Ontogenesis of the tear drainage system requires Prickle1-driven polarized basement membrane deposition

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MS TITLE: Ontogenesis of the tear drainage system requires Prickle 1-driven polarized basement membrane (BM) deposition

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

As you will see, the referees express considerable interest in your work, but have some 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 may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

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

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Dianlei Guo et al. report experiments seeking to define the temporal and cellular parameters governing development of the tear duct drainage system in mid-gestation mouse embryos. Despite numerous in-depth investigations into the genetic pathways and morphogenetic processes that generate mid-face structures during embryonic/fetal development, knowledge remains scant on when, where and how the tear drainage system forms in mice and humans. To rectify this deficiency, and also determine whether the mouse provides a suitable model for understanding congenital blockage of tear ducts in human, the authors performed 3D-reconstructions of immuno-stained primordial tear ducts (PTD) at different gestational days from E11-to-E16.5. In addition, the manuscript includes a set of experiments that pursue the authors' in-situ hybridization data revealing PTD-restricted expression of Prickle 1, a WNT/PCP pathway gene. Using 3D reconstructions of serially sectioned PTDs from Prickle 1 heterozygous <+/-> and homozygous <-/-> embryos on E11-E14, the authors find that the growth/elongation of the PTD requires Prickle 1. To assess whether loss of Prickle 1 affects cell polarity-related behaviors, the authors examined basement membrane formation in the PTD of mutant embryos and in embryoid bodies derived from prickle 1<-/-> iPS cells.
Formation of the primordial tear duct occurs concomitantly with upper lip primary palate and nose morphogenesis; additionally both sets of structures arise at an interface between the nasal and maxillary processes. Numerous publications investigating the origins of cleft lip/palate (CL/P) have documented a requirement for WNT signaling in the development of mid-face structures (lip, palate, nose). Yet, with a focus on CL/P, these studies largely neglected morphogenesis of the primordial tear duct. Thus the significance of this manuscript lies in the knowledge it will contribute on the development of the tear drainage system, an understudied facial structure with clinical relevance to congenital birth defects and diseases afflicting the human eye.
However, as detailed in the specific comments below, deficiencies in the design and execution of the experiments, as well as in the presentation and interpretation of the data, argue against considering this submission for publication. The authors' studies overlook fundamentally important questions about the time and site at which tear duct formation initiates and about the cell population that contributes to the growing duct. Inadequate controls and insufficient characterization of the visceral endoderm layer generated in the iPS-embryoid body system raise concerns about the conclusions the authors draw regarding the role of Prickle 1 in basement membrane deposition during PTD morphogenesis.

Comments for the author

1 - Reviewing the authors' data presented in Figures 1-3 proved exceedingly cumbersome as the introduction did not clearly define existing knowledge and experimentally unanswered questions about the formation of the tear duct drainage system in mouse.
1(i) - Particularly helpful would be a description of the mature tear duct drainage system in mouse, with a diagram illustrating the location and structure of its component parts.
1(ii) – In addition, the introduction should place the development of the primordial tear duct (PTD) in the temporal and spatial context of concurrent morphogenetic processes generating the lip, palate and nose structures of the mid-face. To aid visualization of the problem investigated in this manuscript the authors should consider including of a 3D figure pointing out the predicted position the epithelial junction giving rise to the PTD relative to that of the lambdoidal junction, the site at which three-epithelia [medial nasal (mnp), lateral nasal (lnp) and maxillary process (mxp)] fuse during formation of the lip, palate and nose.
2 – In the experiments presented in Figure 1, the authors sought to define the developmental time at which PTD formation initiates. Yet, the PTD is already morphologically detectable at the earliest stage examined, E11. Development of the facial primordia begins ~ E9.5; thus the authors need to incorporate E9.5-E10.5 embryos into their analyses to identify the last stage before the PTD first visibly emerges.
3 – Is there experimental evidence that the PTD emerges from fusing maxillary and nasal plate ectoderm? On page 5 the authors comment that in Fig 1A the "tear duct was seen to initiate from the epithelial junction of fusing maxillary and nasal plate ectoderm" On page 14 in the Discussion the authors comment that mouse tear ducts "are from joining surface ectoderm of maxillary and

nasal plates. Fusion of the two is a zipping process from frontal/nasal to orbital/rare, leaving an orbital epithelial notch for PTD outgrowth". No references are cited supporting these statements on epithelial fusion.
Moreover, the authors do not cite Ferretti, E et al.2011 which found that epithelial fusion at the lambdoidal junction proceeds by a distinctly different mechanism requiring apoptosis.
4 –The format of the imaging data in Figure 1 precludes an unambiguous interpretation.
4(i) The legend to Figure 1A, as well as the methods section, indicate that the authors performed the 3D-reconstructions on p63-stained E11 heads. The main text, however, states that the E11 heads were stained with E-cadherin. Please clarify.
4(ii) To orient the reader in the 3D reconstructions in Fig 1A, the authors should include a 3D model (with frontal and lateral views) showing the relative placement of the medial nasal process (mnp), lateral nasal process (lnp), maxillary process (mxp) and mandibular process (mdp) at E11. To aid in the interpretation of the panels in Fig 1A, indicate whether the images show the lateral or medial nasal process; also label the images to clearly distinguish the epithelium of the nasal process from that of the maxillary process. Do the images show the right or left eye?
4(iii) One cannot unambiguously decipher the histological sections shown in Figure 1 B-D, F-H, J-L, N-P, R-T and V-X. Each of the six rows should include a panel diagraming the angle and level of the histological section on a 3D model of the facial primordia; the current 2D drawings are not sufficiently informative. In addition, each E-cadherin stained epithelial structure should be labeled and identified. As for Fig 1A, the images should depict the boundaries/limits of the mnp, lnp and mxp. Similarly the authors should add a 3D model diagraming the level and angle at which the histological section cuts through the facial primordia for the following: Figure 2(A-C), 2(D-E), 2(F-H), 2(I-J), 2(K-M), 2(N-O), 2(P-R) & 2(S-U); Figure S1(A-C), S1(D-F) & S1(G-I); and Figure S2(A-D). All visible structures and epithelia should be labeled on the image.
4(iv) Fig 1 (B-D) While this section shows an elongating PTD, one cannot conclude from the image that the PTD emerges from fusing nasal and maxillary process epithelia. As commented above (2 & 3) the authors need to examine sections from earlier stages (E9.5 -E10.5) that depict clearly defined nasal and maxillary process epithelia before appearance of the PTD and just upon emergence. Panels C & D do not distinguish the nasal process and maxillary process epithelia from the fused epithelium. Based on a single section, cut at an unknown angle and level, one cannot determine whether the PTD initiates as a bud growing out of an epithelium or as an epithelial invagination.
4(v) – The authors provide no evidence in support of the statement on page 5 that formation of an epithelial cord "was likely achieved partially through epithelial-mesenchymal transition (EMT), which generated a cell mass with multipolar protrusions observed on sections (Fig.1B-E, F-I)". Assessment of cell shape will require higher magnification images that depict individual cells stained for nuclear & membrane markers.
4(vi) – Deciphering the data in Figure 1(J-X) proved quite difficult, and unnecessarily so. Without any background information on the progression of development from the primordial tear duct to the fully formed tear drainage system, the authors start discussing PTD target tissues and introducing new nomenclature (NLD, LCL, UCL). It would be most helpful if the diagram suggested above in 1(i) would align the epithelia of the facial primordia, as well as structural features of the developing tear duct, with the location of their derivatives in the different components of the mature tear duct drainage system.
5 – Figure 3 presents a cogent and informative comparison of tear duct development between E11 and E14 in wild-type and Prickle1 <b/b> mutants. Yet it also raises a number of questions about the morphogenetic processes involved in PTD formation that the authors do not directly address.
5(i) - Do cells emerging from the epithelium to form the PTD express Prickle 1?
If yes, do epithelial cells at or adjacent to the site of PTD emergence express Prickle 1? Do these cells have apical-basal polarity?
5(ii) - How do the authors define the stalk/duct versus the tip of the PTD? Do they think both cell types co-emerge from the epithelium or that one gives rise to the other? f
(iii) - Does detachment of the stalk from the epithelium occur before coincident with or after branching to generate the UCL and LCL?
6 – The question about how the authors define the stalk/duct versus the tip also bears on the data presented in Figures 4 and S3 comparing the patterns of expression for cell adhesion and basement membrane markers in wild type and Prickle 1 mutants. In Figure 4 and S3, the authors compare the mutant PTD to the stalk region of wild-type PTD. Yet, it is not clear that these are analogous regions. What is known about the mechanisms of PTD growth and elongation?

6(i) - Does cell proliferation drive extension of the duct? Where are these proliferating cells located; in the tip, stalk/duct or throughout the PTD?
6(ii) - When does the lumen form in the duct?
Figure 3 suggests that the PTD in prickle 1 mutants consists predominantly of "tip" cells and that it does not generate a duct. Can the authors rule out the possibility that in wild-type cell proliferation in the tip drives elongation by producing cells that undergo shape changes to incorporate into the duct? Such a model suggests that the focus of the studies in Figures 4 and S3 should be on a comparison of the mutant PTD with the tip of the wild type PTD. Would such a comparison lead to different conclusions about the role of Prickle 1 and BM deposition?
7 – The selection of embryoid body generated visceral endoderm as a model for elucidating Prickle 1 function in the PTD raises some concerns.
7(i) – What is the rationale for using VE, other than it is a polarized epithelium? Why do the authors believe that a WNT/PCP gene has a requisite function in VE?
7(ii) - The authors should examine the expression of known VE genes to characterize the outer layer generated on the Prickle 1<+/b> and Prickle 1<b/b>
embryoid bodies (including apically expressed proteins such as Cubilin, Amn, & Lrp2, as well as Gata4, Gata6, Afp). The variability of Prickle 1-driven GFP expression in the Prickle 1<b/b> embryoid bodies raises the possibility that the loss of Prickle 1 affects the differentiation of VE from iPS cells.
7(iii) – The authors used the lentivirus rtTA/TRE system to generate the iPS cells; thus the iPS cells used in the rescue experiments carry the TetO-FUW-OSKM. Did the authors perform the control to ask whether the OSKM cassette is activated when they add doxycycline to induce Prickle 1?

Reviewer 2

Advance summary and potential significance to field

Dry eye is a major problem worldwide, yet few studies have investigated cell and developmental biology of tear duct formation, especially using mouse genetics.

This interesting study reports a detailed time course of tear duct development in mouse and a significant requirement of PCP Prickle 1 in tear duct elongation by regulating polarized secretion and deposition of basement membrane (BM). Mutant EBs with loss of prickle1 show BM phenotype that could be rescued by prickle 1 expression. These results suggest distinct functions of prickle 1 in BM formation and establishment of cell polarity.

Comments for the author

The data are clean, extensive and nicely presented. Conclusions are justified.
The manuscript is easy to follow. I have a few comments/suggestions

1. The distinction between tear duct and nasolacrimal duct is confusing and should be clearly mentioned in the introduction.
2. The authors mentioned the role of FGF signaling in diseases affecting nasolacrimal duct. They should look at a beautiful paper published in Development (by late David Beebe's lab, Chen et al. 141, 2691, 2014) that shows activation of Sox9 by FGF in regulating lacrimal gland differentiation. It would be good to explore possible relationship of PCP/Prickle 1 to Sox9 in future studies. This should be included in discussion. See papers on Prickle 1 in limb growth.
3. Sentence construction and grammar can be improved at places. One example:
Second sentence in Intro: 'Disturbing' should be 'Disrupting'
4. The EB work shows the role of Prickle 1 in BM formation, supporting the mouse tear duct defects in the mutant. This link can be clarified better.
5. Discussion is somewhat incoherent and can be tightened.

First revision

Author response to reviewers' comments

Reviewer 1 Advance summary and potential significance to field
Dianlei Guo et al. report experiments seeking to define the temporal and cellular parameters governing development of the tear duct drainage system in mid-gestation mouse embryos. Despite numerous in-depth investigations into the genetic pathways and morphogenetic processes that generate mid-face structures during embryonic/fetal development, knowledge remains scant on when, where and how the tear drainage system forms in mice and humans. To rectify this deficiency, and also determine whether the mouse provides a suitable model for understanding congenital blockage of tear ducts in human, the authors performed 3D-reconstructions of immuno-stained primordial tear ducts (PTD) at different gestational days from E11-to-E16.5. In addition, the manuscript includes a set of experiments that pursue the authors' in-situ hybridization data revealing PTD-restricted expression of Prickle 1, a WNT/PCP pathway gene. Using 3D reconstructions of serially sectioned PTDs from Prickle 1 heterozygous <+/-> and homozygous <-/-> embryos on E11-E14, the authors find that the growth/elongation of the PTD requires Prickle 1. To assess whether loss of Prickle 1 affects cell polarity-related behaviors, the authors examined basement membrane formation in the PTD of mutant embryos and in embryoid bodies derived from prickle 1<-/-> iPS cells. Formation of the primordial tear duct occurs concomitantly with upper lip, primary palate and nose morphogenesis; additionally both sets of structures arise at an interface between the nasal and maxillary processes. Numerous publications investigating the origins of cleft lip/palate (CL/P) have documented a requirement for WNT signaling in the development of mid-face structures (lip, palate, nose). Yet, with a focus on CL/P, these studies largely neglected morphogenesis of the primordial tear duct. Thus the significance of this manuscript lies in the knowledge it will contribute on the development of the tear drainage system, an understudied facial structure with clinical relevance to congenital birth defects and diseases afflicting the human eye.
However, as detailed in the specific comments below, deficiencies in the design and execution of the experiments, as well as in the presentation and interpretation of the data, argue against considering this submission for publication. The authors' studies overlook fundamentally important questions about the time and site at which tear duct formation initiates and about the cell population that contributes to the growing duct. Inadequate controls and insufficient characterization of the visceral endoderm layer generated in the iPS-embryoid body system raise concerns about the conclusions the authors draw regarding the role of Prickle 1 in basement membrane deposition during PTD morphogenesis.

Response:
We thank the reviewer for the favorable comments and insightful suggestions about our manuscript. We revised the manuscript accordingly, placing the current work in the context of mid-face development, and address the reviewer's concerns point by point below.

Reviewer 1 Comments for the author
1 - Reviewing the authors' data presented in Figures 1-3 proved exceedingly cumbersome as the introduction did not clearly define existing knowledge and experimentally unanswered questions about the formation of the tear duct drainage system in mouse.
1(i) - Particularly helpful would be a description of the mature tear duct drainage system in mouse, with a diagram illustrating the location and structure of its component parts.

Response:
We thank the reviewer for the advice. A mature tear duct system has yet to be illustrated in mouse. We performed new experiments constructing a tear duct at P1 (new Figure 1), when tear drainage has started (data not shown). Diagrams were added illustrating path and arrangement of the drainage system with reference marks of some facial anatomical structures. We provided additional 3D movies (Movie S1, S2) to aid visualization. The new data description is highlighted in the Result section.

1(ii) – In addition, the introduction should place the development of the primordial tear duct (PTD) in the temporal and spatial context of concurrent morphogenetic processes generating the lip, palate and nose structures of the mid-face. To aid visualization of the problem investigated in this manuscript, the authors should consider including of a 3D figure pointing out the predicted position

the epithelial junction giving rise to the PTD relative to that of the lambdoidal junction, the site at which three-epithelia [medial nasal (mnp), lateral nasal (lnp) and maxillary process (mxp)] fuse during formation of the lip, palate and nose.

Response:
We thank the reviewer for the great suggestions. We revised the introduction according to these suggestions, placing tear duct development in the context of midface development (highlighted in "Introduction", paragraph 3). We added diagrams illustrating where the mxp-lnp epithelial junction gives rise to the PTD relative to lambdoidal junction. Based on our understanding of the literature (de la Cuadra-Blanco et al., 2006), we adopted the terms "NLG (nasolacrimal groove)" for describing mxp-lnp junction, and "LL (lacrimal lamina)" used in humans for describing the thickening NLG, which we believe would facilitate to locate PTD in this manuscript. We also created a 3-D image of E11 embryonic face in Attached Figure 1E (see last page) to show relative locations of PTD, lambdoidal junction, LL, NLG, mnp, and lnp.

2 – In the experiments presented in Figure 1, the authors sought to define the developmental time at which PTD formation initiates. Yet, the PTD is already morphologically detectable at the earliest stage examined, E11. Development of the facial primordia begins ~ E9.5; thus the authors need to incorporate E9.5-E10.5 embryos into their analyses to identify the last stage before the PTD first visibly emerges.

Response:
According to the reviewer's suggestion, we added new data of E10.25 and E10.5 embryos showing 3D reconstructed images (New Figure 3) and serial horizontal sections (new Figure 4). According to these data, PTD is recognizable ~E10.25.

3 – Is there experimental evidence that the PTD emerges from fusing maxillary and nasal plate ectoderm? On page 5 the authors comment that in Fig 1A the "tear duct was seen to initiate from the epithelial junction of fusing maxillary and nasal plate ectoderm" On page 14 in the Discussion the authors comment that mouse tear ducts "are from joining surface ectoderm of maxillary and nasal plates. Fusion of the two is a zipping process from frontal/nasal to orbital/rare, leaving an orbital epithelial notch for PTD outgrowth". No references are cited supporting these statements on epithelial fusion. Moreover, the authors do not cite Ferretti, E et al.2011 which found that epithelial fusion at the lambdoidal junction proceeds by a distinctly different mechanism requiring apoptosis.

Response:
We thank the reviewer for the helpful comments. we acknowledge that the statements regarding "fusion" in the manuscripts are inaccurate due to lack of direct experimental supporting data. To avoid making confusions, we omitted these statements in this revision. Nevertheless, the apoptosis at the junction of lnp and mxp of the conjunctiva suggests that there is probably fusion occuring (New Supplemtal Fig S1C), which we wrote in the text as well in this revision. We apologized that we missed the beautiful work by Ferretti, E et al., 2011 and cited it now in places where demand.

4 –The format of the imaging data in Figure 1 precludes an unambiguous interpretation.
4(i) The legend to Figure 1A, as well as the methods section, indicate that the authors performed the 3D-reconstructions on p63-stained E11 heads. The main text, however, states that the E11 heads were stained with E-cadherin. Please clarify.

Response:
We used both E-cadherin and p63 staining for 3D reconstructions. We initially used the E-cadherin staining (new Figure 3B-F and Figure 6), later wanted to develop high-quality imaging using DISCO tissue clearance (Renier, N. et al. 2014). We found that that E-cadherin antibody does not survive this method in our hand, but p63 does (new Figure 3G-S). Regardless, information obtained from both methods sufficiently supports the major points in this manuscript.

4(ii) To orient the reader in the 3D reconstructions in Fig 1A, the authors should include a 3D model (with frontal and lateral views) showing the relative placement of the medial nasal process (mnp), lateral nasal process (lnp), maxillary process (mxp) and mandibular process (mdp) at E11. To aid in the interpretation of the panels in Fig 1A, indicate whether the images show the lateral or medial

nasal process; also label the images to clearly distinguish the epithelium of the nasal process from that of the maxillary process. Do the images show the right or left eye?

Response:
We thank the reviewer for this suggestion. Now Figure 1A becomes new Figure 3G-I with modifications. We added digrams to indicate mxp, mnp and lnp as well as lambdoidal junction and NLG. The images were all taken from a left eye.

4(iii) One cannot unambiguously decipher the histological sections shown in Figure 1 B-D, F-H, J-L, N-P, R-T and V-X. Each of the six rows should include a panel diagraming the angle and level of the histological section on a 3D model of the facial primordia; the current 2D drawings are not sufficiently informative. In addition, each E-cadherin stained epithelial structure should be labeled and identified. As for Fig 1A, the images should depict the boundaries/limits of the mnp, lnp and mxp. Similarly the authors should add a 3D model diagraming the level and angle at which the histological section cuts through the facial primordia for the following: Figure 2(A-C), 2(D-E), 2(F-H), 2(I-J), 2(K-M), 2(N-O), 2(P-R) & 2 (S-U); Figure S1(A-C), S1(D-F) & S1(G-I); and Figure S2(A-D). All visible structures and epithelia should be labeled on the image.

Response:
We thank the reviewer for the helpful suggestions. Figure 1 B-D, F-H, J-L, N-P, R-T and V-X now become as part of new Figure 2 now. We added drawings of the facial primordia and gave the cutting planes for each stage. We labeled all epithelial structures on the images. We did the same for original Figure 2 (now is Figure 5 in this revision) and Figure S1(now Figure S2) and Figure S2 (now Figure S3).

4(iv) Fig 1 (B-D) While this section shows an elongating PTD, one cannot conclude from the image that the PTD emerges from fusing nasal and maxillary process epithelia. As commented above (2 & 3) the authors need to examine sections from earlier stages (E9.5 -E10.5) that depict clearly defined nasal and maxillary process epithelia before appearance of the PTD and just upon emergence. Panels C & D do not distinguish the nasal process and maxillary process epitheli¬a from the fused epithelium. Based on a single section, cut at an unknown angle and level, one cannot determine whether the PTD initiates as a bud growing out of an epithelium or as an epithelial invagination.

Response:
Figure 1 (B-D) becomes part of new Figure 2 now. As mentioned previously, we omitted statements on “fusion” of nasal and maxillary processes, which is unclear at this point. As suggested by the reviewer, we looked into more details of PTD initiation, and added data from serial horizontal sections of E10.25 and E10.5 (new Figure 3A-F & Figure 4). From these images, PTD appear to initiate from the orbital LL/NLG formed by mxp and lnp, especially the cells of mxp epithelium that expresses Prickle 1 (new Figure 4). However, we can not exclude that lnp of the LL also contribute to PTD.

4(v) – The authors provide no evidence in support of the statement on page 5, that formation of an epithelial cord “was likely achieved partially through epithelial-mesenchymal transition (EMT), which generated a cell mass with multipolar protrusions observed on sections (Fig.1B-E, F-I)”. Assessment of cell shape will require higher magnification images that depict individual cells stained for nuclear & membrane markers.

Response:
We acknowledge that EMT is a speculation. The reduced E-cadherin staining of the PTD cells now provided in new Figure 4 and Supplemental Figure 1A hints at an EMT. We changed “cell mass with multipolar protrusions” to “multipolar-shape cell mass” to avoid confusion. To clarify, by shape, we are referring a group of cells.

4(vi) – Deciphering the data in Figure 1(J-X) proved quite difficult, and unnecessarily so. Without any background information on the progression of development from the primordial tear duct to the fully formed tear drainage system, the authors start discussing PTD target tissues and introducing new nomenclature (NLD, LCL, UCL). It would be most helpful if the diagram suggested above in 1(i) would align the epithelia of the facial primordia, as well as structural features of the

developing tear duct, with the location of their derivatives in the different components of the mature tear duct drainage system.

Response:
Figure 1J-X becomes a part of new Figure 2 now. As addressed in the comment 1(i), we added new data of 3D reconstruction of a fully formed tear duct at P1, showing structral features and locations of tear duct derivatives (new Figure 1). In trying to keep uniform nomenclature, we changed the terms LCL, UCL to ILC (inferior lacrimal canaliculus) and SLC(superior lacrimal canaliculus) respectively, which describe canaliculi in humans.

5 – Figure 3 presents a cogent and informative comparison of tear duct development between E11 and E14 in wild-type and Prickle1 mutants. Yet it also raises a number of questions about the morphogenetic processes involved in PTD formation that the authors do not directly address.
5(i) - Do cells emerging from the epithelium to form the PTD express Prickle 1? If yes, do epithelial cells at or adjacent to the site of PTD emergence, express Prickle 1? Do these cells have apical-basal polarity?

Response:
As shown in the new Figure 4, the emerging PTD at the beginning does not, or only wealy expresses Prickle 1 at E10.25. In contrast, strong Prickle 1 expression is detected in mxp epithelium at E10.5 continuous with the outgrowing PTD, which shows weaker but distinct Prickle 1 expression. We have not looked at apical-basal polarity, but probably the initiating PTD does not have it from its appearance (Figure 4).

5(ii) - How do the authors define the stalk/duct versus the tip of the PTD? Do they think both cell types co-emerge from the epithelium or that one gives rise to the other? f

Response:
The stalk versus tip of PTD were defined partly by shape and partly by geographic location of its connection with the conjunctiva. We do not have genetic evidence the two parts are of different cell types. From multiple sections, we believe the stalk and PTD are all part of the LL (lacrimal lamina)---As PTD cells growing out of the LL at the edge, the LL base depressed in dorsal-ventral direction and narrowed in lateral to medial direction to form a stalk connecting with conjunctiva, and eventually detached from it due to apoptosis at the joining point of lnp and mxp of the conjunctiva (Supplemental Fig. S1C).¬

(iii) - Does detachment of the stalk from the epithelium occur before, coincident with or after branching to generate the UCL and LCL?

Response:
The complete stalk detachment occurs between E11.5~E12 (Supplemental Figure. S1A, B, Figure 6D). The branching appears to occur around E11.5 with two separate cell mass at the PTD lateral extreme, predicted to be future SLC and ILC (Supplemental Fig. S1B). Thus, stalk detachment might overlap with the branching process.

6 – The question about how the authors define the stalk/duct versus the tip also bears on the data presented in Figures 4 and S3 comparing the patterns of expression for cell adhesion and basement membrane markers in wild type and Prickle 1 mutants. In Figure 4 and S3, the authors compare the mutant PTD to the stalk region of wild-type PTD. Yet, it is not clear that these are analogous regions. What is known about the mechanisms of PTD growth and elongation?

Response:
Figure 4 and S3 become new Fig. 7 and S4, respectively. The molecular differences in different regions of PDT is not known. To our best knowledge, little is known about the mechanisms of PTD growth and elongation as stated in introduction and abstract.

6(i) - Does cell proliferation drive extension of the duct? Where are these proliferating cells located; in the tip, stalk/duct or throughout the PTD?

Response:
It is very possible that cell devision drives PTD tubule extension. Currently we are not able to do time-lapse imaging of dividing cells specifically in elongating PTD, a highly embeded structure in embryonic head. It appears that proliferation occurs uniformly throughout PTD (attached Figure 1A, B1-3).

6(ii) - When does the lumen form in the duct? Figure 3 suggests that the PTD in prickle 1 mutants consists predominantly of “tip” cells and that it does not generate a duct. Can the authors rule out the possibility that in wild-type cell proliferation in the tip drives elongation by producing cells that undergo shape changes to incorporate into the duct? Such a model suggests that the focus of the studies in Figures 4 and S3 should be on a comparison of the mutant PTD with the tip of the wild type PTD. Would such a comparison lead to different conclusions about the role of Prickle 1 and BM deposition?

Response:
Figure 3 becomes Figure 6 now. The lumen/calvitation is not uniformly throughout the tube, but the lumen surface may appear trailing the tip advancement. In fact, the mutant PTD does generate local discontinous lumens (attached Figure 1C, D), and the the outer layer cells are organized as more like a tube wall. The molecular property of the tip cells is currently unknown. We do not intend to rule out possiblity that the tip cells drive elongation by newly divided cells regaining polarity to incorporate into duct. Indeed, we proposed that Prickle 1 -regulated polarized BM deposition might fasciliate regaining polarity allowing new cells to incorporate into and stay within the duct.

The comparasion of the mutant PTD with the tip of the wild-type PTD would not offer extra information regarding the role of Prickle 1 in BM deposition. The BM is less deposited in normal PTD tip than rest of the tube (Figure S5), similarly as in the mutants. Such comparision would not differentiate the possibilities whether Prickle 1 has no role in tip BM deposition (and even such, does not exclude its BM regulation in rest of the tube), or the tip local enviroment promotes BM degradation (for tubule advancing) disguising Prickle 1’s role in tip BM but not in other regions. The universal Prickle 1 expression throughout the whole PTD hints at the later possibility for the tip BM deposition.

7 – The selection of embryoid body generated visceral endoderm as a model for elucidating Prickle 1 function in the PTD raises some concerns.
7(i) – What is the rationale for using VE, other than it is a polarized epithelium? Why do the authors believe that a WNT/PCP gene has a requisite function in VE?

Response:
We apologize that we did not make it clear why we used EBs to study BM. A study (Tao et al., 2009) reported that Prickle 1 is required for epiblast apicobasal polarity in ECM deposition during early embryogenesis. We reasoned that the mechanism of Prickle 1-regulated BM deposition in PTD might be similar as that of the early embryogenesis, which the vitro EB system could be conveniently used to obtain further mechanistic insights. We added relevant statements of the rationale for using EBs to the result section and revised the abstract accordingly. Our study found that the VE instead of epiblast highly expresses Prickle 1, and underwent polarity changes in Prickle 1 mutant EBs.

7(ii) - The authors should examine the expression of known VE genes to characterize the outer layer generated on the Prickle 1<+/b> and Prickle 1 embryoid bodies (including apically expressed proteins such as Cubilin, Amn, & Lrp2, as well as Gata4, Gata6, Afp).The variability of Prickle 1-driven GFP expression in the Prickle 1 embryoid bodies raises the possibility that the loss of Prickle 1 affects the differentiation of VE from iPS cells.

Response:
Limited by our lab resources and the impact of current Covid-19, we were only able to perform Gata4 immunostaining among the list of VE markers suggested by the reviewer. Representative data was presented in Supplemental Fig7G-N, showing most outer layer cells expressing Gata4 and GFP. We hope the reviewer agree that VE fate issue is beyond the scope of the current manuscript. Additonally, the EB system has been successfully used in many previous BM studies (Li et al., 2002, 2003; Miner and Yurchenco, 2004; Xu et al., 2007; Liu et al., 2008; He et al., 2010; Li et al., 2016).

The differentiation efficiency of EBs is evaluated by appearance of the outer layer cells expressing Prickle 1 (Figure 9B), and now validated by Gata4 expression for VE (Supplemental Fig. 7H, L). We agree that loss of Prickle 1 may affect differentiation, however, that would be rather secondary and compounded with general imperfections of the in vitro system. The polarity modules have not been reported involving fate changes directly.

7(iii) – The authors used the lentivirus rtTA/TRE system to generate the iPS cells; thus the iPS cells used in the rescue experiments carry the TetO-FUWOSKM. Did the authors perform the control to ask whether the OSKM cassette is activated when they add doxycycline to induce Prickle 1?

Response:
We agree that reactivation of OSKM may have some impacts on EB differentiation. Nonetheless, the efficiency of EB formation/differentiation within our experimenting windows is sufficient to serve our purposes. We thank the reviewer for the great point, which should be carefully considered for future experiments.

Reviewer 2- Advance summary and potential significance to field
Dry eye is a major problem worldwide, yet few studies have investigated cell and developmental biology of tear duct formation, especially using mouse genetics.This interesting study reports a detailed time course of tear duct development in mouse and a significant requirement of PCP Prickle 1 in tear duct elongation by regulating polarized secretion and deposition of basement membrane (BM). Mutant EBs with loss of prickle1 show BM phenotype that could be rescued by prickle 1 expression. These results suggest distinct functions of prickle 1 in BM formation and establishment of cell polarity.

Reviewer 2 Comments for the author
The data are clean, extensive and nicely presented. Conclusions are justified. The manuscript is easy to follow. I have a few comments/suggestions:

Response:
We thank the reviewer for these encouraging comments and endorsement of our work.

1.The distinction between tear duct and nasolacrimal duct is confusing and should be clearly mentioned in the introduction.

Response:
We apologize for making such confusion. We now clarify in first paragraph of the introduction: “The excretory lacrimal system, or tear duct (TD) in general, consisting of lacrimal canaliculi (LC) and nasolacrimal duct (NLD) canals…..”

2.The authors mentioned the role of FGF signaling in diseases affectingnasolacrimal duct. They should look at a beautiful paper published in Development (by late David Beebe’s lab, Chen et al. 141, 2691, 2014) that shows activation of Sox9 by FGF in regulating lacrimal gland differentiation. It would be good to explore possible relationship of PCP/Prickle 1 to Sox9 in future studies. This should be included in discussion. See papers on Prickle 1 in limb growth.

Response:
We thank the reviewer for the insightful suggestions on investigation of potential interactions between PCP/Prickle 1 and Fgf signaling. This is certainly very interesting to be looked into in future. We cited Beebe’s work on ocular glands and the Prickle 1 limb works, adding to discussion section: “ Additionally, downstream of Prickle 1, Fgf/Sox9 appears to play a role in limb growth (Yang et al., 2013). Fgf/Sox9 is also crucial for development of the ocular glands (Chen et al., 2014). It is likely that Prickle 1-regulated PTD growth also involves Fgf signaling, which is of interest for future investigation”

3.Sentence construction and grammar can be improved at places. One example: Second sentence in Intro: ‘Disturbing’ should be ‘Disrupting’

Response:
We thank the reviewer for this suggestion. We have carefully examined the wordings and grammars to improve the clarity.

4.The EB work shows the role of Prickle 1 in BM formation, supporting the mouse tear duct defects in the mutant. This link can be clarified better.

Response:
We thank the reviewer's suggestion. We have revised part of the abstract and the result section, adding the rationales why we used EB to study PTD--- A study (Tao et al., 2009) reported that Prickle 1 is required for epiblast apicobasal polarity in ECM deposition during early embryogenesis. We reasoned that the mechanism of Prickle 1-regulated BM deposition in PTD might be similar as that of the early embryogenesis, which the vitro EB system would be conveniently used to obtain further mechanistic insights.

5.Discussion is somewhat incoherent and can be tightened.

Response:
We rewrote many parts of the discussion incorporating the reviewers' suggestions, and it is more coherent now.

Second decision letter

MS ID#: DEVELOP/2020/191726

MS TITLE: Ontogenesis of the tear drainage system requires Prickle 1-driven polarized basement membrane (BM) deposition

AUTHORS: Dianlei Guo, Jiali Ru, Fuxiang Mao, Kaili Wu, Hong Ouyang, Yizhi Liu, and Chunqiao Liu

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

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. As rightly pointed out by Reviewer 1 the inclusion of the embryoid body data seems superfluous to the study, and indeed distracting, as the data are not necessarily helpful. I would recommend that you remove this somewhat problematic data and rather focus exclusively on the in vivo data. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

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

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing

how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

To respond to the reviewers' concerns, the authors incorporated a number of substantive revisions into their manuscript. These include new figures and movies reporting results from recent additional experiments; new sections of text in the introduction and results providing important background information; and schematic diagrams of the developing midface to guide interpretation of imaging data. Through these extensive changes the authors have successfully met a key challenge posed by the dearth of experimental studies on tear duct formation: effective communication of their findings to an audience of developmental biologists who lack background knowledge of tear duct anatomy and of the intriguing, unexplored aspects of its development.

The new experiments and revised presentation of data tackled head-on a major deficiency in the original manuscript, namely an inadequate investigation of fundamentally important questions about the time and site at which tear duct formation initiates. The additional 3D reconstruction and imaging studies carefully and convincingly document the location of the emerging progenitor tear duct at the earlier developmental stage of E10.25 - E10.5. Importantly the authors' findings, as well as their clarification of terminology, provide information crucial for defining experimental models of tear duct development that will empower future efforts to understand the etiology of congenital birth defects and diseases afflicting the human eye.

However, this manuscript is overly long. It reports 7 lines of experimentation: (a)-defining the anatomy of the postnatal day 1 mouse tear duct; (b)-determining the timing of crucial events during tear duct development; (c)-establishing the site of PTD formation; (d)-documenting the expression of Prickle1 during the emergence and elongation of the PTD; (e) -demonstrating a requisite role for Prickle 1 in tear duct outgrowth; (f) -examining cell adhesion, BM formation, and organization of the cytoskeletal and vesicular systems in Prickle1 deficient tear ducts; and (g)-investigating a connection between polarity and BM deposition in control and Prickle1 mutant iPSC derived Embryoid Bodies. As discussed below, a number of issues confound the studies using iPSC-derived embryoid bodies. While the incorporation of an in vitro system is an important complementary approach for any tissue/organ system, in the present case, the embryo analyses are sufficient to support the major conclusions of the manuscript. Thus this reviewer recommends eliminating the sections on control and Prickle1 mutant iPSC derived Embryoid Bodies.

Comments for the author

Major Comments
1 - Although the authors significantly improved their analysis and discussion of the origin the progenitor tear duct, a few ambiguities remain.
Clarification will further strengthen their seminal study on tear duct development.
1(i) Page 7: The authors' response to the reviewers included a very helpful statement: "Based on our understanding of the literature (de la Cuadra- Blanco et al., 2006), we adopted the terms "NLG (nasolacrimal groove)" for describing mxp-lnp junction, and "LL (lacrimal lamina)" used in humans for describing the thickening NLG, which we believe would facilitate to locate PTD in this manuscript." The section of results titled "The timing of crucial events for tear duct development" should begin with a similar statement to clarify that the terms, NLG and LL, have not been previously used in the mouse and that the authors used their knowledge of human anatomy to guide the search for the mouse PTD.
1 (ii) For readers lacking specific expertise in craniofacial morphogenesis the authors should clearly define conjunctiva relative to the epithelial layer of the maxillary and nasal processes. Both derive from surface ectoderm, but what are the temporal and spatial relationships of these two tissues? Do the conjunctiva develop from mxp and/or np epithelia? When the conjunctiva appear as morphologically distinct layers, are they still considered components of the mxp and/or np

epithelia? Knowing the temporal and spatial relationship of these two structures will help clarify the authors conclusions regarding differences between mouse and human in the origin of the PTD.
1 (iii) Continuing the confusion over conjunctiva versus mxp epithelium, the Discussion states on page 18, “the orbital ingress of LL [is] continuous with the conjunctiva outgrow[ing] PTD” and then on page 19, “Additionally, the mouse PTD appears to start with cells emigrate from mxp epithelium expressing Prickle 1”.
1 (iv) The examination of Prickle1 expression at E10.25 and E10.5, 18-12 hours before the earliest time point included in the previous submission, yielded new data in Figure 4 supporting a role for Prickle1 in tear duct development.
However the lack of defined criteria for labelling epithelial structures as mxp, lnp, cj, or NLG further compounds the confusion over the proposed tissue layer giving rise to the PTD. For an accurate interpretation of their findings, the authors should label all E-cadherin expressing epithelial structures depicted in the images in Fig 4. For example, the images in panels (G, H, I) and (P, Q, R) show the PTD emerging adjacent to an apparent juxtaposition of cj and mxp epithelia; yet this region is ambiguously labeled as “cj”. Since 2D sections can be misleading regarding the arrangement of adjacent epithelia, the authors should discuss these observations in context of their 3D reconstructions at E10.25 and E10.5.
2 (i) To validate the iPSC-EB system for studies on Prickle1 function, the authors must account for the discrepancy in phenotype between the embryos homozygous for the Prickle1<tm1Nue> allele used by Tao H et al (2009) and those homozygous for the Prickle1 **allele used by the authors. The former arrest before gastrulation, whereas the latter survive to P2. Both alleles inactivate exon2 and are purported to be functional nulls. Have the authors examined the mutant phenotype after crossing the Pric**kle1<b> allele onto different genetic backgrounds? The absence of wide, background-dependent variability in the Prickle1<b/b> embryonic phenotype will raise questions about the structure of the targeted allele. For example, did rearrangements (deletions, duplications)
occurring during targeting go undetected because they fall outside the region assayed by the genotyping protocol?
2 (ii) Based on the methods section, the authors generated the Prickle1 control and <b/b> iPSCs from the same colony of mice that generated the wild type and mutant embryos used for their studies on tear duct development. The embryonic viability of the Prickle1<b> allele argues that on this background, Prickle1 does not play an obligate role in the formation of the epiblast and visceral endoderm epithelial layers. If the iPSC-EB assay accurately recapitulates epithelialization events in the early mouse embryo, then one would expect to observe normal differentiation of the Prickle<b/b> iPS-EBs. This undermines the authors rationale for applying the EB system.
2 (iii) The authors observe Prickle1 expression in the outer endoderm layer of the EBs, but not in the inner epiblast-like cells. Yet both Tao H et al. and Crompton LA et al. (2007) detect Prickle1 transcripts exclusively in the epiblast.
Comments I, ii, iii combined suggest that iPSCs achieve efficient-EB differentiation in vitro by exploiting and adapting available genetic pathways. Thus one cannot conclude that Prickle1’s functions during EB differentiation recapitulate its role during tear duct development.
3 - As the manuscript is replete with errors in grammar and word choice, the authors should seek the assistance of an editor well versed in written English.
Minor Comments 4 – Figure 1: 3D-reconstruction of P1 tear duct from p63 stained images. Placing current panels I & J as the first two panels will enhance the impact of this figure by facilitating interpretation of the 2D images and 3D reconstructions.
5 – Page 7: Should “and” be used in place of “or” in the next to the last sentence. “Both conjunctival end of the stalk [or] the PTD cell mass underwent apoptosis (Supplemental Fig. 1C, D)”
6 – Figure 2: Define nm, nictitating membrane, a structure referred to in panels Q-X.
7 – Figure 2: What is the canthus?
8 - Supplemental Figure 1E: The figure or legend should include a color code indicating which cell populations/structures the pink, blue, yellow and white shading represent. The current labeling adds confusion. The LL arrow points to the white layer at E10.5 but to yellow cells at E11; the “cj” arrow points to pink cells at E10.5 but to the white layer at E11 and then light pink at E11.5 9 – Movies S4-S7: Adding unnecessary confusion, the left-right orientation of movies 4 & 5 is flipped relative to that of the images in panels H-L of Figure 3. Similarly, the left-right orientation of movies 6 & 7 is flipped relative to that of the images in panels N-O and Q-S of Figure 3.
10 - Figure 4: The orientation and cutting region depicted by the red dashed lines do not align with the structures present in the serial sections rendering this figure unnecessarily confusing. The authors state that B-H and K-R depict “Horizontal serial sections cutting through NLG region from

dorsal to ventral". However the arrow showing the direction of sectioning runs anterior to posterior, not dorsal to ventral. In addition, horizontal sections cut between the two red dashed lines appear inconsistent with the eye becoming more prominent in each successive section. Please clarify.
11 – Figure 5: Addition of schematic diagrams of lateral and frontal views of the developing face greatly facilitated interpreting the images. However, the E11.5 frontal schematic shows the incorrect location for the λ junction. To further clarify the authors' conclusions on Prickle1 expression in the elongating tear duct, the authors should identify and label the E-cadherin labeled epithelia (conjunctiva, mxp, lnp).

Reviewer 2

Advance summary and potential significance to field

This manuscript reports the role of Prickle 1 in polarized basement membrane formation during tear duct development and provides fundamental molecular insights into a poorly-studied, though clinically important, system.

Comments for the author

The authors have made extensive revisions in response to the reviews and included additional data/figs. I am satisfied with the response.

Second revision

Author response to reviewers' comments

Italic: Reviewer's comments
Arial: Response

Reviewer 1 Advance Summary and Potential Significance to Field:
To respond to the reviewers' concerns, the authors incorporated a number of substantive revisions into their manuscript. These include new figures and movies reporting results from recent additional experiments; new sections of text in the introduction and results providing important background information; and schematic diagrams of the developing midface to guide interpretation of imaging data. Through these extensive changes the authors have successfully met a key challenge posed by the dearth of experimental studies on tear duct formation: effective communication of their findings to an audience of developmental biologists who lack background knowledge of tear duct anatomy and of the intriguing, unexplored aspects of its development.
The new experiments and revised presentation of data tackled head-on a major deficiency in the original manuscript, namely an inadequate investigation of fundamentally important questions about the time and site at which tear duct formation initiates. The additional 3D reconstruction and imaging studies carefully and convincingly document the location of the emerging progenitor tear duct at the earlier developmental stage of E10.25 - E10.5. Importantly, the authors' findings, as well as their clarification of terminology, provide information crucial for defining experimental models of tear duct development that will empower future efforts to understand the etiology of congenital birth defects and diseases afflicting the human eye.
However, this manuscript is overly long. It reports 7 lines of experimentation:
(a)-defining the anatomy of the postnatal day 1 mouse tearduct;
(b)-determining the timing of crucial events during tear duct development;
(c)-establishing the site of PTD formation;
(d)-documenting the expression of Prickle1 during the emergence and elongation of the PTD;
(e)-demonstrating a requisite role for Prickle 1 in tear duct outgrowth;
(f)-examining cell adhesion, BM formation, and organization of the cytoskeletal and vesicular systems in Prickle1 deficient tear ducts; and (g)-investigating a connection between polarity and

BM deposition in control and Prickle1 mutant iPSC derived Embryoid Bodies. As discussed below, a number of issues confound the studies using iPSC-derived embryoid bodies. While the incorporation of an in vitro system is an important complementary approach for any tissue/organ system, in the present case, the embryo analyses are sufficient to support the major conclusions of the manuscript. Thus this reviewer recommends eliminating the sections on control and Prickle1 mutant iPSC derived Embryoid Bodies.

We thank the reviewer for the encouraging comments on our revised manuscript. We further revised the manuscript according to the reviewer's suggestions. We hope this revision will satisfy the reviewer.

Reviewer 1 Comments for the Author:

Major Comments
1 - Although the authors significantly improved their analysis and discussion of the origin the progenitor tear duct, a few ambiguities remain. Clarification will further strengthen their seminal study on tear duct development.
1(i) Page 7: The authors' response to the reviewers included a very helpful statement: "Based on our understanding of the literature (de la Cuadra- Blanco et al., 2006), we adopted the terms "NLG (nasolacrimal groove)" for describing mxp-lnp junction, and "LL (lacrimal lamina)" used in humans for describing the thickening NLG, which we believe would facilitate to locate PTD in this manuscript." The section of results titled "The timing of crucial events forb tear duct development" should begin with a similar statement to clarify that the terms, NLG and LL, have not been previously used in the mouse and that the authors used their knowledge of human anatomy to guide the search for the mouse PTD.

We thank the reviewer for this suggestion. We revised the text accordingly.

1 (ii) For readers lacking specific expertise in craniofacial morphogenesis, the authors should clearly define conjunctiva relative to the epithelial layer of the maxillary and nasal processes. Both derive from surface ectoderm, but what are the temporal and spatial relationships of these two tissues? Do the conjunctiva develop from mxp and/or np epithelia? When the conjunctiva appear as morphologically distinct layers, are they still considered components of the mxp and/or np epithelia? Knowing the temporal and spatial relationship of these two structures will help clarify the authors conclusions regarding differences between mouse and human in the origin of the PTD.

These are great questions. The spatiotemporal relationship between the conjunctiva epithelium and mxp/np is largely defined by the embryonic facial features, mostly in humans. Molecular territories have yet to be characterized, which may involve lineage tracing and finding a new set of early markers. The conjunctiva consists of two parts—palpebral (inner surface of the eyelid) and bulbar (eyeball) conjunctiva. The two are contiguous and fold at conjunctiva fornix. In human embryos at approximately 6 weeks of gestation, the frontonasal process extends as the upper eyelid fold, whereas the maxillary extends as the lower eyelid fold (Sevel, Eye 1988, 2, 123-129; Tawfik et al., Ophthal Plast Reconstr Surg 2016;32:407–414). Thus, it is intuitive to think that palpebral conjunctiva originates from the mxp/np epithelia. In mice, the relationship between the palpebral conjunctiva/eyelid and the facial processes has not been described within the same research contexts. However, similarities in the topographical arrangement of the facial processes between humans and mice, particularly the apparent folding of mxp/lnp over the eyeball surface in both species predict that the mouse palpebral conjunctiva/eyelid, like that of humans, also originates from the mxp/lnp. The bulbar conjunctiva is generally believed from the periocular surface ectoderm surrounding the corneal epithelium.

Recognizable conjunctiva fold (distinct layers referred by the reviewer) is observed between E10~11 in mouse (e.g. from this study) with depression/invagination/denting of periocular surface ectoderm and formation of the eyelid fold from the mxp/np. Eyelid specification is reported even early at E9 with Foxl2 defining the dorsal and ventral lid mesenchyme (Huang et al., Development 2009, 136(10): 1741–1750; Swindell, Dev. Biol. 2008, 322(1): 56-64). Nonetheless, distinct keratin markers for conjunctiva, cornea, and eyelid epithelia only start to express at the late embryonic stage around E14.5 (Zhang et al., Jpn J Ophthalmol, 2005). There have not been any studies on

when conjunctiva should or should not be considered as components of the mxp or np epithelia from either field of ophthalmology or developmental biology.

A direct comparison of PTD origins between mice and humans proved to be difficult, since the most detailed study of the human PTD development (de la Cuadra-Blanco et al., 2006) did not clearly address PTD location and its developmental process, either on serial tissue sections or by 3D reconstruction.

We hope the above explanations would further clarify the spatiotemporal relationship between the conjunctiva and the mxp/lnp. We revised the figures and discussion section accordingly to reflect these points.

1 (iii) Continuing the confusion over conjunctiva versus mxp epithelium, the Discussion states on page 18, "the orbital ingress of LL [is] continuous with the conjunctiva outgrow[ing] PTD" and then on page 19, ""Additionally, the mouse PTD appears to start with cells emigrate from mxp epithelium expressing Prickle 1".

We apologize for these confusing sentences. We have revised the above sentences as follows: "PTD outgrows from orbital invagination of the LL, which is contiguous with the presumptive palpebral conjunctiva of the eyelid folds that are formed by mxp and lnp epithelia"; and "PTD progenitors appear to be those of *Prickle 1*-expressing mxp epithelial cells, which constitute and later grow out from the LL".

1 (iv) The examination of Prickle1 expression at E10.25 and E10.5, 18-12 hours before the earliest time point included in the previous submission, yielded new data in Figure 4 supporting a role for Prickle1 in tear duct development. However the lack of defined criteria for labelling epithelial structures as mxp, lnp, cj, or NLG further compounds the confusion over the proposed tissue layer giving rise to the PTD. For an accurate interpretation of their findings, the authors should label all E-cadherin expressing epithelial structures depicted in the images in Fig 4. For example, the images in panels (G, H, I) and (P, Q, R) show the PTD emerging adjacent to an apparent juxtaposition of cj and mxp epithelia; yet this region is ambiguously labeled as "cj".

We appreciate the reviewer's comments about the ambiguous figure labeling. The ambiguity is generated because the palpebral conjunctiva is a part of the eyelid fold, which is, in turn, a part of mxp/lnp in early embryos. Nevertheless, we have now labeled all epithelial structures in Figure 4 and defined each part of the presumptive conjunctiva in relation to the mxp/lnp, which pertains to this research context.

Since 2D sections can be misleading regarding the arrangement of adjacent epithelia, the authors should discuss these observations in context of their 3D reconstructions at E10.25 and E10.5.

We thank the reviewer for this helpful suggestion. Due to intensive Prickle 1 signal in the lnp and mxp mesenchymes, weak E-cadherin signal in the PTD, and tissue displacement on prefixed frozen sections (Figure 4), 3D reconstruction proved to be difficult. Nevertheless, we hope the reviewer finds it helpful in understanding the arrangement of adjacent epithelia with newly added labeling in the Figures and interpretations for the relationship between mxp/lnp and cj in the text.

2 (i) To validate the iPSC-EB system for studies on Prickle1 function, the authors must account for the discrepancy in phenotype between the embryos homozygous for the Prickle1 allele used by Tao H et al (2009) and those homozygous for the Prickle1 allele used by the authors. The former arrest before gastrulation, whereas the latter survive to P2. Both alleles inactivate exon2 and are purported to be functional nulls. Have the authors examined the mutant phenotype after crossing the Prickle1 allele onto different genetic backgrounds? The absence of wide, background-dependent variability in the Prickle1 embryonic phenotype will raise questions about the structure of the targeted allele. For example, did rearrangements (deletions, duplications) occurring during targeting go undetected because they fall outside the region assayed by the genotyping protocol?

We have demonstrated Prickle 1 protein is undetectable using western blot (Liu et al., 2013, HMG, Figure 2; Liu et al., 2014, Biology Open, Figure 1; Guo et al., 2018, IOVS, Supplemental Figure 1B),

suggesting the targeted *Prickle 1* allele is null. We crossed *Prickle 1* mutant alleles from Sv129/C57B1 to C57B1/6J background for many generations (>7) and observed invariable phenotypes.

We used locus-specific long-range PCR, Southern analysis, and targeted sequencing to examine targeted alleles. We did not detect any unexpected deletions or duplications. Additionally, the phenotypic presentations in several different Prickle 1 lines are similar (Liu et al., 2014, Biology Open; Yang et al, Dev. Dyn. 2013; Gibbs et al., 2016, Biology Open), suggesting the probability of off-targeting is low.

We noticed that Tao et al (2009) used the TT2 ES cell line, which is C57BL/6 and CBA mixed background (Yagi et al., 1993, Anal Biochem), to target both Prickle 1 and Prickle 2 genes. They reported that embryonic lethality of Prickle 2 mutants prior to gastrulation solely depends on CBA background. In sharp contrast, Prickle 2 mutants are completely normal in the pure C56BL/6 background (backcrossed over 6 generations, Tao et al., 2012, Dev Biol. and our unpublished data). It is unclear from Tao's paper (Tao et al., 2009, PNAS) whether Prickle 1 mutant allele was bred onto other genetic backgrounds; however, based on their Prickle 2 work (Tao et al., 2012, Dev Biol.), we speculate that the early lethality of Prickle 1 mutants may also be related to CBA background.

We used R1 ES line (Sv/129 background) to target Prickle 1 allele and later cross onto C57/B1. Thus, as correctly pointed out by the reviewer, variable phenotypical presentations in Prickle 1 mutants are likely attributed to different genetic backgrounds.

2 (ii) Based on the methods section, the authors generated the Prickle1 control and iPSCs from the same colony of mice that generated the wild type and mutant embryos used for their studies on tear duct development. The embryonic viability of the Prickle1 allele argues that on this background, Prickle1 does not play an obligate role in the formation of the epiblast and visceral endoderm epithelial layers. If the iPSC-EB assay accurately recapitulates epithelialization events in the early mouse embryo, then one would expect to observe normal differentiation of the Prickle iPS-EBs. This undermines the authors rationale for applying the EB system.

We understand the reviewer's concern about the suitability for using the EB system to recapitulate epithelialization events in the early mouse embryos and leave relevant sections out of the manuscript according to the reviewer's suggestion.

2 (iii) The authors observe Prickle1 expression in the outer endoderm layer of the EBs, but not in the inner epiblast-like cells. Yet both Tao H et al. and Crompton LA et al. (2007) detect Prickle1 transcripts exclusively in the epiblast. Comments I, ii, iii combined suggest that iPSCs achieve efficient-EB differentiation in vitro by exploiting and adapting available genetic pathways. Thus one cannot conclude that Prickle1's functions during EB differentiation recapitulate its role during tear duct development.

We agree with the reviewer over this concern. We omitted results from the EB experiments. For clarification, Crompton LA et al's study showed clear epiblast expression in primitive streak (PS) at E7.5 (Fig. 1D, 2007). The EBs we used are of much earlier ages than this stage. Crompton et al also stated in their paper: "Prickle and Flamingo orthologues are expressed in a dynamic manner in tissues undergoing morphogenetic movements, such as the migrating AVE (anterior visceral endoderm) and primitive streak" (Crompton LA et al. (2007, Dev. Dyn). Indeed, looking at the Figure 1D' picture of in situ section of E6.5+ embryos by Tao et al (2009), the signal is located mostly in the superficial endodermal layer and the primitive streak (PS), and clear in situ signal is exhibited in anterior visceral endoderm (AVE) as well.

3 - As the manuscript is replete with errors in grammar and word choice, the authors should seek the assistance of an editor well versed in written English.

We thank the reviewer's suggestion and have had the manuscript edited by an English professional editor.

Minor Comments

4 - Figure 1: 3D-reconstruction of P1 tear duct from p63 stained images. Placing current panels I & J as the first two panels will enhance the impact of this figure by facilitating interpretation of the 2D images and 3D reconstructions.

We changed the figure and relevant text of the manuscript according to the reviewer's suggestion.

5 - Page 7: Should "and" be used in place of "or" in the next to the last sentence. "Both conjunctival end of the stalk [or] the PTD cell mass underwent apoptosis (Supplemental Fig. 1C, D)"

We changed "or" to "and".

6 - Figure 2: Define nm, nictitating membrane, a structure referred to in panels Q-X.

We defined it in the figure legend: Nictitating membrane: vestigial transparent third eyelid for moistening cornea while maintaining vision.

7 - Figure 2: What is the canthus?

Canthus (Canthi, plural): The corner of the eye where the junction of upper and lower lid meets.

8 - Supplemental Figure 1E: The figure or legend should include a color code indicating which cell populations/structures the pink, blue, yellow and white shading represent. The current labeling adds confusion. The LL arrow points to the white layer at E10.5 but to yellow cells at E11; the "cj" arrow points to pink cells at E10.5 but to the white layer at E11 and then light pink at E11.5

We redrew these diagrams and added consistent color code.

9 - Movies S4-S7: Adding unnecessary confusion, the left-right orientation of movies 4 & 5 is flipped relative to that of the images in panels H-L of Figure 3. Similarly, the left-right orientation of movies 6 & 7 is flipped relative to that of the images in panels N-O and Q-S of Figure 3.

We apologize for the confusion. We reoriented these videos to be consistent with the images in Figure 3.

10 - Figure 4: The orientation and cutting region depicted by the red dashed lines do not align with the structures present in the serial sections, rendering this figure unnecessarily confusing. The authors state that B-H and K-R depict "Horizontal serial sections cutting through NLG region from dorsal to ventral". However the arrow showing the direction of sectioning runs anterior to posterior, not dorsal to ventral. In addition, horizontal sections cut between the two red dashed lines appear inconsistent with the eye becoming more prominent in each successive section. Please clarify.

We apologize for making these confusions. We repositioned orientations of the drawn embryos to more accurately reflect the actual cutting directions. We eliminate the statements for guiding cutting directions referring to embryonic axes. We simply used the lines and arrows relative to the drawn embryos to indicate the cutting directions in this revision.

11 - Figure 5: Addition of schematic diagrams of lateral and frontal views of the developing face greatly facilitated interpreting the images. However, the E11.5 frontal schematic shows the incorrect location for the λ junction. To further clarify the authors' conclusions on Prickle1 expression in the elongating tear duct, the authors should identify and label the E-cadherin labeled epithelia (conjunctiva, mxp, lnp).

We thank the reviewer for the helpful comments. We correct the labeling of λ junction. We added labeling of conjunctiva, mxp and lnp to Figure 5.

Reviewer 2 Advance Summary and Potential Significance to Field:

This manuscript reports the role of Prickle 1 in polarized basement membrane formation during tear duct development and provides fundamental molecular insights into a poorly-studied, though clinically important, system.

Reviewer 2 Comments for the Author:
The authors have made extensive revisions in response to the reviews and included additional data/figs. I am satisfied with the response.

We are grateful that the reviewer is satisfied with our revised manuscript.

Third decision letter

MS ID#: DEVELOP/2020/191726

MS TITLE: Ontogenesis of the tear drainage system requires Prickle 1-driven polarized basement membrane (BM) deposition

AUTHORS: Dianlei Guo, Jiali Ru, Fuxiang Mao, Rong Ju, Hong Ouyang, Kaili Wu, Yizhi Liu, and Chunqiao Liu
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The additional modifications incorporated by the authors into their second revision resulted in a cogently organized and comprehensible manuscript that defines key, previously undocumented stages in the development of the tear drainage system in mice. Additionally it provides important insights into the mechanisms for elongation of the primordial tear duct that lay a firm foundation for future studies. Accept without any further revision.

Comments for the author

This reviewer greatly appreciates the thought, meticulous attention and effort that the authors devoted to their response to all comments voiced in the reviews of the first and second submission of the manuscript.

Reviewer 2

Advance summary and potential significance to field

This is a revision. I described it previously.

Comments for the author

I am satisfied with the response.