Single-cell transcriptomic signatures and gene-regulatory networks modulated by Wls in mammalian midline facial formation and clefts

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MS TITLE: Single-cell transcriptomics and gene-regulatory networks modulated by Wntless in mammalian midline facial formation and clefts

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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 to interrogate the identified gene regulatory networks, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

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

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

Reviewer 1

Advance summary and potential significance to field

The authors have used single-cell RNA-Seq and network analyses to define the molecular processes regulated by Wntless during mammalian midface formation and pathogenesis of midfacial clefts. The study provides transcriptomic atlases of mid-facial primordial and gene regulatory networks modulated by Wntless during mid-facial formation and fusion. These novel data will contribute to an overall understanding of molecular control of mid-facial development.

Comments for the author

1. In the introduction the authors outline the phenotypes of facial ectoderm (Foxg1-Cre) and neural crest/dorsal neuroepithelium-specific (Wnt1-Cre) Wis mutant and indicate that the role of Wis “in the fusion or merging process of the midfacial primordia remain undetermined”. Without any explanation, they proceeded to use Pax3-Cre in the current study. The rationale for this choice is missing. In addition, the authors do not give any indication on spatial and temporal patterns of recombination induced by Pax3Cre (in ‘discussion’ they indicate that it is also active in the surface ectodermal lineage cells of the dorsal nasal pit).
It would be helpful if a rationale for using Pax3Cre would be provided, and the detailed information on cell types recombined by Pax3-Cre in craniofacial processes would be given, as well.
2. Genetic fate mapping: whole-mount images do not provide enough information on cell types and the efficiency of recombination. Analysis on serial sections would be needed to clarify this aspect.
3. The investigators identified 18 gene clusters in facial primordia harvested at E11.5. How do these findings relate to those published by Li et al., Development 2019, 146(12):dev174888. doi: 10.1242/dev.174888 (Both studies used tissues harvested at E11.5 , and both show mesenchymal cell clustering)?
4. Subsequently the authors compared the clustering between controls and Wis mutants. They discovered that one specific cluster showed a notable reduction in cell numbers in mutants and that two clusters were most affected in terms of differentially expressed genes. They also validated changes in different classes of genes using ISH & qPCR and compare these data to their single-cell data. The investigators conclude that their results show that “Wis modulate and integrates a core gene regulatory network and their downstream targets in a mesenchymal subpopulation of MNP which are responsible for midline facial formation and fusion”. Have the investigators considered the fact that the facial processes of Wis mutants at E11.5 are phenotypically very different than those of controls? For instance, their own results show excessive apoptosis already a day earlier, i.e., at E10.5. How confident are the authors that by comparing differences of expression profiles in different cell populations harvested from primordia that are inherently different (E11.5), they can deduce the critical Wntless-regulated gene regulatory networks that clearly play critical roles already before E10.5?

Minor:
Labeling of violin plots at fig. 3 too small – almost impossible to see the

Reviewer 2

Advance summary and potential significance to field

This manuscript by Gu et al examined the midfacial defects caused by the loss of Wls. The phenotypes were similar to those reported earlier but the authors used additional scRNA-sequencing to identify subpopulations in the midfacial region and attempted to reveal new regulatory mechanisms. The clustering of subpopulations from scRNA-sequencing data was validated

by in situ and qRT-PCR analyses. The data presented were of good quality. However, there was a concern about scientific rigor and reproducibility as the pertinent information was missing. In addition the authors mainly performed correlative studies after obtaining the scRNA-seq data. The inclusion of important functional analysis for the identified gene regulatory networks would greatly enhance the current study.

Comments for the author

Fig 1:
1. The Wls mutants seem to have smaller midfacial primordia in general. So the statement of an enlarged midline gap may not be true (Fig 1D). The relative positions of MNPs, the distal portion of the non-neural nasal epithelium, and the distance between the bilateral junction zone, also appear to be narrower in the mutants. Quantitation with the actual measurements and statistics will improve Fig 1C-F.
2. In Fig 1C, D, it stated “epithelial cells in the bilateral junction zones are clearly EGFP negative” suggesting these cells did not come from the Cre expressing cells deficient for Wls. Could the authors explain how Wls disruption contributes to the defects? This phenotype cannot be attributed to the increased apoptosis in the EGFP positive cells deficient for Wls. There might be an expansion of these EGFP negative cells with normal Wls.
3. It stated “ a cluster of apoptotic cells at the dorsal junction zone between LNP and MNP in the normal control, which is not apparently altered in the Wls-cKO at E10.5”. This statement is incorrect based on the image presented in Fig 1I, J. The apoptotic cells seem to be increased in E10.5. Again, quantification analyses should be conducted to provide a better assessment of the number of apoptotic cells in E10.5 and E11-11.25.
4. The efficacy of Wls deletion is not determined but needs to be assessed in the mutants. The Cre-expressing cells are not equivalent to Wls altering cells because not all cells express Wls. The authors should also perform double staining of TUNEL and Wls.
Fig 2:
5. Due to the severe deformities in Wls mutants, it is difficult to tell the remaining structure solely based on histology. Molecular characterizations will ensure the defective tissues indicated in the images are indeed maxilla, nasal septum, etc. The osteoblast gene markers and von Kossa staining also need to examine if ossification defects are due to osteoblast differentiation and/or mineralization.
Fig 3:
6. The in situ of Alx3 is not limited to m1 and m5, and m9 but also in m8 (Dlx5 Msx2; Alx3 not indicated as a defined gene). For this presentation, it may be better to have three marker genes listed for all 18 subpopulations – a strategy generally used for this type of assessment.
7. What does “low quality” mean for m15? There is no explanation for this. The authors should define these cells based on their gene expression profile.
Fig 4:
8. Does the increase of m10 (nasal epithelial cells) correlate well with the expansion of EGFP negative cells observed in Fig 1D? The authors should consider doing in situ for this population to confirm this correlation.
9. Along the same line, authors’ speculation of altered cell proliferation and fate determination of m10 caused by Wls deficiency should be determined by experimental analyses. These data, easily assessable experimentally, will support their claims and strengthen this work. The scRNA-seq data may provide some clues to decipher the process of fate determination. Understanding the associated mechanistic insight could be an innovative aspect yet to explore in the study.
10. While this reviewer agreed that the m5 population is most significantly reduced, the others, e.g., m2 and m7, are also highly reduced in the Wls mutants. The analyses of these two populations should also be incorporated into Fig 4.
11. Please correct the legend which may be incorrectly stated for Fig 4B.
Fig 5:
12. The loss of Wnt5a expression specifically in the dorsal MNPs of Wls mutants is striking. However, this raised a question about the ventral MNPs not affected by the mutation. The authors did not provide any explanation on the difference between the two. For instance, could the difference be due to Wls-dependent and independent mechanisms? Some kind of information to explain this would provide insight into the role of Wnt5a.
13. The analysis of Wnt5a and Wnt9b showed the contrasting difference. However the presentation is biased and insufficient for examination of Wnt signaling especially Wls regulating all Wnt

proteins. Therefore, it is much more informative to include violin plots of scRNA-seq for all 19 Wnt genes. These results could be done easily but were missing here. Please include the data in a supplemental figure.
14. Interestingly, Wnt signaling pathways are not shown in the GO analysis of Table S1. The author may want to use GO to examine the effects of Wnt signaling pathways to get an unbiased assessment.
15. Along the same line, the conclusion stated “mesenchymal Wls ablation prevents both canonical and noncanonical Wnt signaling pathways” is not supported by any data. The expression of Wnt ligands is not equivalent to Wnt signaling pathways. While Lef1 reduction may suggest the effect of canonical Wnt signaling, there is no evidence supporting noncanonical Wnt signaling is altered. In addition, Wnt5a has been shown to signal via both noncanonical and canonical pathways. To make such a claim, the authors need to examine the outcome of noncanonical signaling effects.
Fig 6, 7:
16. Msx and Pax factors also are only affected in the dorsal but not ventral MNPs. Together with the study of Wnt5a, the results raise concerns about Wls mutation mainly affecting dorsal MNPs. Therefore, it is important to use dorsal and ventral specific makers to assess the Wls mutant phenotypes.
Fig 8:
17. The analyses of Alx genes were a bit more complicated and unable to yield meaningful conclusions. There is a concern about the “publishing notebook”
strategy for building the storyline. Instead of pointing out these facts, the author should consider doing additional investigations to understand what the results mean, to make some sense out of them, and to enhance the impact of this study.
Fig 9, 10:
18. Here are more analyses of previously known genes involved in midfacial development. The scRNA-seq data confirmed the results of in situ and qRT-PCR and show the specific subpopulation was affected. The in situ results already show spatial information so scRNA-seq data provide limited new information.
Fig 11:
19. The SCENIC predicts a GRN affected by Wls mutation. This prediction is novel and should be examined and validated by causative effects rather than correlative studies. Unfortunately, the authors did not pursue it further.
Discussion:
20. On page 18, line 8, “The current study demonstrates that these molecules formed a functionally interactive network are regulated by Wls in midline facial formation and fusion”. The functional interactive network was only suggested but not demonstrated in this study. To demonstrate the GRN, the authors need to provide functional analyses of this network, e.g., alteration of one upstream driver upstream to affect the downstream ones. There are only correlative data presented in this study.
21. The analysis of Kif26b and demonstration of its involvement in midfacial development suggested by the authors sounds like a novel direction to go.
Others:
22. The number of aminals examined for all figures, e.g., in situ, histology scRNA-seq, is not indicated. Do all experiments perform only once? Please also provide the relevant information with statistics, e.g., p-value, SD/SEM, to indicate the images are representative of the experiments throughout the entire paper. The quality control for scRNA-seq contains limited information, e.g., reads, mapped rates, total genes detected, the authors should provide additional information, e.g., exonic and intronic mapping rates, intergenic mapping rate principal component analysis, and hierarchical clustering. These are highly relevant to scientific rigor and reproducibility.
Summary:
The study confirmed many previously known genes and Wls deficient phenotypes associated with midfacial development using a fancier scRNA-seq analysis.
However, there is limited discovery on the mechanistic details concerning what’s known about Wnt signaling regulation in midfacial development. In addition, how to use the scRNA-seq with the division of subpopulations to elucidate the mechanism underlying midfacial development and disease? This seems to be a confirmatory study with shortcomings in innovation despite the use of signal cell analysis. The authors simply use scRNA-seq analysis as another tool to characterize the phenotypes without gaining much new knowledge. One potential use of the scRNA-seq is to predict novel genes and regulatory processes followed by investigations and demonstrations on their link to midfacial deformity. In addition to Wnt signaling, Msx, Pax, Alx, and other transcription factors

known to regulate midfacial development, the authors may want to provide new information to address this important question. Alternatively, the authors can design experiments to perform the functional analyses of the GRN, thus providing new mechanistic insight for an impactful study. Overall, this is an interesting but preliminary study with room for improvement.

First revision

Author response to reviewers' comments

Reviewer 1 Advance summary and potential significance to field
The authors have used single-cell RNA-Seq and network analyses to define the molecular processes regulated by Wntless during mammalian midface formation and pathogenesis of midfacial clefts. The study provides transcriptomic atlases of mid-facial primordial and gene regulatory networks modulated by Wntless during mid-facial formation and fusion. These novel data will contribute to an overall understanding of molecular control of mid-facial development.

Authors' Reply: We appreciate Reviewer 1's overall positive comments on this study.

Reviewer 1 Comments for the author

1. In the introduction the authors outline the phenotypes of facial ectoderm (Foxg1-Cre) and neural crest/dorsal neuroepithelium-specific (Wnt1-Cre) Wis mutant and indicate that the role of Wis "in the fusion or merging process of the midfacial primordia remain undetermined". Without any explanation, they proceeded to use Pax3-Cre in the current study. The rationale for this choice is missing. In addition, the authors do not give any indication on spatial and temporal patterns of recombination induced by Pax3Cre (in 'discussion' they indicate that it is also active in the surface ectodermal lineage cells of the dorsal nasal pit). It would be helpful if a rationale for using Pax3Cre would be provided, and the detailed information on cell types recombined by Pax3-Cre in craniofacial processes would be given, as well.

Reply: We appreciate Reviewer 1's insightful comments and guidance. We have included the rational to use Pax3-Cre for this study in the revised manuscript. Different from the previously used Foxg1-Cre that is activated in the surface ectoderm and neuroepithelia, and Wnt1-Cre that is activated in neural crest lineage cells in the facial primordia, Pax3-Cre exhibits recombination activities in facial mesenchymal cells derived from both neural crest and mesodermal cells, and a subpopulation of non-neural epithelial cells as demonstrated in a previous publication (Kasberg et al., Developmental Biology 2013), which actually allowed us to generate distinctly different phenotypes of midline facial clefts in the Pax3-Cre;Wls-cKO mutants as addressed in this study.

2. Genetic fate mapping: whole-mount images do not provide enough information on cell types and the efficiency of recombination. Analysis on serial sections would be needed to clarify this aspect.

Reply: As advised, we have sectioned the embryos for genetic fate mapping in the revised Fig. 1, which shows efficient recombination activities of Pax3-Cre in all facial mesenchymal cells and a subpopulation of non-neural epithelial cells in the heterozygous control embryos.

3. The investigators identified 18 gene clusters in facial primordia harvested at E11.5. How do these findings relate to those published by Li et al., Development 2019, 146(12):dev174888. doi: 10.1242/dev.174888 (Both studies used tissues harvested at E11.5 , and both show mesenchymal cell clustering)?

Reply: Li et al. published a single-cell RNA-seq study at E11.5 from cells isolated restrictively from the bilateral upper lip junction zones, which did not cover single-cell transcriptomics of the majority midfacial primordia. Especially, that study did not include single-cell transcriptomics of the dorsal medial nasal prominences that are responsible for midline facial formation and fusion process. Nevertheless, we have cited it in Discussion as a relevant study in the revised manuscript.

4. Subsequently the authors compared the clustering between controls and Wis mutants. They discovered that one specific cluster showed a notable reduction in cell numbers in mutants and that two clusters were most affected in terms of differentially expressed genes. They also validated changes in different classes of genes using ISH & qPCR and compare these data to their single-cell data. The investigators conclude that their results show that "Wis modulate and integrates a core gene regulatory network and their downstream targets in a mesenchymal subpopulation of MNP which are responsible for midline facial formation and fusion". Have the investigators considered the fact that the facial processes of Wis mutants at E11.5 are phenotypically very different than those of controls? For instance, their own results show excessive apoptosis already a day earlier, i.e., at E10.5. How confident are the authors that by comparing differences of expression profiles in different cell populations harvested from primordia that are inherently different (E11.5), they can deduce the critical Wntless-regulated gene regulatory networks that clearly play critical roles already before E10.5?

Reply: We appreciate the insightful comments and questions. Yes, the facial processes of Wls mutants are phenotypically different than those of controls at E11.5 visualized by genetic fate mapping at the microscopic level. We also showed that excessive apoptosis already happened a day earlier at E10.5. Indeed, our initial analyses were focused on E10.5 but we were struggling by getting insightful mechanistic results at E10.5 as it did not show clear phenotypic changes and very few gene alterations other than excessive apoptosis in the forehead region. Therefore, we moved mechanistic studies for the midline clefts from E10.5 to E11.5 during the midline facial merging process, which shows specific gene alterations without alterations in cell identity determined by transcriptomics in the medial nasal prominences of the Wls mutants. In the revised manuscript, we conducted additional analyses and found that Fgf8 is significantly diminished in Wls-deficient nasal epithelia at E10.5, which is a potential cause of excessive apoptosis in the forehead region, but not in the medial nasal prominences that are responsible for midline facial merging process. Because the cell identities that are determined by transcriptomics with thousands of gene profiles in each cell cluster are not altered by Wls deficiency, we are confident that the altered specific gene expression patterns and GRNs (particularly, the Msx and Pax regulons) in the medial nasal prominences (particularly in mesenchymal cluster 9) is the cause of the midline clefts in the mesenchymal Wls mutants.

Minor:
Labeling of violin plots at fig. 3 too small – almost impossible to see the

Reply: There are no violin plots in previous fig. 3. We have used larger labels in revised Fig. 4B if that is relevant to the comment.

Reviewer 2 Advance summary and potential significance to field
This manuscript by Gu et al examined the midfacial defects caused by the loss of Wls. The phenotypes were similar to those reported earlier but the authors used additional scRNA-sequencing to identify subpopulations in the midfacial region and attempted to reveal new regulatory mechanisms. The clustering of subpopulations from scRNA-sequencing data was validated by in situ and RT-qPCR analyses. The data presented were of good quality. However, there was a concern about scientific rigor and reproducibility as the pertinent information was missing. In addition, the authors mainly performed correlative studies after obtaining the scRNA-seq data. The inclusion of important functional analysis for the identified gene regulatory networks would greatly enhance the current study.

Authors' Reply: We appreciate Reviewer 2's positive comments on data quality of this study. Reviewer 2 raised "a concern about scientific rigor and reproducibility as the pertinent information was missing", which seems that Reviewer 2 might overlook relevant information in the previous manuscript, and it is addressed further in replies to specific comment 22 below. The "important functional analysis for the identified gene regulatory networks" has been partially carried out by related publications that provide mechanistic explanation of the midfacial hypoplasia and midline clefts in our Wls-deficient mutants, which include the synergetic roles of Msx1 and Msx2 in facial primordial formation (Ishii et al., Development 2005) and the synergetic roles of Pax3 and Pax7 in midline facial formation and midline clefts (Zalc et al., Dev Cell 2015). Functional analyses of other

novel genes determined in the gene regulatory networks are out of scope of the current study and would be addressed further in future studies by us and other colleagues in the same field.

Reviewer 2 Comments for the author
Fig 1:

1. The Wls mutants seem to have smaller midfacial primordia in general. So the statement of an enlarged midline gap may not be true (Fig 1D). The relative positions of MNPs, the distal portion of the non-neural nasal epithelium, and the distance between the bilateral junction zone, also appear to be narrower in the mutants. Quantitation with the actual measurements and statistics will improve Fig 1C-F.

Reply: We appreciate the comments and we have conducted quantification analyses by measuring 4 facial distances (in new Fig. S1C-E), respectively, between the MNPs (I), the dorsal nasal tips (II), the bilateral junction zones (III), and the lateral edges of the LNPs (IV), which show that only the midline gap (I) is significantly increased in the cKO embryos ($p = 0.0005$), and other distances are also slightly increased but without statistical significance ($p > 0.05$).

2. In Fig 1C, D, it stated “epithelial cells in the bilateral junction zones are clearly EGFP negative” suggesting these cells did not come from the Cre expressing cells deficient for Wls. Could the authors explain how Wls disruption contributes to the defects? This phenotype cannot be attributed to the increased apoptosis in the EGFP positive cells deficient for Wls. There might be an expansion of these EGFP negative cells with normal Wls.

Reply: We apologize for any misunderstanding about “the bilateral junction zones” that is responsible for bilateral upper lip fusion but not for midline facial merging, which are two separate processes. Because Wls is conserved in these EGFP negative junction zone cells, it may explain why mesenchymal Wls mutants have fused upper lip at the bilateral junction zones without bilateral cleft lip phenotypes. As addressed in this study, the midline facial clefts are mainly caused by defective morphogenesis of the dorsal MNPs (medial nasal prominences) during midline facial merging process that does not involve bilateral junction zones.

3. It stated “ a cluster of apoptotic cells at the dorsal junction zone between LNP and MNP in the normal control, which is not apparently altered in the Wls-cKO at E10.5”. This statement is incorrect based on the image presented in Fig 1I, J. The apoptotic cells seem to be increased in E10.5. Again, quantification analyses should be conducted to provide a better assessment of the number of apoptotic cells in E10.5 and E11-11.25.

Reply: We appreciate the comments and have refined the statement as “which is not dramatically increased in the Wls-cKO at E10.5”. We accidently lost the original confocal imaging files of these wholemount TUNEL assays for quantification analyses at higher resolutions and we were also unable to acquire new mutant embryos to repeat the experiments. Nevertheless, our results show massive increase of apoptotic cells in the forehead regions of cKO embryos, which is so clear even without quantifications in this case.

4. The efficacy of Wls deletion is not determined but needs to be assessed in the mutants. The Cre-expressing cells are not equivalent to Wls altering cells because not all cells express Wls. The authors should also perform double staining of TUNEL and Wls.

Reply: The efficacy of Wls deletion is determined by wholemount in situ hybridization, real-time RT PCR, and single cell RNA-seq in Fig. 5. As Wls is nearly ubiquitously expressed as shown in its violin plot, we respectively disagree that double staining of TUNEL and Wls would make additional mechanistic insights in the current study.

Fig 2:

5. Due to the severe deformities in Wls mutants, it is difficult to tell the remaining structure solely based on histology. Molecular characterizations will ensure the defective tissues indicated in the images are indeed maxilla, nasal septum, etc. The osteoblast gene markers and von Kossa staining also need to examine if ossification defects are due to osteoblast differentiation and/or mineralization.

Reply: The molecular characterizations of the disrupted primordial structures were carried out at E11.5 from Fig. 3 to Fig. 10. The facial structures at the later stages are defined by identical anatomic regions derived from the early stage. For instance, maxilla at E16.5 are derived from the maxillary prominences at E11.5, which have relatively conserved molecular markers in the Wls-deficient mutants at E11.5 thus less affected anatomical maxilla structures at later stages, which is no necessary and also no later markers to define that is the maxilla. The nasal septum is defined by its anatomic position and morphology, and again there are no suitable markers for nasal septum at the later stages.

Regarding ossification defects, our GO analysis shows that bone development, osteoblast differentiation, and cartilage development are altered in multiple mesenchymal subpopulations (in revised Fig. 4C; Table S2). Our skeleton preparation results show no or little facial bone formation in the mutants at E18.5 (Fig. 2H), therefore, von Kossa staining for calcium deposits would not add new sights to this aspect. In fact, the role of Wls in bone development has been nicely addressed in multiple studies (Zhong et al., PNAS, 2012; Wan et al., Bone, 2013; Zhong et al., Dev Dyn, 2015), so it is not a focus of the current study.

Fig 3:

6. The in situ of Alx3 is not limited to m1 and m5, and m9 but also in m8 (Dlx5, Msx2; Alx3 not indicated as a defined gene). For this presentation, it may be better to have three marker genes listed for all 18 subpopulations – a strategy generally used for this type of assessment.

Reply: Top 5 markers for each cell cluster are listed in heatmaps (revised supplementary Fig. S3, S4).

7. What does “low quality” mean for m15? There is no explanation for this. The authors should define these cells based on their gene expression profile.

Reply: We apologize for not clearly defining “low quality” for cluster 15. We have clearly defined the low quality cells in the revised manuscript. After clustering, we found that cluster 15 has much lower number of genes detected in each cell (nFeature_RNA) and much lower gene number detected per cell (nCount_RNA) than other cell clusteres (as shown in supplementary Fig. S2), which means cells in cluster 15 are in small library size and contains much fewer expressed genes. Importantly, no unique markers are found except highly expressed ribosomal genes (as shown in additional supplemental panels in Fig. S2) in cluster 15, which indicates RNA degradation in these cells. Therefore, we refer to such cells as low quality. These cells can lead to misinterpretation of the data and therefore were excluded for subsequent analysis as a common practice in single cell RNA-seq analyses.

Fig 4:

8. Does the increase of m10 (nasal epithelial cells) correlate well with the expansion of EGFP negative cells observed in Fig 1D? The authors should consider doing in situ for this population to confirm this correlation.

Reply: We did not observe expansion of EGFP negative cells but possibly reduced EGFP positive cells in mutant cluster 10 (nasal epithelial cells). The increased ratio of mutant cluster 10 can be caused by several other factors. The reduced ratios in mutant m2/m5/m7 will partially contribute to it. To explore additional reasons of the notably increased ratio in the mutant cluster 10, we re-clustered it and found no significant alterations of cell cycle status but significantly fewer cells in the WT compared to that in the cKO (Fig. S5). This unusual phenomenon is likely caused by technical limitations during microdissections of midfacial primordia and scRNA-seq preparations due to altered anatomical structures and significantly reduced cell numbers in the cKO embryos. We have

also conducted additional in situ hybridization analyses of Fgf8, Fgfr2, Sp8, Cldn6, and Krt8 (new Fig. 11), which show dominant expression patterns in the non-neural nasal epithelial cells of the control embryos, with significantly diminished expression of Fgf8 and no significant changes of other genes in the cKO nasal structures. Violin plots (supplementary Fig. S7) of these genes indicate that Cldn6 and Krt6 are DEGs in cluster 10, suggesting defective differentiation of non-neural nasal epithelial cells in the Wls-cKOs. However, the DEG analysis in these epithelial cells are less reliable due to the significantly fewer WT epithelial cells incorporated for scRNA-seq analyses.

9. Along the same line, authors' speculation of altered cell proliferation and fate determination of m10 caused by Wls deficiency should be determined by experimental analyses. These data, easily assessable experimentally, will support their claims and strengthen this work. The scRNA-seq data may provide some clues to decipher the process of fate determination. Understanding the associated mechanistic insight could be an innovative aspect yet to explore in the study.

Reply: As requested, we examined the cell proliferation in cluster 10 and found no significant alterations of G1, G2/M, and S phases in mutants (new supplementary Fig. S5). However, we found that the relatively increased ratio of mutant cluster 10 cells is because of dramatically lower cell number in the control cluster 10, which is mainly caused by technical limitations as explained in reply to comment #8.

10. While this reviewer agreed that the m5 population is most significantly reduced, the others, e.g., m2 and m7, are also highly reduced in the Wls mutants. The analyses of these two populations should also be incorporated into Fig 4.

Reply: We have included all mesenchymal cell clusters (m0-m9) plus clusters 10 (nasal epithelia) and 11 (surface ectoderm) for gene ontology (GO) analyses with additional GO terms in addition to facial development related GO terms, the latter were selected from top significantly enriched processes. The results show that although the cell number ratios are also reduced in m2 and m7, the facial and mesenchymal development-related GO terms in m2/m7 and other cell clusters are much fewer than those in the mutant m5 (with decreased cell ratio) and m9 (with no significant change in cell ratio) (see revised Fig. 4 and Table S2).

11. Please correct the legend which may be incorrectly stated for Fig 4B.

Reply: We have revised Fig. 4B and related legend.

Fig 5:

12. The loss of Wnt5a expression specifically in the dorsal MNPs of Wls mutants is striking. However, this raised a question about the ventral MNPs not affected by the mutation. The authors did not provide any explanation on the difference between the two. For instance, could the difference be due to Wls-dependent and independent mechanisms? Some kind of information to explain this would provide insight into the role of Wnt5a.

Reply: Region-specific disruption of Wnt5a in the Wls-deficient facial primordia is an indication of transcriptomic alterations in the dorsal but not ventral MNPs, as demonstrated by gene ontology (GO) with mostly altered GO terms in m5 and m9 of dorsal MNPs, but little changes in m8 or ventral MNPs as shown in revised Fig. 4. Because the dorsal not the ventral MNPs are responsible for midline facial merging, it explains the phenotypic consequence of midline facial clefts in the mutants. The region-specific role of Wls in the dorsal nasal primordia may reflect a general role of Wnt signaling in the dorsal domains as known in the developing CNS and it may be also linked with defective Fgf8 signaling in the dorsal region of the distal nasal pits as demonstrated in our new results (new Fig. 11). We have included related interpretation in the text.

13. The analysis of Wnt5a and Wnt9b showed the contrasting difference. However, the presentation is biased and insufficient for examination of Wnt signaling, especially Wls regulating all Wnt proteins. Therefore, it is much more informative to include violin plots of scRNA-seq for all 19 Wnt genes. These results could be done easily but were missing here. Please include the data in a supplemental figure.

Reply: We appreciate the advice for including violin plots of scRNA-seq for all 19 Wnt genes, and now we include them in Figure S6 expect those are not detectable. Most of them have low or limited expression levels except that Wnt5a is dominantly expressed in most mesenchymal cell clusters (m0-m9) and Wnt6 is restrictively expressed in the surface ectodermal cells (cluster 11) as expected. We selectively examined Wnt5a and Wnt9b alterations in the mutants because their known roles in facial development and fusion processes.

14. Interestingly, Wnt signaling pathways are not shown in the GO analysis of Table S1. The author may want to use GO to examine the effects of Wnt signaling pathways to get an unbiased assessment.

Reply: We have included additional GO terms including both canonical and noncanonical Wnt signaling pathways in revised Table S2 (previous Table S1). Unexpectedly, the GO analyses revealed that both canonical and noncanonical Wnt signaling pathways are upregulated in the mutant mesenchymal cluster 5, but they are not listed in m9, regardless of the diminished expression of Wnt signaling genes such as Wls and Wnt5a in m9. These may be caused by the default GO analyzing program that classified Wls and Wnt5a in other GO terms other than in Wnt signaling pathways.

15. Along the same line, the conclusion stated “mesenchymal Wls ablation prevents both canonical and noncanonical Wnt signaling pathways” is not supported by any data. The expression of Wnt ligands is not equivalent to Wnt signaling pathways. While Lef1 reduction may suggest the effect of canonical Wnt signaling, there is no evidence supporting noncanonical Wnt signaling is altered. In addition, Wnt5a has been shown to signal via both noncanonical and canonical pathways. To make such a claim, the authors need to examine the outcome of noncanonical signaling effects.

Reply: We changed the statement to “mesenchymal Wls ablation MAY prevent both canonical and noncanonical Wnt signaling pathways” based on the facts of Wls in regulation of secretion of all Wnt proteins. The defective noncanonical Wnt signaling is partially supported by diminished Wnt5a that has a potential role in noncanonical Wnt signaling during craniofacial development. Wnt5a-null mice have a flattened face and other distal outgrowth defects, which are suggestive defects in directional tissue growth regulated by PCP signaling. By rechecking GO results, we found both canonical and noncanonical Wnt signaling pathways that are altered in Wls-deficient m5 but not in m9 as it does not include the common Wnt signaling genes such as Wls and Wnt5a in these GO terms with unclear reasons (Table S2).

Fig 6, 7:
16. Msx and Pax factors also are only affected in the dorsal but not ventral MNPs. Togethe with the study of Wnt5a, the results raise concerns about Wls mutation mainly affecting dorsal MNPs. Therefore, it is important to use dorsal and ventral specific makers to assess the Wls mutant phenotypes.

Reply: This has been explained in the reply to comment 12. The marker genes for facial mesenchymal clusters have multiple expression domains. We have examined several region-specific expression genes in the dorsal and ventral MNPs, such as Tfap2a (high in m5) and Tfap2b (high in m5 and m9), which are disrupted in the dorsal MNP of the mutants (Fig. 9). Dlx5 is a marker gene for the ventral MNPs (m8) that is not altered in the mutant m8, and it is also expressed in m0 of MxPs, upregulated in mutant m5 of dorsal MNPs, and downregulated in nasal epithelia (cluster 10) (Fig. 10).

Fig 8:
17. The analyses of Alx genes were a bit more complicated and unable to yield meaningful conclusions. There is a concern about the “publishing notebook” strategy for building the storyline. Instead of pointing out these facts, the author should consider doing additional investigations to understand what the results mean, to make some sense out of them, and to enhance the impact of this study.

Reply: We examined Alx family genes based on Alx1 alterations detected by scRNA-seq in the current study and their known roles in midline facial formation and midline clefts in related publications as explained in the text. Therefore, it is important to validate the critical transcriptomic results, even some of them do not generate fully clear mechanistic insights as

predicted. Intriguingly, both Alx3 and Alx4 are diminished in dorsal MNPs and Alx4 is further supported by RT-qPCR, but scRNA-seq did not determine these genes as DEGs in any clusters (Fig. 8), which are quite unexpected and hard to explain why at the moment.

Fig 9, 10:
18. Here are more analyses of previously known genes involved in midfacial development. The scRNA-seq data confirmed the results of in situ and qRT-PCR and show the specific subpopulation was affected. The in situ results already show spatial information so scRNA-seq data provide limited new information.

Reply: Same reason for including these genes as explained in reply to comment 17. We used three complementary approaches to demonstrate the same set of critical genes based on the consideration and inclusion of scientific rigor and reproducibility. scRNA-seq provides DEGs in subpopulations of facial primordial cells, which is supported but cannot be replaced by in situ hybridization for the spatial information.

Fig 11:
19. The SCENIC predicts a GRN affected by Wls mutation. This prediction is novel and should be examined and validated by causative effects rather than correlative studies. Unfortunately, the authors did not pursue it further.

Reply: We appreciate the positive comments on the GRN prediction results. It provides integrated mechanistic explanation of midline facial clefts and hypoplasia underlying Wls mutants as addressed in the current study, and it also provides future research directions.

Discussion:

20. On page 18, line 8, “The current study demonstrates that these molecules formed a functionally interactive network are regulated by Wls in midline facial formation and fusion”. The functional interactive network was only suggested but not demonstrated in this study. To demonstrate the GRN, the authors need to provide functional analyses of this network, e.g., alteration of one upstream driver upstream to affect the downstream ones. There are only correlative data presented in this study.

Reply: We largely agree with the comments as the functionally interactive network is suggestive based on currently available data sources and the predicted GRN from this study has not been fully demonstrated. However, this study has demonstrated that three key GRN transcription factors (Msx1, Pax3, and Pax7) are regulated by Wls during midline formation and fusion, and that multiple downstream target genes of these GRN regulators are also diminished in Wls-deficient midfacial mesenchymal cells. To further validate the direct regulation of the predicted downstream target genes by the key GRN regulators, we have carried out new experiments of chromatin immunoprecipitation using Msx1 antibodies to pull down the Msx1 binding motifs in the promoter regions of three selected candidate downstream target genes (Wnt5a, Kif26b, and Smad7) (new Fig. 12C,D).

21. The analysis of Kif26b and demonstration of its involvement in midfacial development suggested by the authors sounds like a novel direction to go.

Reply: We agree with that many new candidates including Kif26b as revealed by our GRN predictions are the new direction of future works to determine their roles in midfacial development. Regarding Kif26b, neonatal lethality with impaired kidney development has been reported in Kif26b-null mice. Although it seems no clear craniofacial defects in these mutants, it would be an interesting direction to go. Other novel candidate genes with unknown phenotypes of their mutants would be also very interesting to explore their functions in midfacial development in future studies.

Others:

22. The number of aminals examined for all figures, e.g., in situ, histology, scRNA-seq, is not indicated. Do all experiments perform only once? Please also provide the relevant information with

statistics, e.g., p-value, SD/SEM, to indicate the images are representative of the experiments throughout the entire paper. The quality control for scRNA-seq contains limited information, e.g., reads, mapped rates, total genes detected, the authors should provide additional information, e.g., exonic and intronic mapping rates, intergenic mapping rate, principal component analysis, and hierarchical clustering. These are highly relevant to scientific rigor and reproducibility.

Reply: The number of animals examined is included either in the Methods or in bar graphs. Two mutants and three littermate controls were used for scRNA-seq. Embryo numbers for semi-quantitative RT-qPCR are reflected by the dots in each graph bar, which have been specifically included in the revised figure legends. Statistical information including SD/SEM and p-value are indicated in each qPCR and related scRNA-seq graph. For non-quantitative in situ imaging analyses, we conducted at least two embryos for each single probe which generated the same results. We would respectively say that this study has been rigorously carried out with reliable reproducibility by employing multiple research approaches of scRNA-seq, in situ hybridization, and RT-qPCR validation for the same set of critical DEGs examined in this study.

“The quality control for scRNA-seq, e.g., reads, mapped rates, total genes detected” is included in the session of Results entitled “Single-cell RNA-seq analyses of the midfacial primordia of normal and Wls-cKO mouse embryos”. The requested additional information is included in new Table S1.

Summary:

The study confirmed many previously known genes and Wls deficient phenotypes associated with midfacial development using a fancier scRNA-seq analysis. However, there is limited discovery on the mechanistic details concerning what’s known about Wnt signaling regulation in midfacial development. In addition, how to use the scRNA-seq with the division of subpopulations to elucidate the mechanism underlying midfacial development and disease? This seems to be a confirmatory study with shortcomings in innovation despite the use of signal cell analysis. The authors simply use scRNA-seq analysis as another tool to characterize the phenotypes without gaining much new knowledge. One potential use of the scRNA-seq is to predict novel genes and regulatory processes, followed by investigations and demonstrations on their link to midfacial deformity. In addition to Wnt signaling, Msx, Pax, Alx, and other transcription factors known to regulate midfacial development, the authors may want to provide new information to address this important question. Alternatively, the authors can design experiments to perform the functional analyses of the GRN, thus providing new mechanistic insight for an impactful study. Overall, this is an interesting but preliminary study with room for improvement.

Reply: We appreciate Reviewer 2’s guidance on the future directions and respectively disagree with the comment that this is a confirmatory or preliminary study. In fact, the role of Wnt signaling in midline facial fusion and its integrative regulation of Msx/Pax and related transcription factors in midfacial development remained unknown until the current study. We believe that this is a significant and comprehensive study evident by the following novel aspects: (1) Conditional ablation of Wls in Pax3-Cre lineage cells generated a new and valuable phenotype of midline facial cleft that phenocopies a rare type of human orofacial clefts; (2) scRNA-seq revealed a unique transcriptomic atlas of midfacial primordia with 10 mesenchymal subpopulations which recaptures anatomical positions of the midfacial primordia and also provides widely useful transcriptomic datasets for studying midfacial morphogenesis and related disorders; (3) Multiple and rigorous experimental approaches generated unbiased results of Wls-modulated genes that are responsible for midline facial formation and fusion; (4) The complementary experimental approaches also revealed a crucial mesenchymal subpopulation that is responsible for the merging process of the dorsal medial nasal prominences; (5) GRN analysis revealed key regulons consisting of Msx and Pax transcription factors that are regulated by Wls signaling in this unique mesenchymal subpopulation, providing novel cellular and molecular mechanisms underlying midline facial merging and midline clefts; (6) GRN analysis revealed further candidate target genes downstream Msx/Pax regulons, pointing new directions to study midline facial formation and midline clefts.

Nevertheless, to gain the proof of concept of the GRN regulation during midfacial morphogenesis, we have carried out new experiments of chromatin immunoprecipitation of three selected downstream target genes in this GRN module and demonstrated that Msx1 as a key regulon can bind to respective promoter motifs of Wnt5a, Kif26b, and Smad7 in the orofacial primordial

cells (new Fig. 12C,D), which enhances the functional significance of this GRN in midfacial development and will be addressed further in future studies.

Second decision letter

MS ID#: DEVELOP/2022/200533

MS TITLE: Single-cell transcriptomics and gene-regulatory networks modulated by Wntless in mammalian midline facial formation and clefts

AUTHORS: Ran Gu, Shuwen Zhang, Subbroto Kumar Saha, Yu Ji, Kurt Reynolds, Moira McMahon, Bo Sun, Mohammad Saharul Islam, Paul Trainor, YiPing Chen, Ying Xu, Yang Chai, Diana Burkart-Waco, and Chengji Zhou

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

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Reviewer 1

Advance summary and potential significance to field

The authors have used single-cell RNA-Seq and network analyses to define the molecular processes regulated by Wntless during mammalian midface formation and pathogenesis of midfacial clefts. The study provides transcriptomic atlases of mid-facial primordial and gene regulatory networks modulated by Wntless during mid-facial formation and fusion. These novel data will contribute to an overall understanding of molecular control of mid-facial development.

Comments for the author

The authors have addressed the concerns I raised in my previous review.

Reviewer 2

Advance summary and potential significance to field

The revised manuscript by Gu et al has addressed the majority of questions raised in the initial review. The revision is much improved but the study remains somewhat descriptive and correlative. Understandably, the authors choose to address these important issues in future studies. Nonetheless, the current study is appropriate for publication consideration in Development. There are a few concerns that can be easily clarified.

Comments for the author

Fig 1:
Point #2, Fig 1C, D: The authors should include a similar statement described in their response to reviewers regarding bilateral junction zones, upper lip, and midfacial cleft in the revised manuscript. It is not clear if these clarification statements are incorporated into the revised text.
Pont #3, Fig 1I, J: Lossing of original confocal data is not an excuse to omit quantitative analyses. Unfortunately, for scientific rigor, the author should complete the quantitation and include the

data in the revised manuscript. The review also is not sure how would the authors justify the publication of this work without the possession of raw images/data (no longer existing)? This seems to be an important ethical issue.
Point #4: The importance of performing double staining of TUNEL and Wls is to examine if any apoptosis occurs in Wls-expressing cells. Alternatively, all apoptotic cells are missing Wls. The result will also provide information on the primary vs. secondary effects, as well as autocrine vs. paracrine effects, thus providing additional mechanistic insights. This pertinent information remains missing in the revised manuscript.
Fig 2:
Point #5: Please include proper citations regarding defects in the bone and cartilage and the differentiation of osteoblasts and chondrocytes in the revised manuscript. As indicated by the authors, these aspects missing from the current study but are better supported by previous discoveries. Also, it seems the authors did not properly select the original papers first describing mouse genetic analyses of Wls in skeletal and craniofacial development.
Fig 4:
Point #9: The authors need to indicate the technical limitations described in their response to reviewers in the Discussion section of the revised manuscript.
Fig 5:
Point #15: The presentation format in Table S2 may not sufficient for the analysis of canonical and noncanonical Wnt signaling effects. The “pathway analysis” format seems to be a better representation for the analysis of these pathways.
Fig 8:
Point #17: Please include their response with regards to Alx genes in the Discussion section of the revised manuscript.

Second revision

Author response to reviewers' comments

Reviewer 1 Advance summary and potential significance to field
The authors have used single-cell RNA-Seq and network analyses to define the molecular processes regulated by Wntless during mammalian midface formation and pathogenesis of midfacial clefts. The study provides transcriptomic atlases of mid-facial primordial and gene regulatory networks modulated by Wntless during mid-facial formation and fusion. These novel data will contribute to an overall understanding of molecular control of mid-facial development.

Reviewer 1 Comments for the author
The authors have addressed the concerns I raised in my previous review.

Authors’ reply: We appreciate Reviewer 1’s acceptance for the revised manuscript.

Reviewer 2 Advance summary and potential significance to field
The revised manuscript by Gu et al has addressed the majority of questions raised in the initial review. The revision is much improved but the study remains somewhat descriptive and correlative. Understandably, the authors choose to address these important issues in future studies. Nonetheless, the current study is appropriate for publication consideration in Development. There are a few concerns that can be easily clarified.

Reviewer 2 Comments for the author

Fig 1:
Point #2, Fig 1C, D: The authors should include a similar statement described in their response to reviewers regarding bilateral junction zones, upper lip, and midfacial cleft in the revised manuscript. It is not clear if these clarification statements are incorporated into the revised text.

Authors reply: We have included these statements in both original and revised text.
Regarding bilateral junction zones, we included the following statement in the first paragraph of Introduction: “Many studies have examined the upper lip and primary palate formation at the bilateral junction zones where the LNP, MNP, and MxP merge and fuse (Abramyan and Richman 2015; Ji et al. 2020). The fusion process involves a rapid expansion of the neural crest-derived mesenchymal cells within the midfacial primordia and the epithelial seam formation and apoptosis at the fusion site. Defective primordial growth and/or fusion at the junction zones may cause bilateral or unilateral cleft lip with or without cleft palate (CL/P) (Mossey et al. 2009; Ji et al. 2020). On the other hand, the two adjacent MNPs merge at the midline (which does not involve epithelial fusion) to form the philtrum, nasal septum, and associated structures (Abramyan and Richman 2015). Although it is relatively rare, midline orofacial clefts, including median cleft lip and bifid nose, also occur in human newborns with unknown pathogenesis (Eppley et al. 2005)”.

Regarding Wls in the bilateral junction zones, we included and revised the following statements in the first session of Results: “It is important to note that the epithelial cells in the bilateral junction zones are EGFP negative (indicating no Pax3-Cre recombination activities) in both heterozygous control and cKO embryos (arrowheads in Fig. 1A,B,E,F), and that the fusion of the upper lip and primary palate among the MNP, LNP, and MxP is not affected in the Wls-cKO embryos (Fig. 1H,J) as a consequence of conserved Wls in the bilateral junction zone epithelial cells.”

Pont #3, Fig 1I, J: Lossing of original confocal data is not an excuse to omit quantitative analyses. Unfortunately, for scientific rigor, the author should complete the quantitation and include the data in the revised manuscript. The review also is not sure how would the authors justify the publication of this work without the possession of raw images/data (no longer existing)? This seems to be an important ethical issue.

Authors reply: We had inaccurate statement regarding our original confocal data in the previous response. Actually, we have the original confocal images in tif files. We have also found the huge ND2 files in external backup drives. As requested, we performed quantification analyses of TUNEL positive cells and confirmed that there are no significant changes in the dorsal junction zones of the mutants compared to the littermate controls at both ages, and that TUNEL positive cells are increased significantly in the forehead regions of the mutants (new Fig. S2). We have accordingly revised the results in the manuscript.

Point #4: The importance of performing double staining of TUNEL and Wls is to examine if any apoptosis occurs in Wls-expressing cells. Alternatively, all apoptotic cells are missing Wls. The result will also provide information on the primary vs. secondary effects, as well as autocrine vs. paracrine effects, thus providing additional mechanistic insights. This pertinent information remains missing in the revised manuscript.

Authors reply: We appreciate Reviewer 2's insightful comments and we respectively disagree with the specific request for double staining in this study. Because Wls is nearly ubiquitously expressed in the control midfacial primordia and it is entirely ablated in the nasal primordia of the mutants as shown by wholemount in situ hybridization in Fig. 4A,B and by violin plot of single-cell analyses in Fig. 4J, it lacks a scientific foundation to perform double staining of TUNEL and Wls in this study. In other words, Wls is gone in the region where ectopic apoptosis occurred in the mutants, so the double labeling will prove nothing in this study. Hope Reviewer 2 will agree with this explanation.

Fig 2:

Point #5: Please include proper citations regarding defects in the bone and cartilage and the differentiation of osteoblasts and chondrocytes in the revised manuscript. As indicated by the authors, these aspects missing from the current study but are better supported by previous discoveries. Also, it seems the authors did not properly select the original papers first describing mouse genetic analyses of Wls in skeletal and craniofacial development.

Authors reply: We have included two key publications (Fu et al., 2011; Goodnough et al., 2014) of Wls in cranial skeletal development in the Introduction. We have included additional publications regarding Wls in skeletal development and homeostasis (Fu et al. 2011; Zhong et al. 2012;

Maruyama et al. 2013; Wan et al. 2013; Goodnough et al. 2014; Zhong et al. 2015) in the Discussion of the re-revised manuscript.
Fig 4:

Point #9: The authors need to indicate the technical limitations described in their response to reviewers in the Discussion section of the revised manuscript.

Authors reply: We have included this technical phenomenon and related interpretations in the session 4 of Results: “The reduced cell numbers in m2, m5, and m7 may increase the ratios in other clusters, such as m3, m8, and cluster 10 (nasal epithelia) in the cKO. To explore additional reasons of the notably increased ratio in the mutant cluster 10, we re-clustered it and found no significant alterations of cell cycle status but significantly fewer cells in the WT compared to that in the cKO (Fig. S5). This unusual phenomenon is likely caused by technical limitations during microdissections of midfacial primordia and scRNA-seq preparations due to altered anatomical structures and significantly reduced cell numbers in the cKO embryos”, which is not repeated or expanded further in Discussion due to space limitations.

Fig 5:

Point #15: The presentation format in Table S2 may not sufficient for the analysis of canonical and noncanonical Wnt signaling effects. The “pathway analysis” format seems to be a better representation for the analysis of these pathways.

Authors reply: We are unclear what exactly this specific comment from Reviewer 2 is talking about, but we additionally performed signaling pathway specific analyses which revealed identical results.

Fig 8:

Point #17: Please include their response with regards to Alx genes in the Discussion section of the revised manuscript.

Authors reply: We have added related discussion by the end of the session with subtitle of “Single-cell transcriptomics of midline facial formation and clefts”: “It is worthy to mention that our scRNA-seq results did not catch up all critical DEGs, such as Alx3 and Alx4 which were clearly diminished in dorsal MNPs of the mutants detected by wholemount in situ hybridization and RT-qPCR, implying a technical limitation of scRNA-seq with unexplained reasons.”

Third decision letter

MS ID#: DEVELOP/2022/200533

MS TITLE: Single-cell transcriptomics and gene-regulatory networks modulated by Wntless in mammalian midline facial formation and clefts

AUTHORS: Ran Gu, Shuwen Zhang, Subbroto Kumar Saha, Yu Ji, Kurt Reynolds, Moira McMahon, Bo Sun, Mohammad Saharul Islam, Paul Trainor, YiPing Chen, Ying Xu, Yang Chai, Diana Burkart-Waco, and Chengji Zhou
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.