E18.5), it is important to consider the strongly Edu-labeled cells in the striola region only since this is where the newly post-mitotic HCs at E11.5 should reside. Therefore, a low power image should be added to panels in Fig. 3C to the location of the EdU and tdTomato double-labeled cells in the utricle. Additionally, the marked cells in Fig. 3C do not appear to be the most heavily labeled cells in the panel. HCs with moderate level of EdU labeling could be from precursor cells that have undergone more than one round of cell divisions between E11.5 and E18.5 and thus would not support the authors' claim that tdTomato signal occurs after cell cycle exit.

The claim that Fgf8 is restricted to the Type I hair cell lineage also depends on a good correlation between endogenous Fgf8 expression and the reporter activity driven by Fgf8-creER. Comparing between the reporter activity in Fig 2A and Fgf8 gene expression in Fig. 2F and 7A, it seems like the cre reporter activity is broader than the endogenous Fgf8 expression, which is predominantly in the striolar region. These patterns, though could be explained based on different sensitivity of detection, do raise a question of whether the cre reporter activity is a good reporter of the endogenous Fgf8 gene expression. For example, is Fgf8 gene expression first detectable only in the striolar hair cells around E12.5 and is never found in the supporting cell layer? Is there a wave of Fgf8-positive expression in hair cells expanding from the center to the periphery of the utricle over time? Answers to these questions will strength the authors' claim.

Other comments:
1) Fig.8. It was not stated in the legend or Materials and Methods how the lateral and medial margins were determined to calculate the lateral extrastriolar/striolar width ratio? It appears from I and J that there may be a reduction in striolar Ocm staining in Pax2-cre; Fgf8 cko.
2) Line 241, "labeled" misspelled.
3) Add reference to the statement in line 451 regarding regenerated hair cells are of type II identity.

Reviewer 2

Advance summary and potential significance to field

This study addresses the role of FGF8 in inner ear development. The authors have generated a new transgenic mouse line (FGF8-mcm) carrying an inducible form of Cre recombinase downstream of the FGF8 coding sequence. Using this line, they perform lineage-tracing experiments of the FGF8-expressing cells after administration of tamoxifen at different stages of embryonic development. Their findings show that FGF8 is transiently expressed in auditory and vestibular neurons. It is also, importantly, an early marker of vestibular hair cells: a subset of hair cells are already labelled by tamoxifen treatment at E11.5, a very early stage in terms of vestibular hair cell specification. One of the most thought-provoking aspects of the study is the suggestion that FGF8 could represent an early and specific marker of "type I" (as opposed to "type II") vestibular hair cells. As discussed by the authors, this would provide some evidence that these two hair cell subtypes are specified independently (perhaps from distinct precursors) and would make this particular line a very useful tool for inner ear biologists investigating the specific contribution of type I hair cells to vestibular function. I believe, however, that some of the conclusions of the authors regarding the specific association of FGF8 to the type I hair cell lineage are not fully supported by their experiments (see below).
The final sections of the study test directly the function of FGF8 by inactivating its expression in the inner ear using an FGF8 conditional allele and two Cre lines: Atoh1-Cre (for hair cell-specific ablation) and Pax2-Cre (for an earlier and more widespread inactivation). None of the lines exhibit a clear phenotypic defect, leaving unresolved the role of FGF8 in vestibular hair cell differentiation or function.

Overall, the manuscript is very well written and illustrated. The FGF8-mcm line is potentially an excellent addition to the current arsenal of Cre lines available to study gene function in a cell type specific manner in the inner ear. The suggestion that FGF8 expression is associated from early stages of development to the specification of type I hair cells only is very interesting. However, one crucial experiment (see suggestion 1) is missing to strengthen this conclusion and validate further use of this line as a type-1 specific Cre line at early stages of inner ear development.

Comments for the author

1)The evidence that FGF8-mcm expressing cells labelled at E17.5 differentiate predominantly into type I hair cell at P14 is very convincing (presence of calyx; expression of osteopontin; absence of ANXA4 – see Fig 5). It is not so clear to me that those labelled at earlier stages (eg E11.5 =, Fig 3) do so, given that the lineage-traced cells were analysed at E18.5, a stage that does not allow the unambiguous identification of type I hair cells. It is a significant evidence gap, which could limit the use of this transgenic line for other experimental purposes and affect some of the conclusions of the study: it is for example possible that the cells expressing FGF8 before E17.5 form both type I and type II hair cells, which would undermine the conclusion of the authors regarding the mechanisms of specification of these two hair cells types (Discussion p15 "Together these observations suggest that vestibular hair cell identity is defined at the earliest stages of their development …". One way to resolve this issue would be to label FGF8-expressing cells at E11.5-E12.5 and examine their progeny at P14, when type I hair cells are well differentiated.

2)Section starting p11, line 297-p12, line 304. One of important criteria advanced by the authors to decide on hair cell subtype identity at E18.5 is the "neck to base (NBR) ratio". They refer to a 1998 study by Rusch et al. in a way that suggests that such NBR ratio have been previously measured in type I and type II hair cells and at the same developmental stage. Having looked at this study in some detail, I could not find any specific reference to such measurements (observation seem qualitative: presence or not of a constricted "neck"). Furthermore, one of the conclusions of Rusch et al. (page 7496, end of Results section) is that "cell shape was not a reliable marker (of cell identity) at early stages… the distinctive flask shape of mature type I cells was more frequently seen beginning at P7". Unless I have missed something, the Rusch et al. study should not be cited in support of the NBR analysis and its results at E18.5. In fact, the NBR results should be interpreted with caution given the conclusions of the Rusch et al. study.

Reviewer 3

Advance summary and potential significance to field

This study from the laboratory of Michael Deans describes a new Fgf8 reporter mouse and then uses that mouse to examine the expression of Fgf8 in the vestibular system of the inner ear. The images are very nice and do a good job of illustrating the author's conclusions. However, in contrast with the images, quantification is almost non-existent and the primary conclusion of the study, that Fgf8 is a marker for Type I hair cells, while probably true, is forced in several places in the study with limited evidence to support the conclusion. Finally, the overall depth of the study is limited. The images are very nice, but the experiments can be summarized as an Fgf8 fate mapping study in the vestibular system and the conclusion that deletion of Fgf8 does not lead to phenotypes in the vestibular system.

Comments for the author

The following specific issues should also be addressed:

The title suggests that Fgf8 plays a role in the specification of Type I hair cells, but there is not evidence for this, especially considering that deletion of Fgf8 does not lead to a change in Type I hair cell development.

Expression of a gene in a particular cell type does not automatically equate to importance in the development of that cell type.

Line 51: "engulfs" is a bit excessive, the surface of the hair cell is not surrounded by the calyx

Line 61, the discrepancies between these studies are not as stark as presented. McInturff et al was a fate mapping study and so noted when Type I hair cells are generated under normal conditions but did not look at the developmental potential of these cells. The Sox2 deletion studies demonstrate that cell fate is not set until considerably later. Therefore the two studies are not contradictory as they examine different developmental issues.

Line numbers were cut off preventing using them for comments from this point on and the pages are not numbered either.

Results, first paragraph. This is largely a recapitulation of the methods section and not necessary here.

It would be nice to use qPCR in a few tissues to confirm that there is in fact no change in the level of expression of Fgf8. It's clear that there is no phenotypic change in known phenotypes, but a quantitative approach would be more accurate.

Line 141 maybe, "abeled" should be "labeled".

Line 144 maybe: it's stated that increasing the dosage does not impact the patterns of expression, but was the number of cells labeled impacted?

Line 153 maybe: isn't this more accurately "fate mapping" than "lineage tracing"? as virtually all the labeled cells are post-mitotic at the time of labeling?

Line 155 maybe: in looking at figure 3A, there appear to be as many labeled cells that are OCM negative as are OCM positive. It seems wrong to state that labeled cells are predominantly in the striola. Especially without any quantification.

Line 157 maybe: it's unclear what the point of the BrDU studies was? How does this result add to the conclusions of the study?

Line 181 maybe: as decribed below, the evidence that Fgf8 "selectively" labels Type I hair cells is weak.

Line 188 maybe, it's not clear how the NFH labeling indicated can be indicative of a calyx as opposed to two individual bouton branches.

Line 188 maybe: while the results shown in 3B do appear to show a bias towards a lower NBR in Fgf8+ cells, it would be nice to see the actual values that were used to generate the T-test results. Moreover, there are clearly Fgf8+ cells with NBRs that are more consistent with Type II hair cells (NBR greater than 0.5). so again, suggesting selective labeling of Type I's is not appropriate.

Figure 5C, there are many cells that are Fgf8+ but OCM negative, again suggesting that they are not Type I hair cells, at least not at this developmental stage.

Line 220 maybe: The introduction to this paragraph about postnatal lineage tracing doesn't make any sense. Any hair cell specific gene expressed embryonically is not preferentially labeling Type I hair cells, it would simply label more type I hair cells because those are the cells that are predominantly generated embryonically.

Figure 6: it would be very helpful to include some ortho views as well as some quantification of these results. While the images show predominant calyceal labeling, the plane of confocal section is very low in the tissue such that any type II hair cells might not even be visible. Similarly, in panel 6C there does appear to be at least one cell that is Fgf8+ but not Osteopontin positive.

First revision

Author response to reviewers' comments

Editors comment

As you will see, all the referees express great interest in your work, but they also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, they comment on the lack of quantification of the data. Referee 2 also requests that you re-analyse cells labelled by FGF8-mcm at an early stage and that you examine their fate at a postnatal stage when type I hair cells can be unambiguously identified. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their major concerns. Please also note that *Development* will normally permit only one round of major revision.

Thank you for considering our manuscript. We have integrated or responded to each Reviewer's comments and made substantial improvements to the manuscript based upon these recommendations. Specifically, we have included quantification in the primary figures to support our experimental observations and have included a summary of the primary data used for quantification as supplemental figures. We have also completed the experiment recommended by Reviewer 2 in which hair cells labeled during early embryogenesis are evaluated at postnatal day 14 when the defining feature of a type I hair cell, the synaptic calyx, has formed. We have also updated the figures, in particular with regards to color formatting, to be in line with *Development* guides. These are strong additions and I believe you will find that our resubmission is significantly improved by the changes. Individual responses to the Reviewer's specific comments can be found below.

Reviewer 1 Comments for the Author:

One does not get a good sense of percentages of Type I HCs or lack of the Type II HCs in the Fgf8-lineage as the manuscript currently stands. Using neck to base width ratio to analyze HC morphology at a developing stage is not clear enough. Adding quantifications to the staining results in the mature/postnatal stages would be useful.

We have added quantification including the percentage of Type-I or Type-II cells marked by Ffg8^{mcm} to support the conclusions drawn from immunofluorescent micrographs. This includes the frequency

with which labeled hair cells are contacted by a calyx when tamoxifen is delivered at different developmental stages (Fig.6), the frequency of colocalization with Type-I and Type-II molecular markers (Fig.7), and a quantification of hair cells expressing Type-I markers in CKOs and controls (Fig.8). The raw data used for these quantification figures are presented as supplemental data (Figs. S2-S4).

In order to claim that Fgf8 lineage starts after HCs exited from the cell cycle based on the results shown in Fig 3 (Edu administration at E11.5, tamoxifen at E12.5, and analysis at E18.5), it is important to consider the strongly Edu-labeled cells in the striola region only since this is where the newly post-mitotic HCs at E11.5 should reside. Therefore, a low power image should be added to panels in Fig. 3C to the location of the EdU and tdTomato double-labeled cells in the utricle.

We have made several changes to strengthen the EdU birthdating experiments and ensure that the evaluation is focused upon newly post-mitotic hair cells. This includes a low-magnification image showing the location of EdU and tdTomato double-labeled cells in the striolar region of the utricle (Fig.3C).

Additionally, the marked cells in Fig. 3C do not appear to be the most heavily labeled cells in the panel. HCs with moderate level of EdU labeling could be from precursor cells that have undergone more than one round of cell divisions between E11.5 and E18.5 and thus would not support the authors' claim that tdTomato signal occurs after cell cycle exit.

The EdU delivery protocol was also improved, resulting in more robust labeling of post-mitotic hair cells (Fig.3D,E). We find that heavily EdU labeled cells, in the striolar region, contain the tdTomato reporter.

The claim that Fgf8 is restricted to the Type I hair cell lineage also depends on a good correlation between endogenous Fgf8 expression and the reporter activity driven by Fgf8-creER. Comparing between the reporter activity in Fig 2A and Fgf8 gene expression in Fig. 2F and 7A, it seems like the cre reporter activity is broader than the endogenous Fgf8 expression, which is predominantly in the striolar region. These patterns, though could be explained based on different sensitivity of detection, do raise a question of whether the cre reporter activity is a good reporter of the endogenous Fgf8 gene expression.

We agree with the interpretation that the CreER genetic labeling approach provides significantly greater sensitivity that can be achieved using wholemount ISH protocols. This is likely due to the highly-efficient recombination that occurs at the Rosa26 Ai9 tdTomato allele which requires low levels of CreER activity to activate the tdTomato reporter, a reporter that is also amplified by immunofluorescent detection. Thus, we expect permanent genetic labeling to reveal a broader spectrum of Fgf8 expression than can be identified using wholemount ISH. With this in mind it is striking that Fgf8 genetic-labeling remains restricted to a subset of hair cells and does not label supporting cells in either the vestibule or the cochlea. It is worth noting that, although it is less sensitive, the ISH is qualitative similar and shows a 'salt and pepper' pattern of labeling that is also consistent with Fgf8 expression in a subset of hair cells. More importantly, the genetic labeling results reveal that a transcriptional program is initiated in Type-I hair cells shortly after mitosis that distinguishes them from Type-II hair cells born concurrently or during later stages of development.

For example, is Fgf8 gene expression first detectable only in the striolar hair cells around E12.5 and is never found in the supporting cell layer? Is there a wave of Fgf8-positive expression in hair cells expanding from the center to the periphery of the utricle over time? Answers to these questions will strengthen the authors' claim.

We have done this experiment using the Fgf8 ^{mcm} mice. The delivery of tamoxifen at different days embryonic development results a shallow wave of labeling that initiates in the striolar region following injections at E11.5 or E12.5 and subsequently includes hair cells in the periphery following injections at later embryonic stages. We have added a developmental time course that illustrates this progression, however due to space constraints we have elected to present this data as a supplemental figure.

Other comments:

1) Fig.8. It was not stated in the legend or Materials and Methods how the lateral and medial margins were determined to calculate the lateral extrastriolar/striolar width ratio? It appears from I and J that there may be a reduction in striolar Ocm staining in Pax2-cre; Fgf8 cko.

This has been clarified in the methods section. We have also separated the Oncomodulin labeling (Fig.8C,D) and BII-Spectrin (Fig.8G,H) labeling of hair cells into separate panels because when combined the distribution of Oncomodulin-labeled cells is obscured. There is no reduction in the

number of Oncomodulin-positive hair cells in the *Pax2*-Cre; *Fgf8* CKO utricle and we have added quantification data to support this finding (Fig.8J).
2) Line 241, "labeled" misspelled.
corrected

3) Add reference to the statement in line 451 regarding regenerated hair cells are of type II identity.
corrected

Reviewer 2 Comments for the Author:

1) The evidence that FGF8-mcm expressing cells labelled at E17.5 differentiate predominantly into type I hair cell at P14 is very convincing (presence of calyx; expression of osteopontin; absence of ANXA4 - see Fig 5). It is not so clear to me that those labelled at earlier stages (eg E11.5 =, Fig 3) do so, given that the lineage-traced cells were analysed at E18.5, a stage that does not allow the unambiguous identification of type I hair cells. It is a significant evidence gap, which could limit the use of this transgenic line for other experimental purposes and affect some of the conclusions of the study: it is for example possible that the cells expressing FGF8 before E17.5 form both type I and type II hair cells, which would undermine the conclusion of the authors regarding the mechanisms of specification of these two hair cells types (Discussion p15 "Together these observations suggest that vestibular hair cell identity is defined at the earliest stages of their development …". One way to resolve this issue would be to label FGF8-expressing cells at E11.5-E12.5 and examine their progeny at P14, when type I hair cells are well differentiated.

We agree with the importance of this experiment and have included it in Fig.6 along with quantification of the frequency by which tdTomato-positive hair cells are contacted by calyceal synapses so that the reader may evaluate the specificity of labeling for different ages of tamoxifen administration. It should be noted that for these experiments a lower dose of Tamoxifen was required to prevent miscarriage of the litter and that at E11.5 there are fewer *Fgf8*-mcm expressing cells available for labeling. As a result, only a limited number of tdTomato expressing hair cells were available for analysis.

2) Section starting p11, line 297-p12, line 304. One of important criteria advanced by the authors to decide on hair cell subtype identity at E18.5 is the "neck to base (NBR) ratio". They refer to a 1998 study by Rusch et al. in a way that suggests that such NBR ratio have been previously measured in type I and type II hair cells and at the same developmental stage. Having looked at this study in some detail, I could not find any specific reference to such measurements (observation seem qualitative: presence or not of a constricted "neck"). Furthermore, one of the conclusions of Rusch et al. (page 7496, end of Results section) is that "cell shape was not a reliable marker (of cell identity) at early stages… the distinctive flask shape of mature type I cells was more frequently seen beginning at P7". Unless I have missed something, the Rusch et al. study should not be cited in support of the NBR analysis and its results at E18.5. In fact, the NBR results should be interpreted with caution given the conclusions of the Rusch et al. study.

We would like to the thank the reviewer for bringing this important oversight to our attention and have determined that it would be best to remove this morphometric quantification from the manuscript. Neck to Base Ratio (NBR) measurements were employed twice and have been replaced by other methods or approaches. (1) NBR was used to evaluate hair cell identity at E18.5 following tamoxifen induction at E11.5. This is no longer required now that these cells are being evaluated at P14 using calyx formation as a cell type marker. (2) NBR was to evaluated Fgf9 CKOs, and these analyses have been replaced by quantification of molecular markers.

Reviewer 3 Comments for the Author:

The title suggests that Fgf8 plays a role in the specification of Type I hair cells, but there is not evidence for this, especially considering that deletion of Fgf8 does not lead to a change in Type I hair cell development.
We respectfully disagree since the subject of the title is '*Fgf8 genetic labeling*' and not '*Fgf8*'. Nonetheless, we appreciate this concern and have changed the abstract to state "*..analyses of Fgf8*

conditional knockout mice did not reveal developmental phenotypes" so that we do not mistakenly communicate a functional role for Fgf8 in this process.

Expression of a gene in a particular cell type does not automatically equate to importance in the development of that cell type.

We agree and note that this is consistent with our experimental outcomes though our approach does not rule out the potential for functional redundancy between Fgf ligands.

Line 51: "engulfs" is a bit excessive, the surface of the hair cell is not surrounded by the calyx

We have tempered the description as requested.

Line 61, the discrepancies between these studies are not as stark as presented. McInturff et al was a fate mapping study and so noted when Type I hair cells are generated under normal conditions but did not look at the developmental potential of these cells. The Sox2 deletion studies demonstrate that cell fate is not set until considerably later. Therefore, the two studies are not contradictory as they examine different developmental issues.

We have tempered the paragraph so that these hypotheses are not presented as stark alternatives but rather mechanisms which may overlap in ways not currently appreciated. Specific text includes the following, "*The commonalities and differences between these alternative etiologies have been difficult to resolve …*"

Results, first paragraph. This is largely a recapitulation of the methods section and not necessary here.

This section describes engineering strategy that was employed to ensure that the expression of MCM matched that of endogenous Fgf8 and therefore addresses a concern raised by Reviewer 1. Results presented in this section also demonstrate that the genetic modification does not impact endogenous Fgf8 expression nor result in haploinsufficiency or mutant phenotypes that are well known for Fgf8 outside of the inner ear.

It would be nice to use qPCR in a few tissues to confirm that there is in fact no change in the level of expression of Fgf8. It's clear that there is no phenotypic change in known phenotypes, but a quantitative approach would be more accurate.

We appreciate the logic and there may be subtle decrements in *Fgf8* transcript levels due to insertion of the IRES MCM cassette as is observed for this strategy however, since none of the phenotypes that result from small decreases in Fgf8 are observed, any difference in transcript levels would be minor and not functionally relevant. We have revised the text to acknowledge this possibility "*Since even a modest decrease in Fgf8 function has phenotypic consequences in many systems, these results indicate that the presence of the IRES:mER:Cre:mER insertion into the 3'UTR of Fgf8 does not significantly affect gene function. "*

Line 141 maybe, "abeled" should be "labeled".

corrected

Line 144 maybe: it's stated that increasing the dosage does not impact the patterns of expression, but was the number of cells labeled impacted?

The number of cells labeled is dose dependent and increased tamoxifen labeled more cells however this does not impact the type of hair cell labeled. The 'pattern of expression' is constant and appears to label a subset of cells in a salt and pepper pattern that is similar between genetic labeling and Fgf8 ISH.

Line 153 maybe: isn't this more accurately "fate mapping" than "lineage tracing"? as virtually all the labeled cells are post-mitotic at the time of labeling?

We have consulted with colleagues and experts in developmental biology and have concluded that neither term accurately describes the experimental technique and how it has been applied. The more general term 'genetic labeling' will be used instead. This change has been in the title and throughout the manuscript.

Line 155 maybe: in looking at figure 3A, there appear to be as many labeled cells that are OCM negative as are OCM positive. It seems wrong to state that labeled cells are predominantly in the striola. Especially without any quantification.

corrected

Line 157 maybe: it's unclear what the point of the BrDU studies was? How does this result add to the conclusions of the study?
This set of experiments demonstrates that the Fgf8 promoter is active in hair cells shortly after terminal mitoses and, since Fgf8 CreER activity is restricted to type I hair cells, tells us that hair cell identity is emerging at this stage.

Line 181 maybe: as decribed below, the evidence that Fgf8 "selectively" labels Type I hair cells is weak.
This concern has been addressed in our responses to Reviewers 1&2. "Selective" labeling of Type I hair cells is supported by additional quantification (Rev1) and the P14 analysis of hair cells labeled at E11.5 (Rev2).

Line 188 maybe, it's not clear how the NFH labeling indicated can be indicative of a calyx as opposed to two individual bouton branches.
Presuming that this concern is leveled at Fig.5A, the concern of distinguishing afferent synapse types at this age using a general neuronal marker is appreciated. For this reason we were careful not to call these neuronal structures calyxes and instead say "..*appeared to be nascent calyceal synapses*" which reflects our interpretation but invites the reader to do the same.

Line 188 maybe: while the results shown in 3B do appear to show a bias towards a lower NBR in Fgf8+ cells, it would be nice to see the actual values that were used to generate the T-test results. Moreover, there are clearly Fgf8+ cells with NBRs that are more consistent with Type II hair cells (NBR greater than 0.5). so again, suggesting selective labeling of Type I's is not appropriate.
Unfortunately, cell shape is not a reliable marker of cell identity, particularly at embryonic stages. As result we have removed NBR as an analyses criterion. See response to Reviewer 2. The concern about sharing actual values is appreciated and raw data has been provided as supplemental figures.

Figure 5C, there are many cells that are Fgf8+ but OCM negative, again suggesting that they are not Type I hair cells, at least not at this developmental stage.
Type I hair cells can be subdivided into at least two subsets; (1) those that express Oncomodulin (OCM), are contacted by a complex calyx and are found in the striolar region and (2) those that do not express OCM but express Osteopontin instead, and are contacted by a simple calyx. Therefore, the presence of tdTomato in OCM-negative cells does not suggest that they are not Type I hair cells nor does it reveal that they are Type II hari cells. Our subsequent experiments (fig.7) show genetic labeling of hair cells that express the type I marker Osteopontin and do not express the type II markers Calb2 and Anxa4.

Line 220 maybe: The introduction to this paragraph about postnatal lineage tracing doesn't make any sense. Any hair cell specific gene expressed embryonically is not preferentially labeling Type I hair cells, it would
simply label more type I hair cells because those are the cells that are predominantly generated embryonically.
The text in this paragraph has been edited and improved to clarify the rationale for postnatal induction experiments.

Figure 6: it would be very helpful to include some ortho views as well as some quantification of these results. While the images show predominant calyceal labeling, the plane of confocal section is very low in the tissue
such that any type II hair cells might not even be visible. Similarly, in panel 6C there does appear to be at least one cell that is Fgf8+ but not Osteopontin positive.
As suggested by reviewers 1&3 we have quantified these experimental results.

Second decision letter

MS ID#: DEVELOP/2020/192849

MS TITLE: Fgf8 Genetic Labeling Reveals the Early Specification of Vestibular Hair Cell Type in Mouse Utricle

AUTHORS: Evan M Ratzan, Anne M Moon, and Michael R Deans
ARTICLE TYPE: Research Article

I am delighted 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

The revised manuscript is acceptable for publication.

Comments for the author

The revised manuscript is acceptable for publication.

Reviewer 2

Advance summary and potential significance to field

The authors have performed the early genetic labelling experiment I suggested in my initial review. There is a low number of cells analyzed, but these show a consistent trend towards the type I subtype, strenghtening the original conclusion of the authors. I recognize the technical issue with administration of Tamoxifen at early embryonic stages and congratulate the authors for performing these experiments at these unusual times.
My other comment (NBR analysis) has been addressed adequately.

Comments for the author

The authors have performed the early genetic labelling experiment I suggested in my initial review. There is a low number of cells analyzed, but these show a consistent trend towards the type I subtype, strenghtening the original conclusion of the authors. I recognize the technical issue with administration of Tamoxifen at early embryonic stages and congratulate the authors for performing these experiments at these unusual times.
My other comment (NBR analysis) has been addressed adequately.

Reviewer 3

Advance summary and potential significance to field

The authors very nicely demonstrate that virtually all cells that express Fgf8 develop as Type I vestibular hair cells. They use this observation to examine the timing of Type I vestibular hair cell specification, demonstrating that Type I hair cells are generated embryonically and during the early post-natal period.

Comments for the author

The authors have done a nice job of addressing most of the specific comments in this manuscript. However, based on this line in the Development review criteria "paper should make a significant and novel contribution to our understanding of developmental mechanisms" I believe that this

manuscript is not suitable for publication in development. The study fails to provide any mechanistic insight into vestibular development. The suggestion that there is functional redundancy with other Fgfs is possible, but equally possible is the fact that Fgfs are not required for Type I hair cell development.

Minor issues

Line numbers are cut off on the pdf and there are no page numbers making it hard to indicate positions of comments

Mouse strains and husbandry
Line ending in 7: there is a typo, two “not” in same sentence

Results
Line ending in 9: typo “Genetic genetic labeling”

Results indicating labeling of some auditory and vestibular neurons in Figure 4
- was this pattern of expression confirmed by in situ?