A developmental stage-specific network approach for studying dynamic co-regulation of transcription factors and microRNAs during craniofacial development

Fangfang Yan, Peilin Jia, Hiroki Yoshioka, Akiko Suzuki, Junichi Iwata and Zhongming Zhao
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Original submission

First decision letter

MS ID#: DEVELOP/2020/192948

MS TITLE: A developmental stage specific network approach for studying dynamic transcription factor-microRNA co-regulation during craniofacial development

AUTHORS: Fangfang Yan, Peilin Jia, Junichi Iwata, Akiko Suzuki, and Zhongming Zhao

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 interest in your work, but have some significant criticisms (please see the Editor's note appended below) and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

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

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

Editor's Note:

The critique highlights the biological validity of the predicted targets and network, as the microRNA prediction programs are not consistently accurate and potentially misleading. There are examples that the network information was used on the presumption that it is proven, but found not to hold true subsequently and causes problems for future studies. It is prudent that the findings are validated either experimentally or by drawing from prior knowledge of the targets and network in other biological models. If the restriction of access is a barrier for conducting experimental validation, it would be imperative to drill into the other published data to find corroborating evidence for the networks, which do not require further laboratory work. The merit of this study may be enhanced by the outcome of the validation.

Please address reviewer 2's comments in sufficient detail in your response/revision.

Reviewer 1

Advance summary and potential significance to field

Journal: Development
MS ID#: DEVELOP/2020/192948
MS title: A developmental stage specific network approach for studying dynamic transcription factor-microRNA co-regulation during craniofacial development
Authors: Fangfang Yan, Peilin Jia, Junichi Iwata, Akiko Suzuki, and Zhongming Zhao
Article type: Research Article
The authors use specific databases to identify gene-microRNA networks that control stage-specific craniofacial development. They employ several bioinformatic databases and techniques to find nodes and specific factors expressed during the different stages of development. They focused on a few regulatory networks and found that Wnt-FoxO-Hippo pathway and Col1a1 were important. miR-129-5p and miR-340-5p were identified as potential regulators of gene expression. This is an excellent use of the abundant murine database information available and bioinformatic programs. It is very clear and easy to understand and nice study. The concept that there are TF-microRNA interactions and networks is not new, but the manuscript does not validate any of the bioinformatics. Some of the prediction software has been shown to be only close to 45% accurate. Without testing and validation of the networks some of the conclusions may be misleading. This is inherent in using these prediction programs. But this is not the author's fault they have used these tools effectively.

Comments for the author

Overall a nice use of current murine databases for gene expression and microRNA expression analyses. Identification of TF networks and microRNA profiles is important and provides the readers with information on how these two processes work together to regulate tissue remodeling and development of the craniofacial region. The authors also determine potential pathways for CL/P. While this is an interesting report it lacks in fundamental validation of the networks. One concern is that the study does not demonstrate much new information, ideas or concepts. Most of these pathways and microRNAs have been shown to play a role in craniofacial development. Some data was gleaned from cell-based informatics and experiments and not developmental stages of craniofacial development, such as gene and miR targets. The networks, nodes and microRNA associations could be misleading without some type of validation experiments. These do not have to be in mice, but at least shown in cell-based assays. There are many reports showing in vivo association between TF's, factors and microRNAs, demonstrating networks and processes. Many of these were not discussed and could have been used to bolster the conclusions in the manuscript. Although beyond the scope of this report it would have been beneficial to included several murine models in the analyses.
An excellent use of current databases and bioinformatics and the report provides some data analysis information that other researchers could potentially use for their research projects. My main concern is that this report does not increase our knowledge of the networks beyond

interpretation of gene data sets, microRNA profiles and prediction software and the conclusions may be insufficient.
The main criterion for publication of a Research Article or Report in Development is that a paper should make a significant and novel contribution to our understanding of developmental mechanisms. The authors are well trained in bioinformatics and have published these types of data previously.

Reviewer 2

Advance summary and potential significance to field

In the manuscript by Yan, et al., the authors suggest a novel way of identifying gene regulatory modules during craniofacial development by building networks using combined mRNA and miRNA sequencing. Gene ontology and KEGG analyses were used to identify the function of the modules at several stages of mouse development (E10.5-E14.5). The authors identified function consistent with tissue re-organization and remodeling that is known to occur during murine craniofacial development between E10.5-E14.5. The authors suggest that miR-129 targets Col1a1 and that miR-340 targets FoxM to regulate craniofacial development.
While the focus of this study is the assembly of novel regulatory modules, the gap in knowledge addressed and conceptual advance was not clearly conveyed.

Comments for the author

Major Comments:
- The abstract and introduction are vague and non-specific and might benefit from known examples of modules during development.
- The manuscript does not clearly identify which cells were sequenced.
- More details should be provided to describe differences between the feedforward loops.
- The authors should give examples in the results/intro to give their readers some context to their feed forward loops (e.g. as to how transcription factors inhibit expression of miRNAs) and how this is important for craniofacial development.
- The authors should provide more biological context during the time period of interest for the regulatory modules identified in figure 2.
- The manuscript is organized such that the figures are difficult to follow in linear order.
- The authors obtain relatively few GO terms.
- The tables in figure 5 are confusing. How do the Venn diagrams correspond to the tables on the right?
- No evidence for direct miRNA targeting is provided (e.g. targeting of Col1a1 by miR-129).
- Some biological function of the regulatory hubs identified should be explored further.
- Better figure legend titles could help focus the attention of the reader.

Minor Comments:
- The introduction mentions that there is no biological mega data for CL/P and CPO. The meaning of this statement is unclear.
- Several typos and grammar problems throughout. The manuscript would be significantly improved with thorough editing.
- Grouping the data by time point might be easier for the reader to understand.

Reviewer 3

Advance summary and potential significance to field

Yan et al. presents a novel network approach for studying co-regulation between transcription factor, mRNA and microRNA in craniofacial development. They downloaded mouse data mice from the FaceBase Portal, analyzed the raw data and queried several transcription factor databases. They examined regulation between TF and gene/miRNA, regulation between miRNA and gene/TF, and constructed three-node feed-forward loops among miRNA, gene, and TF. They identified some novel stage-specific regulators and pathways, which provide a novel angle and clue for understanding craniofacial development. The paper is well written and easy to follow The approach

is novel and statistically sound. The results will advance our knowledge in craniofacial development.

Comments for the author

I have a few comments below to further strengthen the manuscript.

1. Instead of pairwise comparison between different stages, it is also worthwhile (may be more powerful) to perform DE analysis between each stage and others (treat all other stages as one group).

2. The co-regulation network is likely tissue or cell-type specific. It is unclear what type of tissue or cell types used and how the results can be generalized to other tissues and to human.
3. Pearson correlation is calculated based on a limited number of samples, so coefficient and pvalue may be unstable. Some discussion is needed. The rationale to use threshold 0.3 should be given as well.

4. Can authors comment if the newly identified co-regulation network can help interpret findings from human omics study such as genome-wide association or transcriptomic studies of craniofacial diseases.

First revision

Author response to reviewers' comments

Response to Reviewers

MS ID: DEVELOP/2020/192948

MS TITLE: A developmental stage specific network approach for studying dynamic transcription factor- microRNA co-regulation during craniofacial development

Journal: Development

Editor's Note

The critique highlights the biological validity of the predicted targets and network, as the microRNA prediction programs are not consistently accurate and potentially misleading. There are examples that the network information was used on the presumption that it is proven but found not to hold true subsequently and causes problems for future studies. It is prudent that the findings are validated either experimentally or by drawing from prior knowledge of the targets and network in other biological models. If the restriction of access is a barrier for conducting experimental validation, it would be imperative to drill into the other published data to find corroborating evidence for the networks, which do not require further laboratory work. The merit of this study may be enhanced by the outcome of the validation.

Please address reviewer 2's comments in sufficient detail in your response/revision.

Response: We thank the editor for the advice and all reviewers for their constructive comments. Even though COVID-19 has caused us a lot of inconvenience, we tried our best to perform such experimental validation work and our experiments supported our network findings. Because of substantial experimental work, we added a new author, Hiroki Yoshioka, who performed the experiments, and changed Dr. Junichi Iwata as the co-corresponding author (regarding dental biology and experimental work). In addition, we reorganized the figures in linear order to make it easier for the reader to understand.

In the revised version, we have added experimental validation results, which allowed us to justify and enhance the outcomes. We also added more details and revised other parts based on reviewers' comments. Please see our detailed point-by-point response below. The substantial changes in the revised manuscript were labeled in red.

Response to Reviewer #1

The authors use specific databases to identify gene-microRNA networks that control stage-specific craniofacial development. They employ several bioinformatic databases and techniques to find nodes and specific factors expressed during the different stages of development. They focused on a few regulatory networks and found that Wnt-FoxO-Hippo pathway and Col1a1 were important. miR-129-5p and miR- 340-5p were identified as potential regulators of gene expression. This is an excellent use of the abundant murine database information available and bioinformatic programs. It is very clear and easy to understand and nice study. The concept that there are TF-microRNA interactions and networks is not new, but the manuscript does not validate any of the bioinformatics. Some of the prediction software has been shown to be only close to 45% accurate. Without testing and validation of the networks some of the conclusions may be misleading. This is inherent in using these prediction programs. But this is not the author's fault they have used these tools effectively.

Major Comments:
Overall a nice use of current murine databases for gene expression and microRNA expression analyses. Identification of TF networks and microRNA profiles is important and provides the readers with information on how these two processes work together to regulate tissue remodeling and development of the craniofacial region. The authors also determine potential pathways for CL/P. While this is an interesting report it lacks in fundamental validation of the networks. One concern is that the study does not demonstrate much new information, ideas or concepts. Most of these pathways and microRNAs have been shown to play a role in craniofacial development. Some data was gleaned from cell-based informatics and experiments and not developmental stages of craniofacial development, such as gene and miR targets. The networks, nodes and microRNA associations could be misleading without some type of validation experiments. These do not have to be in mice, but at least shown in cell-based assays.

Response: We thank the reviewer for this critical comment. We agree that while TF-miRNA co-regulation network is an effective approach to identify potential regulators during craniofacial development, such results may either overlap with the existing knowledge or false positives. To validate and enhance our outcomes, we conducted quantitative RT-PCR analyses after overexpression of the genes and microRNAs of interest in O9-1 cells, a mouse cranial neural crest cell line. As shown in revised Figures 7 and 8, our network findings could be generally supported by experiments. Due to the scope of this work and COVID-19 pandemic, we only selected a few top candidates for experimental validation.

In the revised manuscript, pages 13-14, we added a new subsection "Experimental validations". Briefly, the experimental results supported the regulatory roles of miR-129-5p and Foxm1 in the Wnt-FoxO-Hippo subnetwork, as well as the role of miR-340-5p in the miR-340-5p-*Col1a1* subnetwork. It provides supporting evidence of our putative network (see revised Figure 2D), where Foxm1 and miR-340-5p act as promising contributors and may be involved in the pathogenic mechanism of orofacial clefts. The putative model and potential regulators are new findings in the study.

There are many reports showing in vivo association between TF's, factors and microRNAs, demonstrating networks and processes. Many of these were not discussed and could have been used to bolster the conclusions in the manuscript. Although beyond the scope of this report it would have been beneficial to include several murine models in the analyses.

Response: We thank the reviewer for pointing this out. We cited some reports that used normal murine models or xenografts models to show the regulation of TFs or miRNAs. Because our manuscript is to introduce the promising use of developmental stage specific network approach, we only cited a few references to make the manuscript neat. In the Introduction part of the revised manuscript, page 4, we added "Thomason and his colleagues used mouse models to show the

cooperation of TF p63 and gene *IRF6* in cleft palate (Thomason et al., 2010). In addition, He et al. reported the regulation of miRNA-375 in AEG-1 in hepatocellular carcinoma xenograft mouse models (He et al., 2012)."

An excellent use of current databases and bioinformatics and the report provides some data analysis information that other researchers could potentially use for their research projects. My main concern is that this report does not increase our knowledge of the networks beyond interpretation of gene data sets, microRNA profiles and prediction software and the conclusions may be insufficient.
The main criterion for publication of a Research Article or Report in Development is that a paper should make a significant and novel contribution to our understanding of developmental mechanisms. The authors are well trained in bioinformatics and have published these types of data previously.

Response: We thank this reviewer for this critical point. While network approach has been applied in many studies, this study represents the first developmental stage specific network approach by integrating two critical regulators, transcription factor (TF) and microRNA (miRNA), to study their co- regulation during craniofacial development. As more and more omics data have been generated recently (e.g. FaceBase project), and more will be generated in the near future (e.g. single cell level gene expression and enhancer data for craniofacial development), it is important to develop and apply novel analytical approaches to mine such complex and often heterogeneous data. Our approach in this work is expected to be effective for this purpose, and it can be applied to more time points during craniofacial development.

In revision, we further enhanced the outcomes and conclusions through experimental validations. We pointed out several promising contributors of orofacial clefts and a putative model that may play a role in the pathogenic mechanism of orofacial clefts. We believed this work introduces a new stage specific network approach that is important for craniofacial development data, and our study has increased current knowledge and understanding of developmental mechanisms.

Response to Reviewer #2

In the manuscript by Yan, et al., the authors suggest a novel way of identifying gene regulatory modules during craniofacial development by building networks using combined mRNA and miRNA sequencing. Gene ontology and KEGG analyses were used to identify the function of the modules at several stages of mouse development (E10.5-E14.5). The authors identified function consistent with tissue re-organization and remodeling that is known to occur during murine craniofacial development between E10.5-E14.5. The authors suggest that miR-129 targets Col1a1 and that miR-340 targets FoxM to regulate craniofacial development. While the focus of this study is the assembly of novel regulatory modules, the gap in knowledge addressed and conceptual advance was not clearly conveyed.

Major Comments:
- *The abstract and introduction are vague and non-specific and might benefit from known examples of modules during development.*

Response: We thank the reviewer for this valuable comment. In response, we have revised abstract and introduction to make them more specific. Known examples of modules during lung and craniofacial development were also added. In the revised manuscript, page 5, we added "In addition to these diseases, FFLs have also been applied in lung and craniofacial development. Fibroblast growth factor (FGF) forms an FFL with canonical molecules *Wnt2a* and *Wnt7b* in the WNT signaling to regulate lung development (Yin et al., 2011). The transcription factor MEF2C has been found to be involved in a feed- forward transcriptional circuit with Dlx5/6 and Hand2 and exhibit unanticipated role in craniofacial development (Verzi et al., 2007). Li et al. showed that the miRNA hsa-mir-27b represses the expression of transcription factor SMAD1 and accordingly activates WNT pathway core gene *WNT3A* through FFL during lip development (Li et al., 2020)."

- *The manuscript does not clearly identify which cells were sequenced.*

Response: We are sorry for not making this clear in the previous manuscript. The sequenced cells come from the maxillary processes of C57BL/6J mouse embryos. In the first paragraph of the Methods section, page 20, we wrote "The mouse gene expression data of 30 C57BL/6J samples isolated from the maxillary processes at embryonic days 10.5, 11.5, 12.5, 13.5 and 14.5 (E10.5-E14.5) was downloaded from the FaceBase Portal."

- *More details should be provided to describe differences between the feedforward loops*.

Response: As suggested by the reviewer, in the revised manuscript, page 7, we added "For example, if a gene is activated by a TF and repressed by a miRNA, then the regulation type between this TF and miRNA can only be repression. The classifications of FFLs depend on the regulation type between TF, gene, and miRNA. In TF-FFL loop, TF is the master regulator and dominates expression of gene and miRNA. In miRNA-driven FFL loop, miRNA's expression affects gene and TF. In composite-based FFL, in which TF and miRNA repress each other, gene's expression is mainly affected by the regulator in the FFL with higher expression change (Sun et al., 2012)."

- *The authors should give examples in the results/intro to give their readers some context to their feed forward loops (e.g. as to how transcription factors inhibit expression of miRNAs) and how this is important for craniofacial development.*

Response: As suggested by the reviewer, in the revised manuscript, page 5, we added an example of FFL in cancer, see "Jiang et al. recognized frequently dysregulated TF-miRNA FFLs across multiple tumor types (Jiang et al., 2016)". We also added an example of FFL in craniofacial development, see "Li et al. showed that the miRNA hsa-mir-27b represses expression of transcription factor SMAD1 and accordingly activates WNT pathway core gene *WNT3A* through FFL during lip development (Li et al., 2020)."

The figures representing feed forward loops in the above-mentioned examples are shown below for the reviewer's convenience.

We have removed unpublished data provided for the referees in confidence.

- *The authors should provide more biological context during the time period of interest for the regulatory modules identified in figure 2.*

Response: We thank the reviewer for this valuable suggestion. As suggested by the reviewer, we added more biological context for the regulatory modules. Please see pages 9, 10, and 11 of the revised manuscript.

- *The manuscript is organized such that the figures are difficult to follow in linear order.*

Response: We are sorry for this problem. In revision, we attempted to enhance it. The figures have been reorganized and can be followed in linear order now. See revised Figures 2-6.

- *The authors obtain relatively few GO terms.*

Response: We thank the reviewer for raising this concern. Actually, only those GO terms with less than 300 genes were retained in this study because large GO terms tended to be general or in high hierarchical level (Jia et al., 2011). We obtained relatively few GO terms only for network from E11.5 to E12.5. We obtained 17, 18, 20 GO terms for E10.5 to E11.5, E12.5 to E13.5, E13.5 to E14.5, respectively.

Reference:
1. Jia, P., Wang, L., Meltzer, H. Y., & Zhao, Z. (2010). Common variants conferring risk of schizophrenia: a pathway analysis of GWAS data. *Schizophrenia Research*, *122*(1-3):38-42.

- *The tables in figure 5 are confusing. How do the Venn diagrams correspond to the tables on*

the right?

Response: We are sorry for the confusion. In revision, we adjusted the figures and highlighted the numbers in *Venn* diagrams that connect to the tables. See the revised Figure 6.

- *No evidence for direct miRNA targeting is provided (e.g. targeting of Col1a1 by miR-129).*

Response: We thank the reviewer for this important question. The miRNA targeting information is curated from databases. To control false discovery rate, we kept only the experimentally validated miRNA-target pairs (miRTarBase) plus the pairs predicted by at least two computational algorithms (PITA, TargetScan, and miRanda). The evidence of miR-129 targeting Col1a1 was computationally predicted by both miRanda and TargetScan. Detailed information can be found on page 21: "The miRTarBase is a comprehensive database including experimentally validated miRNAs-target gene interactions (Chou et al., 2018). It deposits 959 unique miRNAs and 7280 target genes with a total of 40,681 pairs in mice (Release 7.0). In addition to the miRTarBase, computationally predicted miRNA-target gene pairs were collected from PITA (Version 6) (Kertesz et al., 2007), TargetScan (Release 7.2) (Agarwal et al., 2015), and miRanda (Released on August in 2010) (Betel et al., 2008) (Supplemental Table 3). To control false discovery rate, only the experimentally validated miRNA-target pairs were collected in miRTarBase plus the pairs predicted by at least two computational algorithms (PITA, TargetScan, and miRanda). In total, 130,608 unique miRNA-target pairs were retrieved including 1,183 miRNAs and 11,958 targets. Pearson correlation coefficient was calculated for each miRNA-target pair."

- *Some biological function of the regulatory hubs identified should be explored further.*

Response: We thank the reviewers for this valuable suggestion. In revision, more description about the biological function of the regulatory hubs have been added.

On page 9, we wrote "Based on the definition, we pinpointed one hub miRNA (miR-340-5p, degree=35) and three hub TFs (Foxm1, degree=29; Hif1a, degree=20; and Zbtb16, degree=19), where miR-340-5p can reduce mesenchymal traits and cell migration and Foxm1 may be involved in cell proliferation and epithelial-to-mesenchymal transition (EMT) (Kim et al., 2019; Luo et al., 2019), suggesting their important regulatory roles in the network (Fig. 2A)."

On page 10, we wrote" In literature, muscle-specific TFs, Myog and Myod1, play a role in the formation of myofibers (Rosero Salazar et al., 2020)."

On page 11, we added "As muscle-specific TFs, Myog and Myod1 play a role in the formation of myofibers (Rosero Salazar et al., 2020)". We also added "Tcf7 is an HMG box protein that associated with molecules in the nucleus to modulate Wnt signaling pathway (Cadigan & Waterman, 2012)."

- *Better figure legend titles could help focus the attention of the reader.*

Response: We thank the reviewer for this valuable suggestion. In revision, the figure legends and titles have been enhanced. We hope it is clear to the reader, but we are happy to enhance further. See figure legends of the revised Figures 2-6.

Minor Comments:

- *The introduction mentions that there is no biological mega data for CL/P and CPO. The meaning of this statement is unclear.*

Response: We are sorry for the confusion. We have corrected this during revision. Please see page 4, "…., the molecular mechanisms in orofacial clefts still remain unclear due to the lack of appropriate analytical approaches for the analyses during craniofacial development."

- *Several typos and grammar problems throughout. The manuscript would be significantly improved with thorough editing.*

Response: We thank the reviewer for pointing this out. During revision, we have tried our best to correct typos and grammar problems. We carefully read the revised manuscript before submission.

- *Grouping the data by time point might be easier for the reader to understand.*

Response: We thank the reviewer for this valuable suggestion. Our results and regulatory networks at each developmental stage have been reorganized and grouped by time point. See the revised Figures 2- 6.

Response to Reviewer #3

Yan et al. presents a novel network approach for studying co-regulation between transcription factor, mRNA and microRNA in craniofacial development. They downloaded mouse data mice from the FaceBase Portal, analyzed the raw data and queried several transcription factor databases. They examined regulation between TF and gene/miRNA, regulation between miRNA and gene/TF, and constructed three-node feed-forward loops among miRNA, gene, and TF. They identified some novel stage-specific regulators and pathways, which provide a novel angle and clue for understanding craniofacial development. The paper is well written and easy to follow. The approach is novel and statistically sound. The results will advance our knowledge in craniofacial development.

Major Comments

1. Instead of pairwise comparison between different stages, it is also worthwhile (may be more powerful) to perform DE analysis between each stage and others (treat all other stages as one group).

Response: We thank the reviewer for this constructive suggestion. During revision, we have performed DE analysis between each stage and others, and the significant genes did not change much, especially the hub nodes of the regulatory networks. Since the results are similar, we decided not to add these new results in the manuscript to avoid redundancy (and it is already 8 figures in the revised manuscript).

2. The co-regulation network is likely tissue or cell-type specific. It is unclear what type of tissue or cell types used and how the results can be generalized to other tissues and to human.

Response: We thank the reviewer for this important point. The node connections were curated from several databases. The construction of network is based on the gene expression file of certain tissue at certain time point, in our case, maxillary process at embryonic days in mouse strain C57BL/6J. The analysis pipeline is generalizable, and it can be applied to any other tissues and to other organisms (e.g., human). The results will be different If the gene expression data comes from other tissues or other organisms.

In revised manuscript, Discussion, page 15, we revised “This approach can be generally applied to any developmental or disease stage specific research to decode molecular regulation; it can be applied to other organisms such as humans, and it can be extended to the single cell level as well.”

3. Pearson correlation is calculated based on a limited number of samples, so coefficient and p-value may be unstable. Some discussion is needed. The rationale to use threshold 0.3 should be given as well.

Response: We thank the reviewer for this valuable suggestion. Discussion on coefficient and p-value has been added. The rationale to use threshold 0.3 has been provided. On the Results section of page 7, we added “Sample size of 6 is sufficient for detecting a correlation coefficient of 0.9 with power of 0.8 (Bujang & Baharum, 2016). Li et al. applied the PCC greater than 0.3 to nominate the co-expression (Li et al., 2019). Thus, we only retained the significant pairs with a coefficient greater than 0.3 ($p < 0.05$), which result in a total of 34,198 significant TF-miRNA pairs.”

On Discussion part of page 19, we wrote “Pearson correlation restrictions were applied to reduce the false positive rate in our studies (e.g., Pearson’s correlation coefficient > 0.3 & $p < 0.05$, and

also see “FFL assembling and network construction” in Results). However, the calculation is based on a limited number of samples, which may result in unstable coefficient and p-values and existence of false interaction pairs.”

4. Can authors comment if the newly identified co-regulation network can help interpret findings from human omics study such as genome-wide association or transcriptomic studies of craniofacial diseases.

Response: We thank the reviewer for this interesting question. Yes, this is definitely the direction of cross-species and cross-domain analysis. We think our regulatory network approach is uniquely appropriate to such future analysis, e.g. mapping human GWAS or transcriptomic data to mouse regulatory networks. Recently, NIH NIDCR announced such research program as well. The newly identified co-regulation network can help the interpretation of omics study. For example, the genome-wide association studies (GWASs) significant variants/genes can be mapped to mouse homologous genes, and then we can use our network to perform enrichment of such human signals. Importantly, there are network tools that are developed for network enrichment of GWAS or both GWAS/transcriptomic signals, including our tools dmGWAS (Jia et al., 2011) and EW_dmGWAS (Wang et al., 2015). In addition, our recent work demonstrated TF-miRNA network modules are conserved for cleft genes in humans and mice (Li et al, 2020). In the revised manuscript, pages 19 and 20, Discussion section, we added a paragraph to discuss this issue.

References:
1. Jia P, Zheng S, Long J, Zheng W, Zhao Z (2011) dmGWAS: dense module searching for genome-wide association studies in protein-protein interaction networks. *Bioinformatics*, 27(1):95-102
2. Wang Q, Yu H, Zhao Z, Jia P (2015) EW_dmGWAS: Edge-Weighted dense module search for genome-wide association studies and gene expression profiles. *Bioinformatics*, 31:2591-2594
3. Li A, Jia P, Mallik S, Fei R, Yoshioka H, Suzuki A, Iwata J, Zhao Z (2020) Critical microRNAs and regulatory motifs in cleft palate identified by a conserved miRNA-TF-gene network approach in humans and mice. *Briefings in Bioinformatics*, 21(4):1465-1478

Second decision letter

MS ID#: DEVELOP/2020/192948

MS TITLE: A developmental stage specific network approach for studying dynamic transcription factor-microRNA co-regulation during craniofacial development

AUTHORS: Fangfang Yan, Peilin Jia, Hiroki Yoshioka, Akiko Suzuki, Junichi Iwata, and Zhongming Zhao

I have now received 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.

I am satisfied with your response to Review 1 and the corresponding revision of the manuscript. In light of the critique of the depth of experimental validation, I am prepared to consider publishing this manuscript as a Technology and Resource article if you are able to address, to your best ability, the issues raised by Reviewer 2. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so

within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 2

Advance summary and potential significance to field

This paper identifies feed forward loops that have the potential to be important regulators of craniofacial development.

Comments for the author

We thank the authors for an improved manuscript, which has been presented in a more straightforward and clear manner. The remaining concerns involve impact and experimental design.
Concerns:
Can the authors include the exact location of cells that were isolated for analysis in the main text? It is not clear where the expression data came from in the text below which is very important for spatial context in the embryo and interpretation of the data. Perhaps a diagram would help? See the first sentence of the results section below:

“In this study, we developed a novel developmental stage specific approach to study expression of genes TFs, and miRNAs, and their co-regulation in a phenotype or disease. Specifically, we applied this approach to the mRNA and miRNA gene expression data from E10.5-E14.5 mouse embryonic tissues. ”

We previously suggested the authors provide context as to how feedforward loops impact craniofacial development. The additions to the revised manuscript still lack context as to how these loops affect development. Do the FFLs discussed in this study promote/inhibit developmental processes (i.e. growth morphogenesis, differentiation) and what is known or relevant about FFLs at specific timepoints being studied here? See an example below:

“Li et al. showed that the miRNA hsa-mir-27b represses expression of transcription factor SMAD1 and accordingly activates WNT pathway core gene WNT3A through FFL during lip development (Li et al., 2020).”

Additionally, the authors validated experiments in vitro using a multipotent neural crest cell line, however, the authors are analyzing FFLs in tissue after neural crest have been specified into craniofacial mesenchymal progenitors. At a minimum the authors can acknowledge this limitation.

The authors have softened their language throughout the manuscript with respect to direct targeting of mRNA by specific miRNAs and the novelty of their findings. However, evidence of direct targeting, preferably in the context of relevant cells/tissue, would add impact to this manuscript.

In summary, although the network approach used here has the potential to deliver important hypothesis-generating ideas, in it's current form the manuscript provides limited advance or resource to the field. Considering the limitations of the validation experiments, there is little evidence to support a role for FFLs during development of craniofacial tissue.

Reviewer 3

Advance summary and potential significance to field

This paper advances our knowledge of regulatory mechanism for craniofacial development using bioinformatics approach followed by experimental validation.

Comments for the author

Authors have addressed all of my comments and I am pretty happy with the added experimental validation by RT-PCR.

Second revision

Author response to reviewers' comments

Response to Reviewers

MS ID: DEVELOP/2020/192948

MS Title: A developmental stage specific network approach for studying dynamic transcription factor- microRNA co-regulation during craniofacial development

Journal: Development

Editor's Note

I am satisfied with your response to Review 1 and the corresponding revision of the manuscript. In light of the critique of the depth of experimental validation, I am prepared to consider publishing this manuscript as a Technology and Resource article if you are able to address, to your best ability, the issues raised by Reviewer 2. 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.

Response: We thank the editor for the advice and the reviewers for their constructive comments. We are okay to publish it as a Technology and Resource article. In the second revised version, we have tried our best to address reviewer 2's comments. We also revised it by following editorial check list. Please see our detailed point-by-point response below. The changes in the revised manuscript were labeled in red.

Response to Reviewer #2

This paper identifies feed forward loops that have the potential to be important regulators of craniofacial development. We thank the authors for an improved manuscript, which has been presented in a more straightforward and clear manner. The remaining concerns involve impact and experimental design.

Concerns:

- *Can the authors include the exact location of cells that were isolated for analysis in the main text? It is not clear where the expression data came from in the text below which is very important for spatial context in the embryo and interpretation of the data. Perhaps a diagram would help? See the first sentence of the results section below:*
"In this study, we developed a novel developmental stage specific approach to study expression of genes, TFs, and miRNAs, and their co-regulation in a phenotype or disease. Specifically, we applied this approach to the mRNA and miRNA gene expression data from E10.5-E14.5 mouse embryonic tissues."

Response: We thank the reviewer for this specific question. In the second revised manuscript, we added the exact location of cells that were isolated for analysis in the main text. In the first paragraph of the Results section, page 6, we wrote "Specifically, we utilized the gene expression data from the maxillary processes of C57BL6/J mouse embryos (E10.5, E11.5, E12.5, E13.5, and E14.5) available at the FaceBase Portal (Affymetrix Mouse Genome 430 2.0 Array, n=6 per developmental stage, Accession ID: FB00000804/GSE67985) and miRNA expression data from the

frontonasal prominence of 129S6 mouse embryos (E10.5, E11.5, E12.5, E13.5, and E14.5) available at the FacaBase portal (RNA-Seq, n=2, Accession ID: FB00000663.01- FB00000666.01)."

- *We previously suggested the authors provide context as to how feedforward loops impact craniofacial development. The additions to the revised manuscript still lack context as to how these loops affect development. Do the FFLs discussed in this study promote/inhibit developmental processes (i.e. growth, morphogenesis, differentiation) and what is known or relevant about FFLs at specific timepoints being studied here? See an example below:*
"Li et al. showed that the miRNA hsa-mir-27b represses expression of transcription factor SMAD1 and accordingly activates WNT pathway core gene WNT3A through FFL during lip development (Li et al., 2020)."

Response: We thank the reviewer for this valuable comment. We are sorry for not describing clearly how these loops affect development. In the revised manuscript, we added relevant context.

On page 9, we added "The transcription factor Foxm1 may repress expression of miRNA miR-340-5p through FFL and accordingly affect EMT, cell migration, and disappearance of medial edge epithelium (MEE) during lip formation."

On page 10, we added "Abnormal regulation of Foxm1 and miR-340-5p may lead to the alteration of gene expression in Wnt, FoxO, and Hippo signaling pathways, which may result in orofacial clefts."

On page 12, we added "For instance, in the motif Tcf7 – miR-340-5p – *Igf1*/*Pdk4*/*Spp1*, Tcf7 regulates several important genes for tissue remodeling (*Igf1, Pdk4,* and *Spp1*) by binding to the promoter regions of these genes during palatogenesis, while it also regulates them through the suppression of miR-340- 5p."

On page 13, we added "These TFs may suppress the expression of *miR-129-5p* to alleviate its inhibition to *Col1a1*, which may promote the cell invasion and proliferation during development process."

We agree with the reviewer that discussion of known FFLs that have been studied can be helpful. However, even though FFL has been widely applied to other diseases (schizophrenia, glioblastoma, prostate cancers, gastric cancers, tuberculosis process, among others), there are limited studies that applied FFL in craniofacial development at specific stage. Li et al. showed FFL of human miRNA and transcription factors (Li et al., 2019; Li et al., 2020) and did not involve time-series specific FFLs, which is our focus on this work. We have discussion of molecules in our newly discovered FFLs and listed some examples below.

On page 16, we wrote "Foxo6, which expressed particularly in craniofacial tissues, activates Hippo signaling to control the growth of the craniofacial complex (Sun et al., 2018)." We also wrote "For example, Tgfbr1, a transmembrane receptor for TGF ligands and ubiquitously expresses in craniofacial epithelium and mesenchyme, plays crucial roles in palate development (Iwata et al., 2011)."

On page 17, we wrote "*Hif1a* knockout mouse is embryonic lethal by E11.0 with severe developmental defects such as open neural tube and cardiovascular defects (Iyer et al., 1998). Zbtb16 (aka PLZF), a transcriptional repressor which works with histone deacetylase, is involved in the maturation of myeloid and organogenesis."

On page 19, we wrote "A previous study has shown that overexpression of miR-340-5p may reduce mesenchymal traits in Glioblastoma multiforme (Kim et al., 2019)."

- *Additionally, the authors validated experiments in vitro using a multipotent neural crest cell line, however, the authors are analyzing FFLs in tissue after neural crest have been specified into craniofacial mesenchymal progenitors. At a minimum the authors can acknowledge this limitation.*

Response: We thank the reviewer for being critical on this point. We tried our best to find the most appropriate cell lines during revision. As suggested by the reviewer, we acknowledged this limitation in the Discussion section of the second revised manuscript. On pages 19 and 20, we wrote "Lastly, the *in vitro* experimental validations were conducted using O9-1 cells, a multipotent cranial neural crest cell line established from mouse embryo E8.5. However, the FFLs in our study were constructed in tissues at stages E10.5 to E14.5 when the neural crest have been specified into craniofacial mesenchymal progenitors. Thus, the usage of O9-1 cells is limited in the conservation of regulatory networks."

- *The authors have softened their language throughout the manuscript with respect to direct targeting of mRNA by specific miRNAs and the novelty of their findings. However, evidence of direct targeting, preferably in the context of relevant cells/tissue, would add impact to this manuscript.*

Response: We thank the reviewer for this valuable suggestion. We agree with the reviewer that providing evidence of direct targeting in the context of relevant tissue will increase the impact of this manuscript.
However, it is a challenge as well to culture primary cells isolated from the developing maxillary prominence at each developmental stage, especially at the early stage. To make things worse, these primary cells are not available to conduct gene- and miRNA-editing experiments, for example, the overexpression and knockdown of the genes and miRNAs of interest. Therefore, we utilized established O9-1 cells in our experimental validation.

- In summary, although the network approach used here has the potential to deliver important hypothesis generating ideas, in its current form the manuscript provides limited advance or resource to the field. Considering the limitations of the validation experiments, there is little evidence to support a role for FFL during development of craniofacial tissue.

We appreciate the reviewer for critical thinking on this specific mechanism. As we discussed above and stated in the manuscript, TF and miRNA regulation on gene expression has been well recognized in the biological and biomedical fields, and recently their coregulation has been found more informative to explore gene function and regulation in the developmental and cellular system. That is why we performed this study. Based on your kind suggestions, we added context regarding how our discovered feedforward loops impact craniofacial development at a specific stage. We acknowledged the limitation of the experiments and provided the justification for choosing neural crest cell lines for experiments. Hope our revisions have addressed the reviewers' concerns. In our study, we pointed out several promising regulators that may contribute to orofacial clefts through FFLs. We also proposed a putative model that may play a role in the pathogenic mechanism of orofacial clefts. We believed this work introduces a new stage specific network approach that is important for craniofacial development data, and our study has increased current knowledge and understanding of developmental mechanisms. Accordingly, we accepted the editor's suggestion to publish it as a Technology and Resource article.

Third decision letter

MS ID#: DEVELOP/2020/192948

MS TITLE: A developmental stage specific network approach for studying dynamic transcription factor-microRNA co-regulation during craniofacial development

AUTHORS: Fangfang Yan, Peilin Jia, Hiroki Yoshioka, Akiko Suzuki, Junichi Iwata, and Zhongming Zhao
ARTICLE TYPE: Techniques and Resources Article

I am satisfied with the revision of the manuscript. Your paper has been accepted for publication in Development, pending our standard ethics checks.