Cleft lip and cleft palate in *Esrp1* KO mice is associated with alterations in epithelial-mesenchymal crosstalk

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Original submission

First decision letter

MS ID#: DEVELOP/2019/187369

MS TITLE: Cleft lip and cleft palate (CL/P) in Esrp1 KO mice is associated with alterations in Wnt signaling and epithelial-mesenchymal crosstalk

AUTHORS: Russ P. Carstens, Sungkyoung Lee, Matthew J. Sears, Hong Li, Imad Salhab, Philippe Krebs, Zijun Zhang, Yi Xing, Hyun-Duck Nah, and Trevor Williams

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.

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

Reviewer 1

Advance summary and potential significance to field

In this paper, the authors characterize the cleft lip and palate phenotype observed in mice in which the epithelial-specific splicing factor Esrp1 has been ablated in the ectoderm. The paper extends previous research on Esrp1 (and Esrp2) by demonstrating that the effects are epithelial-specific (two new mouse lines have been generated) and by characterizing the downstream consequences. Specifically, the authors demonstrate that: the effects on lip and palate development are distinct; that mesenchymal proliferation is reduced in both the lip and palate; that cell death in epithelial seams is affected; and that, in addition, to numerous splicing events being disrupted, several cell signalling pathways are affected. In the latter case, the authors have documented that the WNT and SHH signaling are down-regulated. The paper is well-written, the conclusions drawn are well-founded, and the discussion is appropriate. Unfortunately, the paper has not been able to elucidate the mechanism(s) by which WNT and SHH signalling are affected.

Comments for the author

I have a few comments that the authors might like to consider.
The abstract is rather general in nature; more specific detail would help to 'sell' the paper!
A word appears to have been omitted on line 112 In figure 1, the nomenclature used to denote the Esrp1 genotypes is not consistent The quality of fig. 1C is lower than that of other parts of the same figure.
In fig 3, is the reduced cell proliferation observed in the epithelium and mesenchyme of Esrp1-/- mice statistically-significant?
Are the gene expression changes presented in fig. 5A statistically-significant?

Reviewer 2

Advance summary and potential significance to field

The manuscript by Lee et al examines the morphological, cellular, and molecular bases of cleft lip and palate malformations resulting from loss of the epithelial-specific splicing factor Esrp1. This is an important area of investigation as orofacial clefts are common human birth defects and because our understanding of cleft lip in particular is limited by the relative scarcity of mouse models that recapitulate this outcome. The present manuscript follows this group's report of cleft lip and palate resulting from Esrp1/2 deletion and represents a substantive advance in describing mechanisms by which aberrant splicing may result in orofacial clefts including a number of key insights enumerated below. The manuscript is well crafted and the data are presented clearly and interpreted carefully and evenly. Enthusiasm is diminished by the mechanistic gap between altered splicing and downstream changes in gene expression that likely drive well documented cell proliferation and fusion defects. Addressing this gap experimentally and attending to several other minor issues noted below would strengthen this manuscript and increase its impact in the field.

Manuscript highlights
1. The evidence supporting the importance of epithelial-mesenchymal crosstalk in this model is compelling. The authors show that Esrp1 expression is epithelial-specific and that its deletion results in reduced mesenchymal proliferation and attenuated outgrowth of facial growth centers that form the upper lip.
2. Identification of epithelial versus mesenchymal splice variants and overlap of Esrp1 null epithelial splice forms with wild-type mesenchyme-specific variants.
3. The tissue-specific RNA-seq experiments, while somewhat technically challenging, appear to have yielded robust results that are anchored by careful validation of known epithelial and mesenchymal specific transcripts.

Comments for the author

Essential revision
1. The authors show that deletion of epithelial Esrp1 alters expression levels of key genes in the adjacent mesenchyme and that disruption of this crosstalk likely plays a major role in clefting pathogenesis in this model.
The authors also provide evidence implicating Wnt signaling and, to a lesser extent, Shh signaling as key factors potentially involved in this pathogenic mechanism. Of the many changes observed, focusing on Wnt and Shh is a reasonable choice as these signaling pathways have been shown to act as central mediators of epithelial-mesenchymal crosstalk in orofacial morphogenesis. However, whether Esrp1 function directly regulates Wnt and Shh signaling and whether the expression changes observed in Wnt and Shh genes (and reduced Wnt reporter activity) are operational in the pathogenic mechanism of clefting in this mouse model remains unclear. The authors are commended for stating this limitation upfront in the discussion section but should further explore experimental approaches to examine the involvement of these pathways. For example pharmacologic and genetic Wnt pathway activation (in utero administration of small molecule DKK inhibitor or genetic deletion of secreted Wnt antagonists, including Dkk1 or Wise) has been shown to rescue cleft palate resulting from deletion of Pax9, which acts upstream of Wnt signaling in orofacial morphogenesis (PMIDs 28692808 and 28893947). In utero exposure to a pharmacological activator of Shh signaling (PMID: 27801979) could also be applied to examine the pathogenic role of Shh signaling changes in this model.

Minor issues
1. The penetrance of cleft subtype phenotypes (CL, CP, CL+CP) should be clearly stated for each newly described mouse model and cross.
2. While the data appear generally to be of high quality, light images depicting facial phenotypes could be clearer, which would allow phenotypes in these animals to be more readily compared to those in other published models of orofacial clefts. For example, simply submerging the heads in PBS should yield much clearer images of upper lip and palate phenotypes.
3. In the same vein, the quality of the images shown in Figure 1C is poor, making qualitative phenotypic comparison of upper lip phenotype difficult. Given that these data are not central to the narrative, the authors may consider downplaying these results. Alternatively, higher quality images with consistent positioning across experimental groups should be provided.
4. Some rationale for selecting the 17 cassette exon events to validate by RT-PCR should be provided.
5. The authors outline data supporting the premise that cleft secondary palate is a distinct manifestation of Esrp1 deletion and not simply secondary to cleft lip and primary palate defects. The authors may want to include in this argument the in vitro palatal shelf fusion assay data that would seem to further strengthen this contention.
6. Figure 4 could be made more intuitive and better described. For example, schematics in 4B and 4D could be better described in the legend and could be reconstructed to be easier to understand. In my opinion, inverting the order of the AS events in WT Ectoderm vs WT mesenchyme and WT ectoderm vs Esrp1 KO ectoderm (left-right in Venn diagram and top-middle of key) would make this figure more intuitive.
7. Nomenclature should be used more consistently. E.g. “CL/P” represents both “cleft lip and/or cleft palate” and “cleft lip with or without palate”, which are typically considered separately.
8. Bebee et al should be cited after the sentence beginning in line 68.
9. Gene and protein nomenclature should be checked for accuracy and consistency.

Noted typos
1. Line 79: “canonical Wnt targets genes and reduced…”
2. Line 112: “null mutation in Esrp1 have clefts of the lip, ….”
3. Line 93: “Conditional ablation…”
4. Line 166: “Esrp1-/-E16.5”
5. Line 266: “Esrp1 KO”
6. Line 703: repeated sentence
7. Line 782: “pairs per replicated”

Reviewer 3

Advance summary and potential significance to field

Lee et al., present a follow up study to the Esrp1-/- mutant analysis that also develop C/LP which is a new model for finding novel genes in C/LP. Here, Lee et al., use the Crect mice to conditionally delete Esrp1 in the surface ectoderm starting at E8.5 and provide the phenotypic analysis followed by gene expression analysis of ectoderm and mesenchyme population RNA-seq. Their rationale was to query the mechanism underlying Esrp1 function in the facial and oral ectoderm and identify splice isoform specific genes that function in face and palate development. This new line of work is based on solid and emerging evidence that tissue restricted splicing regulators can coordinate gene regulatory networks.

Comments for the author

The results are interesting, genetics are thorough (Figure 1-3) and they find a new role of Esrp1 in secondary palate formation using the Triaka allele and the conditional Esrp1 mutant.
My main critique is the following: The phenotypic analysis is qualitative and recapitulate known function of Esrp1 in C/LP and cell proliferation (Figure 1-3) and the genome wide data are not analyzed to reveal extensively new mechanisms and identify new genes (Figure 4-6).

Some other concerns:
Figure1: Efficiency of Esrp1 deletion needs to be shown in the embryos at the mRNA level (from the sequencing data) or other methods.

In Figure 4 can they mine the data for additional novel splice variants other than known candidates such as FGFR2-IIIb and IIIc. They will need to validate these new targets or show that their mutants have C/LP. The KEGG pathway analysis is not informative or followed up. It is far to generic. GSEA analysis might yield more rigorous results with significance values.

Figure 5/6: They focus entirely on Wnt signaling levels. If Wnt signaling is the mediator, what is the mechanism. Are the splice isoform specific variants of Wnt pathway components? How does Esrp1 regulate Wnt signaling levels or Retinoic Acid pathway components such as Aldh1a2 and Aldh1a3. Similarly they identify several other known genes such as Shh and others that are also well known to cause C/LP, but there is no other mechanistic analysis of these differentially expressed genes.

Figure 5: The authors clearly demonstrate the Wnt signaling levels are diminished in the ectoderm and more in the mesenchyme. The relationship of Esrp1, Wnt, Retinoic acid, Shh pathway is tenuous and not well developed.

First revision

Author response to reviewers' comments

We thank the editors and reviewers for their attention and thoughtful critiques on our manuscript that further characterizes cleft lip and cleft palate in Esrp1 mutant mice. Overall we appreciated enthusiasm for the results of these studies and their potential to further understand pathways that, when disrupted, lead to orofacial clefting disorders in human patients. We have carefully reviewed the critiques and made modifications to the manuscript to address them. Below we outline each of the reviewer comments and clarify how they were addressed in the resubmission. Where there were modifications to the manuscript to address the critiques we used red type. In addition, the manuscript was edited to conform to the word limit. Finally, we note that the RNA-Seq data have been submitted to the Gene Expression Omnibus under accession GSE144853 and are now publicly available.

In this paper, the authors characterize the cleft lip and palate phenotype observed in mice in which the epithelial-specific splicing factor Esrp1 has been ablated in the ectoderm. The paper extends previous research on Esrp1 (and Esrp2) by demonstrating that the effects are epithelial-specific (two new mouse lines have been generated) and by characterizing the downstream consequences. Specifically, the authors demonstrate that: the effects on lip and palate development are distinct; that mesenchymal proliferation is reduced in both the lip and palate; that cell death in epithelial seams is affected; and that, in addition, to numerous splicing events being disrupted, several cell signalling pathways are affected. In the latter case, the authors have documented that the WNT and SHH signaling are down-regulated. The paper is well-written, the conclusions drawn are well-founded, and the discussion is appropriate. Unfortunately, the paper has not been able to elucidate the mechanism(s) by which WNT and SHH signalling are affected.

We thank the reviewer for the generally positive comments on our manuscript. With respect to the mechanism by which Esrp1 ablation leads to altered Wnt and Shh (as well as alterations in other signaling molecules) please see the detailed comments addressing this issue at the end of this response letter following the point by point responses to the other reviewer critiques.

Reviewer 1 Comments for the author

I have a few comments that the authors might like to consider.

The abstract is rather general in nature; more specific detail would help to 'sell' the paper!

We have edited the abstract and are confident the reviewer will agree that it is improved.

A word appears to have been omitted on line 112

We have added “clefts” to correct the sentence.

In figure 1, the nomenclature used to denote the Esrp1 genotypes is not consistent

We have corrected the Figure to use consistent genotypes and that directly match those used in the legend.

The quality of fig. 1C is lower than that of other parts of the same figure.

We crossed Esrp1+/- and Esrp1Triaka/+ mice to generate two additional Esrp1Triaka/- heads (in the Carstens lab) and controls in order to obtain higher quality pictures from fresh samples. (Previous images were from samples sent to us from the Krebs lab after a more prolonged period in formalin). We hope the reviewers agree these new images are much higher quality and show more clearly a cleft of the secondary palate, but not of the lip or primary palate.

In fig 3, is the reduced cell proliferation observed in the epithelium and mesenchyme of Esrp1-/- mice statistically-significant?

We have corrected the figure to indicate that both differences are statistically significant based upon 3 replicates each for control and Esrp1 KO palate sections. The legend has been updated to indicate this.

Are the gene expression changes presented in fig. 5A statistically-significant?

We have corrected the figure and legend to indicate statistical significance.

Reviewer 2 Advance summary and potential significance to field

The manuscript by Lee et al examines the morphological, cellular, and molecular bases of cleft lip and palate malformations resulting from loss of the epithelial-specific splicing factor Esrp1. This is an important area of investigation as orofacial clefts are common human birth defects and because our understanding of cleft lip in particular is limited by the relative scarcity of mouse models that

recapitulate this outcome. The present manuscript follows this group's report of cleft lip and palate resulting from Esrp1/2 deletion and represents a substantive advance in describing mechanisms by which aberrant splicing may result in orofacial clefts, including a number of key insights enumerated below. The manuscript is well crafted and the data are presented clearly and interpreted carefully and evenly. Enthusiasm is diminished by the mechanistic gap between altered splicing and downstream changes in gene expression that likely drive well documented cell proliferation and fusion defects. Addressing this gap experimentally and attending to several other minor issues noted below would strengthen this manuscript and increase its impact in the field.

We thank the reviewer for the summary and highlights of our manuscript. With respect to the mechanistic gap between altered splicing and downstream changes in gene expression, please see our response to this critique at the end of our point by point response to each reviewer's specific comments.

Manuscript highlights

1. The evidence supporting the importance of epithelial-mesenchymal crosstalk in this model is compelling. The authors show that Esrp1 expression is epithelial-specific and that its deletion results in reduced mesenchymal proliferation and attenuated outgrowth of facial growth centers that form the upper lip.

2.Identification of epithelial versus mesenchymal splice variants and overlap of Esrp1 null epithelial splice forms with wild-type mesenchyme-specific variants.

3.The tissue-specific RNA-seq experiments, while somewhat technically challenging, appear to have yielded robust results that are anchored by careful validation of known epithelial and mesenchymal specific transcripts.

Reviewer 2 Comments for the author

Essential revision

1.The authors show that deletion of epithelial Esrp1 alters expression levels of key genes in the adjacent mesenchyme and that disruption of this crosstalk likely plays a major role in clefting pathogenesis in this model. The authors also provide evidence implicating Wnt signaling and, to a lesser extent, Shh signaling as key factors potentially involved in this pathogenic mechanism. Of the many changes observed, focusing on Wnt and Shh is a reasonable choice as these signaling pathways have been shown to act as central mediators of epithelial-mesenchymal crosstalk in orofacial morphogenesis. However, whether Esrp1 function directly regulates Wnt and Shh signaling and whether the expression changes observed in Wnt and Shh genes (and reduced Wnt reporter activity) are operational in the pathogenic mechanism of clefting in this mouse model remains unclear. The authors are commended for stating this limitation upfront in the discussion section but should further explore experimental approaches to examine the involvement of these pathways. For example pharmacologic and genetic Wnt pathway activation (in utero administration of small molecule DKK inhibitor or genetic deletion of secreted Wnt antagonists, including Dkk1 or Wise) has been shown to rescue cleft palate resulting from deletion of Pax9, which acts upstream of Wnt signaling in orofacial morphogenesis (PMIDs 28692808 and 28893947). In utero exposure to a pharmacological activator of Shh signaling (PMID: 27801979) could also be applied to examine the pathogenic role of Shh signaling changes in this model.

We agree with the reviewer that further experiments to explore the role of these expression changes will be important. With respect to the experiments carried out in the Pax9 KO model, we agree that these are very interesting and important papers. However, the Pax9 KO model has been well characterized over many years and these papers focus on cleft secondary palate. The Esrp1 KO mice are a new model and our primary focus has been on clefting of the lip and primary palate and indeed our RNA-Seq are related to this timepoint and do not cover gene expression changes during formation of the secondary palate. Furthermore, the efficacy of the rescue procedures used in these papers for primary palate development remains unclear and would be more problematic and technically challenging to use in earlier stage embryos at time points necessary to potentially rescue CL/P. Therefore, although great examples of rescue, it is not clear that this approach is

applicable to the problem we are studying. However, we are excited that we can establish models with cleft lip/primary palate as well as a model with only cleft secondary palate (Esrp1Triaka/- mice) suggesting that different mechanisms, or different thresholds for a common mechanism, must distinguish these processes. We intend to follow up with these studies in the future for the cleft secondary palate model, but that is outside the scope and timeline of the current manuscript.

Minor issues

1.The penetrance of cleft subtype phenotypes (CL, CP, CL+CP) should be clearly stated for each newly described mouse model and cross.

In our initial publication on the Esrp1 KO mice we clarified that bilateral cleft lip associated with cleft palate was fully penetrant based on examination of over 40 Esrp1 KO embryos. We have added the reference to the manuscript that mentions this in the section of the introduction as suggested by reviewer critique # 8. With respect to Esrp1Triaka/- mice, we have added to the results section that all seven of seven such animals examined showed clefting of the secondary palate, but not of the primary palate or lip (including an additional two that were generated in our lab in order to obtain a better image for Fig. 1C). We also have added a brief mention that there was some hypoplasia of the faces in these compound mutant mice.

2.While the data appear generally to be of high quality, light images depicting facial phenotypes could be clearer, which would allow phenotypes in these animals to be more readily compared to those in other published models of orofacial clefts. For example, simply submerging the heads in PBS should yield much clearer images of upper lip and palate phenotypes.

We agree with the reviewer that in retrospect these embryos should have been imaged while submerged to reduce reflection. Unfortunately, we no longer have these embryos available to reimage. Nevertheless, we believe that the general conclusions concerning clefting of the lip and secondary palate are still apparent in the images and we note that this minor point was not raised by reviewers 1 and 3. Please note that we can remake equivalent embryos and reimage them if this is required, but this will take several months.

3.In the same vein, the quality of the images shown in Figure 1C is poor, making qualitative phenotypic comparison of upper lip phenotype difficult. Given that these data are not central to the narrative, the authors may consider downplaying these results. Alternatively, higher quality images with consistent positioning across experimental groups should be provided.

We agree that the images of Esrp1Triaka/- mice in Fig 1C are less clear than desired and have taken new images that we hope the reviewer agrees address this critique, as was also raised by Reviewer 1.

4.Some rationale for selecting the 17 cassette exon events to validate by RT-PCR should be provided.

The validations were largely performed for cassette exons (or skipped exons(SE)) splicing events with FDR values of 0 and the largest changes in Percent Spliced In (PSI). We have added this detail in the text along with an additional directed reference to Table S1 where these events are ranked by these criteria. We also added 2 additional validations (Plekha1 and Inpp4a) as these were also near the top of the ranked list. In addition, we reordered these validations to match their relative rank in Table S1.

5.The authors outline data supporting the premise that cleft secondary palate is a distinct manifestation of Esrp1 deletion and not simply secondary to cleft lip and primary palate defects. The authors may want to include in this argument the in vitro palatal shelf fusion assay data that would seem to further strengthen this contention.

We thank the reviewer for making this excellent point and have added this argument at the end of the paragraph describing these findings.

6.Figure 4 could be made more intuitive and better described. For example, schematics in 4B and 4D could be better described in the legend and could be reconstructed to be easier to understand. In my opinion, inverting the order of the AS events in WT Ectoderm vs WT mesenchyme and WT ectoderm vs Esrp1 KO ectoderm (left-right in Venn diagram and top-middle of key) would make this figure more intuitive.

We have edited the figure legend to help make these figures more intuitive. We also reordered the legend included in Figure 4D to match the order of the Venn diagram from left to right.

7.Nomenclature should be used more consistently. E.g. “CL/P” represents both “cleft lip and/or cleft palate” and “cleft lip with or without palate”, which are typically considered separately.

We have corrected the text to indicate CL/P only in reference to cleft lip with or without cleft palate and used CP or CPO to indicate isolated cleft palate

8.Bebee et al should be cited after the sentence beginning in line 68.

Done.

9.Gene and protein nomenclature should be checked for accuracy and consistency.

We have gone through and corrected all (I believe!) gene and protein nomenclatures.

Noted typos
1.Line 79: “canonical Wnt targets genes and reduced…”
2.Line 112: “null mutation in Esrp1 have clefts of the lip, ….”
3.Line 93: “Conditional ablation…”
4.Line 166: “Esrp1-/-E16.5”
5.Line 266: “Esrp1 KO”
6.Line 703: repeated sentence
7.Line 782: “pairs per replicated”

We have corrected all of these errors.

Reviewer 3 Advance summary and potential significance to field
Lee et al., present a follow up study to the Esrp1-/- mutant analysis that also develop C/LP, which is a new model for finding novel genes in C/LP. Here, Lee et al., use the Crect mice to conditionally delete Esrp1 in the surface ectoderm starting at E8.5 and provide the phenotypic analysis followed by gene expression analysis of ectoderm and mesenchyme population RNA-seq. Their rationale was to query the mechanism underlying Esrp1 function in the facial and oral ectoderm and identify splice isoform specific genes that function in face and palate development. This new line of work is based on solid and emerging evidence that tissue restricted splicing regulators can coordinate gene regulatory networks.

Reviewer 3 Comments for the author

The results are interesting, genetics are thorough (Figure 1-3) and they find a new role of Esrp1 in secondary palate formation using the Triaka allele and the conditional Esrp1 mutant. My main critique is the following: The phenotypic analysis is qualitative and recapitulate known function of Esrp1 in C/LP and cell proliferation (Figure 1-3) and the genome wide data are not analyzed to reveal extensively new mechanisms and identify new genes (Figure 4-6).

In our previous manuscript in which we first identified CL/P in Esrp1 KO mice (Bebee et al., 2015), the analysis was limited to showing gross morphology and bone /cartilage stains of Esrp1 KO heads. In this manuscript we extend the phenotypic analysis to clearly demonstrate reduce proliferation during lip and secondary palate formation. Furthermore, we show that there is also a failure in fusion of the lip and in palatal explants, indicating that reduced proliferation alone does not cause these defects. This manuscript is also the first global analysis of alternative splicing during face formation using this model in which alterations in this post-transcriptional process lead to CL/P.

This analysis indeed identified changes in the expression of numerous genes, including many that function in pathways known to be essential for lip and/or palate formation. Thus, it will be a major undertaking to further interrogate each of these pathways in future studies using this novel CL/P model (as will be addressed in further detail at the end of the critiques).

Some other concerns:
Figure1: Efficiency of Esrp1 deletion needs to be shown in the embryos at the mRNA level (from the sequencing data) or other methods.

The sequencing data was derived from mice with germline deletion of Esrp1 which we previously showed was complete. We suspect that the reviewer is referring to the efficiency of Crect induced conditional ablation. While we did not isolate RNAs from Esrp1flox/flox; Crect +/- embryos at earlier embryonic stages, we did perform RT-qPCR analysis from epidermis of E18.5 embryos to validate conditional ablation in this ectoderm derived cell population. These data, which are shown in new Supplemental Figure S2, show that Crect induced Esrp1 ablation is fairly complete (~93%). Note that the primers for this analysis targeted floxed exons 7-9, whereas even with Esrp1 KO mice, we previously noted that pre-mRNA splice from exon 5 to 10 when these exons are deleted such that total mRNA levels are less completely reduced (but also out of frame to nonetheless still generate non-functional transcripts).

In Figure 4 can they mine the data for additional novel splice variants other than known candidates such as FGFR2-IIIb and IIIc. They will need to validate these new targets or show that their mutants have C/LP.

While we have identified splicing changes in numerous gene transcripts, we have not found direct evidence to direct us towards which of the isoform switches contribute to CL/P. Testing some of these candidates, perhaps using isoform specific rescue of the phenotype or by creating new mouse mutants, will be a longer term goal of future studies.

The KEGG pathway analysis is not informative or followed up. It is far to generic. GSEA analysis might yield more rigorous results with significance values.

Several of us – independently – ran analyses using ENRICHR, DAVID and GSEA on the rMATS, DE and combined datasets. Beyond the enrichment analysis in the manuscript nothing rose to the level of high significance with the exception of rather general terms (e.g. glycoprotein, secreted, developmental protein, multicellular organism development etc.) that were not informative.

Figure 5/6: They focus entirely on Wnt signaling levels. If Wnt signaling is the mediator, what is the mechanism. Are the splice isoform specific variants of Wnt pathway components? How does Esrp1 regulate Wnt signaling levels or Retinoic Acid pathway components such as Aldh1a2 and Aldh1a3. Similarly they identify several other known genes such as Shh and others that are also well known to cause C/LP, but there is no other mechanistic analysis of these differentially expressed genes.

Please see response to the next critique.

Figure 5: The authors clearly demonstrate the Wnt signaling levels are diminished in the ectoderm and more in the mesenchyme. The relationship of Esrp1, Wnt, Retinoic acid, Shh pathway is tenuous and not well developed.

These critiques, which are similar to comments by the other reviewers, illustrate one of the major challenges that we will need to address as we carry these studies forward. It is clear that all three reviewers express enthusiasm for the study and recognize that it represents a substantive advance in showing how tissue restricted splicing factors can coordinate numerous gene regulatory networks. However, the broad effects of Esrp1 ablation on numerous signaling pathways presents a major challenge in understanding the relative contributions of alterations in each of these pathways to the observed phenotype. Our findings that Esrp1 functions in both lip and secondary palate formation provides the field with two new mouse models to further interrogate the roles of each of these pathways. We are also very interested in the mechanism of ESRP1 function during facial development and believe that this manuscript provides significant advances towards addressing this question. However, we agree that we have not definitely answered this question. In

fact, given the complexity of the changes observed in multiple developmentally significant pathways, we expect that this will occupy our labs for the next 5+ years. At this time there are no obvious answers to how altering splicing affects downstream events as we are impacting the regulation of several signaling pathways including Fgf, Wnt, Shh, TGF-beta, Hippo, and RA via changes in isoform expression. Although we could focus on one particular pathway, e.g. Wnt or Shh, at this time, our current hypothesis is that loss of Esrp1 is affecting many pathways and that the overall clefting phenotype reflects the cumulative influence on several signaling mechanisms. It may also turn out that rescue of one pathway to rescue proliferation (e.g. Wnt9b) would not rescue fusion, which might be regulated through another pathway or pathways. In the discussion section of the revision we have added text to highlight these challenges. We appreciate the suggestions of reviewer 2 concerning attempting to rescue the phenotype via pharmacological or genetic intervention. However, the procedures they refer to have not been tested for efficacy in rescuing clefting of the lip and primary palate. We are now designing experiments to test these ideas, but such studies will take considerably longer than the 90 days required to submit a revised manuscript – as they will involve importation of new strains of mice as well as several rounds of breeding to obtain the desired genotypes. For example partial genetic rescue of CL/P has been in some models by ectopically expressing Wnt1 from a Lox-Stop-Lox allele (Ferretti et al., 2011). However, in unrelated studies we note that activation of Wnt1 in the ectoderm causes its own craniofacial developmental defects such that the interpretation of findings is not straightforward. As noted, we do intend to follow up on these studies, but expect that a definitive mechanistic answer concerning how Esrp1 KO impacts CL/P will require many more years of focused research effort. At the same time, we believe that the results as they stand are meritorious and add significant new insight into how alternative splicing impacts facial development – and that these results are timely and of general interest to the wider scientific community.

Second decision letter

MS ID#: DEVELOP/2019/187369

MS TITLE: Cleft lip and cleft palate (CL/P) in Esrp1 KO mice is associated with alterations in Wnt signaling and epithelial-mesenchymal crosstalk

AUTHORS: Sungkyoung Lee, Matthew J. Sears, Hong Li, Imad Salhab, Philippe Krebs, Zijun Zhang, Yi Xing, Hyun-Duck Nah, Trevor Williams, and Russ P. Carstens

I have now received all the referees reports on the above manuscript. 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 from the reviewers' comment, they are positive about the revision and the response to the previous review and expressed interest in seeing this work published. However, they noted that the key issue of the mechanistic connectivity of the activity of Esrp1 on the expression of the spliced transcripts and changes in the function of the spliced genes with WNT signalling activity and lip/palate morphogenesis remained unresolved. I do understand that the constraint of research resource and time would impact on the feasibility/desirability to conduct further experiments to address this issue. In view of that such mechanistic insight will not be provided in this phenomenology study, it may not be appropriate to present WNT signalling as the prime molecular pathway that may mediate the downstream activity of Esrp1 in palate/lip morphogenesis. One suggestion is to highlight instead the potential role/s of the multitude of signalling activities identified in the transcriptome, with some of which known to play a role in palate/lip morphogenesis.
Since the overall evaluation is positive, we are prepared to publish a revised manuscript, provided that the revisions can be made along the lines suggested (see Editor's note and reviewer reports). Please highlight the revision as marked text in the revised manuscript. If you do not agree with any of the suggestions, please explain clearly why this is so.

Editor's note

Edits to be considered:

- Title: Cleft lip and cleft palate (CL/P) in Esrp1 KO mice is associated with alterations epithelial-mesenchymal crosstalk
- Running title: altered tissue crosstalk and cleft lip/palate
- Keywords: Cleft lip; cleft palate; epithelial-mesenchymal crosstalk; lip morphogenesis
- Abstract line 18-19: … altered expression of genes previously implicated in cleft/lip and/por palate, including components of multiple signalling activities.
- Line 216-264: Please provide separate worksheets in Table S3 summarizing the up- and down-regulated genes and alternatively spliced transcripts (which may encode protein with altered function, such as the FGFR) that are components of the signalling pathways: WNT, FGF, SHH, RA and Hippo, and the respective downstream response genes. Refer to these findings in the Results to support the inference that loss of Esrp1 function was accompanied by changes (elevation or reduction) of multiple signalling pathway activities, some of which may, based on prior knowledge, play an essential role in lip/palate morphogenesis.
- Reference to a broader dataset on singaling pathways may help toning down the focus on WNT signalling, where being "most enriched" (line 237) or "most conspicuous" (line 257) may not justify the assertion this is the most critical pathway to mediate the activity of Espr1 in lip/palate morphogenesis (Line 412-421).
- The finding of Lef1 (line 418-424) is interesting, However, the connection between splicing variants and Lef1 expression (by reporter assay) is not known, and ESPR1 interaction with WNT genes had not been established. This part of the discussion may be more suited for the section on the splicing events in the Esrp1-null tissues (Line 331-356)
- Discussion Line 433-439: "Hence, while rescue of Wnt activity….. from our group (Lee et al., 2018)": This commentary on the rescue experiment and the argument for not performing the functional work is superfluous.

Reviewer 1

Advance summary and potential significance to field

This is an amended version of a manuscript documenting the effect of loss of Esrp1 function on facial development. The manuscript is well-written and provides an advance in our understanding of the molecular events underpinning development of the lip and palate. The authors have addressed all the points that I raised in my review of the first submission. While I will leave other reviewers to comment on the responses to the changes that they suggested, I am confident that the data presented provide major new insights into cleft lip and palate.

Comments for the author

No additional comments

Reviewer 2

Advance summary and potential significance to field

The advancements outlined in my original review are maintained in this revised manuscript.

Comments for the author

The authors satisfactorily addressed most of the initial comments and concerns from reviewers. The central critique expressed similarly from each of the three reviewers was not addressed experimentally. Rather, the authors more directly address the gap in mechanism as an important and challenging area of future investigation. I do accept their argument that the findings presented in this manuscript provide a step forward and foundation to attack those challenging

questions. While leaving a large gap in mechanism, I do think that this work is a solid advancement and will be a valuable contribution to the field whether it is published in Development or elsewhere.

Reviewer 3

Advance summary and potential significance to field

Lee et al., present a follow up study to the Esrp1-/- mutant analysis that also develop C/LP which is a new model for finding novel genes in C/LP. Here, Lee et al., use the Crect mice to conditionally delete Esrp1 in the surface ectoderm starting at E8.5 and provide the phenotypic analysis followed by gene expression analysis of ectoderm and mesenchyme population RNA-seq. Their rationale was to query the mechanism underlying Esrp1 function in the facial and oral ectoderm and identify splice isoform specific genes that function in face and palate development.

Comments for the author

I thank the authors most of the minor critiques to improve the presentation of the paper and statistical rigor. However, the revised paper still does not address the main critique from all three reviewers. The revised manuscript still does not show if the effect on Wnt signaling are either direct effect of Esrp1 and functional. Similarly they identify several other known genes that are also well known to cause C/LP, but there is no other mechanistic analysis of these differentially expressed genes. We understand this is a significant undertaking, but some pharmacological or genetic effort is needed for publication here.

Second revision

Author response to reviewers' comments

We appreciate the input from the reviewers and the editor on our resubmission. The reviewers agreed that the study provides new insights into lip and palate development and is a valuable contribution to the field. While it was noted that the revision did not completely identify a clear mechanistic link between Esrp1 ablation and alterations in multiple signaling pathways, it was appreciated that this would be a significant effort and two of the reviewers agreed that the manuscript merited publication despite not resolving this gap. As suggested by the editor, given the complexity of the gene and pathway alterations in Esrp1 KO mice, we have reduced the emphasis on the role of Wnt signaling in the CL/P phenotype to highlight the fact that we observed alterations in several genes and pathways that are known to play a role in lip and palate morphogenesis. Below, we address each of the edits suggested by the editor in a revised manuscript.

Editor’s note

Edits to be considered:

• Title: Cleft lip and cleft palate (CL/P) in Esrp1 KO mice is associated with alterations epithelial-mesenchymal crosstalk

Done

• Running title: altered tissue crosstalk and cleft lip/palate

Done

• Keywords: Cleft lip; cleft palate; epithelial-mesenchymal crosstalk; lip morphogenesis

Done

• Abstract line 18-19: … altered expression of genes previously implicated in cleft/lip and/por palate, including components of multiple signalling activities.

Done

• Line 216-264: Please provide separate worksheets in Table S3 summarizing the up- and down-regulated genes and alternatively spliced transcripts (which may encode protein with altered function, such as the FGFR) that are components of the signalling pathways: WNT, FGF, SHH, RA and Hippo, and the respective downstream response genes. Refer to these findings in the Results to support the inference that loss of Esrp1 function was accompanied by changes (elevation or reduction) of multiple signalling pathway activities, some of which may, based on prior knowledge, play an essential role in lip/palate morphogenesis.

We appreciate this excellent suggestion. However, the transcriptomic changes we focused on included separate supplemental tables for splicing changes in ectoderm, gene expression changes in ectoderm, and gene expression changes in mesenchyme. Therefore, rather than adding a summary of these genes and pathways to a tab in Table S3 (which shows gene expression changes in ectoderm), we instead created a new Table S6 in which we curated a list of genes within several of these pathways that were altered at the level of splicing or gene expression in ectoderm and/or mesenchyme. This table is referenced in the results (lines 258-258) and the discussion (lines 422-423).

• Reference to a broader dataset on singaling pathways may help toning down the focus on WNT signalling, where being “most enriched” (line 237) or “most conspicuous” (line 257) may not justify the assertion this is the most critical pathway to mediate the activity of Espr1 in lip/palate morphogenesis (Line 412-421).

We changed lines 237 and 257 to delete the most enriched or most conspicuous qualifiers and modified the text to refer to multiple pathways including the reference to new Table S6 (lines 256-258). We have also made several changes in lines 412-424 to state that while Wnt is one of the affected pathways, there are several other relevant pathways that are also altered.

• The finding of Lef1 (line 418-424) is interesting, However, the connection between splicing variants and Lef1 expression (by reporter assay) is not known, and ESPR1 interaction with WNT genes had not been established. This part of the discussion may be more suited for the section on the splicing events in the Esrp1-null tissues (Lines 331-356)

Since we presently do not have evidence of a link between the change in LEF1 splicing and the other transcriptomic alterations we have elected to simply delete this sentence.

• Discussion Line 433-439: “Hence, while rescue of Wnt activity….. from our group (Lee et al., 2018)”: This commentary on the rescue experiment and the argument for not performing the functional work is superfluous.

We deleted this sentence.

We also made several additional edits to reduce the emphasis on Wnts to the detriment of other affected genes and pathways in the Introduction (lines 74-76), Results (lines 229-230), and Discussion (lines 369-369 and 379).

Note that all changes to the text (other than the indicated deletions) are highlighted in red.

Third decision letter

MS ID#: DEVELOP/2019/187369

MS TITLE: Cleft lip and cleft palate (CL/P) in Esrp1 KO mice is associated with alterations in epithelial-mesenchymal crosstalk

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ARTICLE TYPE: Research Article

I am satisfied with the revision of the manuscript. This paper has been accepted for publication in the Special Issue of Development on The Origin of Mechanisms of Developmental Disorders pending our standard ethics checks.