Single-cell reconstruction with spatial context of migrating neural crest cells and their microenvironments during vertebrate head and neck formation

Jason A Morrison, Rebecca McLennan, Jessica M Teddy, Allison R Scott, Jennifer C Kasemeier-Kulesa, Madelaine M Gogol and Paul M Kulesa
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Original submission

First decision letter

MS ID#: DEVELOP/2021/199468

MS TITLE: Single-cell reconstruction with spatial context of migrating neural crest cells and their microenvironments during vertebrate head and neck formation

AUTHORS: Jason A Morrison, Rebecca McLennan, Jessica M Teddy, Allison R Scott, Jennifer C Kasemeier-Kulesa, Madelaine M Gogol, and Paul M Kulesa

Thank you for your submission and inclusion of previous referees' reports on the above manuscript. I can see that the referees express considerable interest in your work, but had some significant criticisms. I solicited an additional report (detailed below), taking into consideration the existing comments, whichrecommends a substantial revision of your manuscript before we can consider publication.

Referee comments:
1. Figure 6G-I: Multiple comparisons are being made in these panels, however, the authors have not corrected for multiple hypothesis testing. Given the marginal significance of several of these comparisons, I believe that only a subset of the comparisons would be significant following multiple hypothesis testing correction.

2. While I disagree with Reviewer 1's comment about the design of this study and think that this is an informative strategy, I think the reviewer's concern on using morpholinos as the sole source of gene knockdown is valid, and the author's response in the rebuttal is insufficient, especially given the very small margin of significance they observe in their comparisons. The authors should use CRISPR-Cas9 for knocking down their genes of interest to validate their claims on perturbed mean speed, straightness, and displacement following the loss of their candidate genes.

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3. Figure 3A-D: The authors should label Sox10 transcripts using HCR and overlay the image with HNK1 immunostaining to verify their observation from figure 3A-D. I am concerned that the lack of HNK1/Sox10 overlap could be an artifact of using a suboptimal Sox10 antibody. Moreover, given that they talk about transcript data throughout the manuscript, I believe it would be fair to validate their observations.

4. Transcripts for genes RBM38, KAZALD1, NRSN1, and PODXL should be labeled using an alternative method such as HCR or conventional in situ hybridization. As of now, as rightly pointed out by reviewer 2, it is hard to tell where these genes are really expressed based on the RNA-scope images. The authors' segmentation image doesn't seem to match the actual intensity data, so I am not sure how this analysis was performed. In fact, the authors in their rebuttal say that RNAscope might require optimization, so why it was used in the first place is unclear. I would have thought that the dropout rate of 10x-based scRNA-seq platform would make it less sensitive than a smFISH-based approach, but the authors suggest otherwise. HCR has worked well in chick embryos and has been adapted by several labs across the world, so I think the authors should try to see if that would work better than RNA-scope. They should also consider presenting cross-sections through the branchial arches instead of whole mount images for this section of the manuscript.

5. I believe that the comments made by reviewer 3 regarding the analysis presented in the paper are very valid and should not be addressed by merely making textual changes. The authors should analyze their data with a stringent cut-off to ensure that their claim is in fact supported through the analysis.

6. It is not pointed out by any of the reviewers, but I believe that the authors' analysis presented in figure 4G is faulty. The 'trajectories' that the authors describe seem like cherry picking to me. The authors should use unbiased approaches such as Slingshot or Monocle to perform this analysis, otherwise figure 4G offers little scientific insight into their data. On that note, I am very surprised that the authors chose to map their data to the chicken galgal4 assembly (released in 2011!) instead of the galgal6 assembly (released in 2018). Having personally used the two, I know for a fact that annotations for many genes are missing from galgal4 that were later included in galgal6. Their explanation that they wanted to analyze the data in a way similar to their 2017 paper also concerns me. They should take their old data and map it to the newer assembly if they want to keep the analysis uniform across the two datasets, not go back to an old assembly for their new dataset.

Overall, it is my opinion that this manuscript should be revised significantly before it is accepted for publication in any journal. While I understand that the authors will have to perform some validation experiments, most of the comments above (similar to the reviewers) require analysis that should be possible without needing lab access during the pandemic.

If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.
We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

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First revision

Author response to reviewers' comments

Response to Reviewers

Referee comments:
1. Figure 6G-I: Multiple comparisons are being made in these panels, however, the authors have not corrected for multiple hypothesis testing. Given the marginal significance of several of these comparisons, I believe that only a subset of the comparisons would be significant following multiple hypothesis testing correction.

Response: We believe the Reviewer is commenting on Fig. 5G-I since Fig. 6H-I does not exist. To clarify, Fig. 5G-I presents three distinct measurements of cell speed, direction and displacement all quantified from tracked cell trajectories in control morpholino versus morpholino knockdown for a subset of 5 novel neural crest cell invasion genes. We understand the Reviewer's concern for minimizing false positives within our analysis. We suggest that p-values are easily interpretable by a wide audience and the small number of tests (5 genes) allows those who are concerned with false positives to quickly calculate the FDR. Regarding the marginal statistical significance of these results, we have previously published the cell autonomous nature of our morpholino transfections, in which wildtype neural crest cells were able to presume the role of the Trailblazers despite reduction of individual Trailblazer genes (Morrison et al., 2017). These cell autonomous effects combined with the robustness of neural crest cell invasion signaling, likely contribute to the small statistical significance observed in our explant culture experiments. We previously measured more dramatic changes in the reduction of neural crest cell invasion in vivo (Morrison et al., 2017), however our goal in this paper was to rapidly determine changes in the neural crest cell behaviors using an in vitro time-lapse strategy. We anticipate that small changes in cell speed and direction will be amplified over longer distances and time required for in vivo invasion. We have clarified this in the text.

2. While I disagree with Reviewer 1's comment about the design of this study and think that this is an informative strategy, I think the reviewer's concern on using morpholinos as the sole source of gene knockdown is valid, and the author's response in the rebuttal is insufficient, especially given the very small margin of significance they observe in their comparisons. The authors should use CRISPR-Cas9 for knocking down their genes of interest to validate their claims on perturbed mean speed, straightness, and displacement following the loss of their candidate genes. Response: We agree with the Reviewer that a more thorough functional analysis of any specific neural crest cell invasion gene would require multiple knockdown methods, as we have previously published (Aquaporin-1, McLennan et al., 2020; Angiopoietin-2, McKinney et al., 2016). However, our goal in this study was to rapidly identify whether there are any changes to neural crest cell invasive behaviors in a small subset of genes (n=5) that would provide information towards the prioritization of the >200 genes identified as enhanced in the leading edge of all four BA1-4 neural crest cell migratory streams.In support of this, we have now added functional in vivo data on the knockdown of two genes (Kif26a, Podxl) using siRNA, which show significance in the reduced distance migrated (Fig. 5K).

3. Figure 3A-D: The authors should label Sox10 transcripts using HCR and overlay the image with HNK1 immunostaining to verify their observation from figure 3A-D. I am concerned that the lack of HNK1/Sox10 overlap could be an artifact of using a suboptimal Sox10 antibody. Moreover, given that they talk about transcript data throughout the manuscript, I believe it would be fair to validate their observations. Response: We appreciate the Reviewer's comment. We have previously published a detailed comparison and quantification of Sox10 transcripts with HNK1 immunostaining in the same chick embryo neural crest migratory streams using a novel integrated mRNA expression detection, immunohistochemistry and tissue clearing method we developed (Figs 1 & 3; Morrison et al., 2017 Mech Dev, 148: 100-106). We have clarified this in the text and corresponding figures (Fig. 5, Suppl. Fig. 2).

4. Transcripts for genes RBM38, KAZALD1, NRSN1, and PODXL should be labeled using an alternative method such as HCR or conventional in situ hybridization. As of now, as rightly pointed out by reviewer 2, it is hard to tell where these genes are really expressed based on the RNA-scope

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images. The authors' segmentation image doesn't seem to match the actual intensity data, so I am not sure how this analysis was performed. In fact, the authors in their rebuttal say that RNAscope might require optimization, so why it was used in the first place is unclear. I would have thought that the dropout rate of 10x-based scRNA-seq platform would make it less sensitive than a smFISH-based approach, but the authors suggest otherwise. HCR has worked well in chick embryos and has been adapted by several labs across the world, so I think the authors should try to see if that would work better than RNA-scope. They should also consider presenting cross-sections through the branchial arches instead of whole mount images for this section of the manuscript. Response: We appreciate the Reviewer's comment about using an alternative method such as HCR. However, we do not anticipate that this will be more sensitive than RNAscope since the latest version of HCR is actually based on the RNAscope technology to prevent dubious mis-amplification of signal that was a problem for the original HCR version. We were one of the first laboratories to validate HCR in the avian embryo and have extensive published experience with both HCR and RNAscope, dating back to our use of HCR to confirm the molecular heterogeneities within the cranial neural crest migratory streams (McLennan et al., 2015, Development 142: 2014-2025; Fig. 3). Further, we analyzed n=13 different neural crest invasion genes we discovered in our single cell RNA-seq profiling of the BA2 neural crest cell migratory stream in a detailed analysis combining RNAscope and immunolabeling to visualize and quantify transcripts in lead neural crest cells (Fig. 6; Morrison et al., Elife, 2017). We have revised Fig. 5F,G in this paper to provide a clearer representation of both the spatial heterogeneity of neural crest genes from the leaders (Kazald1) to the followers (Cdh5) and specifically in high resolution Kazald1 expression within the BA2 stream.

5. I believe that the comments made by reviewer 3 regarding the analysis presented in the paper are very valid and should not be addressed by merely making textual changes. The authors should analyze their data with a stringent cut-off to ensure that their claim is in fact supported through the analysis. Response: While we appreciate the Reviewer's intent to improve the overall analysis, the number of cells affected by their suggestion would in fact be quite small and have minimal, if any, effect on the biological interpretation of this data set.

6. It is not pointed out by any of the reviewers, but I believe that the authors' analysis presented in figure 4G is faulty. The "trajectories" that the authors describe seem like cherry picking to me. The authors should use unbiased approaches such as Slingshot or Monocle to perform this analysis, otherwise figure 4G offers little scientific insight into their data. On that note, I am very surprised that the authors chose to map their data to the chicken galgal4 assembly (released in 2011!) instead of the galgal6 assembly (released in 2018). Having personally used the two, I know for a fact that annotations for many genes are missing from galgal4 that were later included in galgal6. Their explanation that they wanted to analyze the data in a way similar to their 2017 paper also concerns me. They should take their old data and map it to the newer assembly if they want to keep the analysis uniform across the two datasets, not go back to an old assembly for their new dataset. Response: We appreciate the Reviewer's comment. We have removed the dashed lines and arrowheads from Fig 4G-I and edited the results section. Regarding our use of galgal4: our Bioinformatician co-author has already reanalyzed the data using galgal6. We are working to show similarity between the two alignments. If we can show congruency between cluster markers derived using galga4 and galga6, then the biological interpretation should be unaffected by the alignment. We agree that alignment of our data set to galgal6 would likely detect additional transcripts and potentially increase the number of markers for subpopulations of interest. However, we contend that a time-intensive reanalysis is not justified when the results would not significantly impact the biological interpretations.

Second decision letter

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

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, 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 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

See previous review.

Comments for the author

Responses to author comments from previous review:

- Previous comment 1. I respectfully disagree with the Authors' suggestion that using "mapped reads" is more understandable to readers than unique molecular identifiers, "UMIs". The term UMI is commonly used when referring to the number of unique molecules detected per cell, both in the literature and the Cellranger pipeline documentation. The Authors also report in the methods section that "Cells were kept for downstream analysis if they had more than 500 genes expressed, less than 20,000 UMIs", rather than referring to a filtering cutoff based on "mapped reads". The Authors should be consistent between the methods section and QC plots in Figure S1.

- Previous comment 2. While I understand the Authors' response that the currently available doublet-detection tools are not perfect for finding true multiplets in all kinds of single cell datasets, I disagree that the number of cells affected is likely to be negligible. Given the number of recovered cells per 10X sample and expected multiplet rate (~7.6% for 10k cells recovered), it is a conservative estimate that >5,000 detected "cells" are in fact multiplets. Even if the authors do not feel that current multiplet detection methods are satisfactory, they should acknowledge the true predicted multiplet rate for their samples in the text.

- Previous comment 3. To clarify, the authors do have a written legend for S1F-H but there is no color legend for the cell cycle signature plots. The described method is insufficient to ascertain precisely how the scores were computed (scaled counts vs raw counts, averaging, etc). The Authors should add this to the methods section and additionally describe the human to chick ortholog conversion process including the number of genes retained post-conversion for each score (G2/M, M/G1 S). Depending on how the signature scores were computed, using such a large number of genes to generate scores can result in biases from a few highly expressed genes; this could result in missing true proliferative signatures for these cells.

One of the advantages of single cell experiments is the ability to capture heterogeneity in cell cycle phase in a population of cells. The result as displayed in Figure S1F-H suggests that all cells express somewhat equal levels of genes from all phases of the cell cycle; this seems biologically implausible. The authors should comment on using other proliferative signatures that have been

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applied to more similar systems (such as those from Kowalczyk, Tirosh et al Genome Research, 2015) and are commonly used for measuring cell cycle phase in single cell RNA-seq data as an orthogonal metric for measuring proliferation in this dataset.

- Previous comment 4. The Authors' clarification of this point is satisfactory.
- Previous comment 5. The Authors' modification of the figure is satisfactory.

Reviewer 2

Advance summary and potential significance to field

This is an scRNA-seq study of the cranial neural crest cells (NCCs) in chick embryos. The authors have separated distal and proximal migrating cranial NCCs and argue that a set of distal marker genes characterizes invasive cells (e.g. "trailblazer") NCCs. They show that some of these genes are expressed in NCCs and that knocking down their expression disrupts NCC migration. They describe similar signatures in human neuroblastoma cells that correlate with invasiveness. scRNA-seq studies of cranial NCCs or cancer cells that can be correlated with migratory behaviors address an important issue in embryonic morphogenesis as well as cancer metastasis.

Comments for the author

While in this revision the authors have made some modifications that make the figures easier to follow and many text changes, major flaws in the data analyses and their interpretation remain. Additional comparative analyses of scRNA-seq signatures of cardiac mesoderm and presumptive invasive NCCs within the cardiac mesoderm cluster have not been provided, so I am not convinced that any of these are NCCs.

Insufficient data are provided to evaluate how the clustering in Figures 1 and 4 was generated and although the paper focuses on invasiveness and migration, rather than differentiation, the proposed transition from cluster 1 to 4 still makes no sense. This relates to comment #6 in the new set of Referee comments included with the Response to Reviewers.

If the genes chosen for functional analysis are also expressed in cells surrounding the NCCs and knockdowns may be causing defects cell non-autonomously as the authors acknowledge, this complicates interpretation of specific roles in NCC invasiveness. I agree with the Referee comment #1 doubting the specificity or statistical significance of the MO results.

Without having resolved the issues with the scRNA-seq analyses of embryonic NCCs interpreting similar invasive signatures in neuroblastoma cells in Figure 6 is problematic.

Reviewer 3

Advance summary and potential significance to field

In this paper, Morrison and colleagues use single-cell RNA-sequencing to profile neural crest cells emerging from the chick hindbrain that innervates branchial arches I-IV. This reviewer appreciates the changes made by the authors in response to the original comments. However, some potential issues still remain as highlighted below.

Comments for the author

Below is this reviewer's response to the rebuttal letter submitted by the authors. The comment number corresponds to the order of the original review points.

1. This reviewer very strongly disagrees with the authors on their statistical approach. Using a simple multiple testing correction method called Bonferroni's where the corrected p-value for significance is calculated by dividing the original p-value (in this case, 0.05) by the number of comparisons made (in this case, 5), only p-values less than 0.01 would be considered "significant,"

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rendering two comparisons from 5H, both comparisons from 5I, one comparison from 5J, and both comparisons from 5K insignificant. In response to the authors' rebuttal to this reviewer's original comment, corrected p-values are also easily interpretable by a wide audience, especially in today's day and age of genomics-based research. Ultimately, this reviewer will leave it to the editor to decide how they would like to handle this disagreement. It is very important that correct statistical methods be used, and unfortunately, that is not the case in this particular instance. This reviewer thinks that the authors can either present their data as a qualitative difference or remove the comparisons that are insignificant from the main figure. Also, this reviewer couldn't find the number of embryos analyzed for the siRNA knockdown data. The plot itself only seems to have one raw data point, yet a widespread distribution is presented.
Can the authors please clarify how many embryos were analyzed?

2. While this reviewer still believes that the authors should have used a more robust method for gene knockdowns, this is now a minor point given the siRNA knockdown data that the authors added in the revised manuscript.

3. Is the Sox10 expression in supplementary figure 2 using HCR? This reviewer was unable to find that information in the text.

4. Can the authors please comment on why they don't present cross-sections through the branchial arches to confirm the expression of their candidate genes?

5. Have the authors performed this analysis? How many cells were affected? Have the authors tested what a minimal effect on the biological interpretation of this data would look like?

6. If the original analysis was performed by the collaborator, they likely have R scripts for the downstream analysis, and therefore should not be time-intensive to re-align the data using a newer assembly. This reviewer appreciates that the authors have changed aspects of this figure by removing the "trajectories". However, if there is a potential to expand the number of genes identified in the data by using a newer assembly, it should be at least tried and presented as a supplementary figure. Once again, this reviewer will leave it to the editor to decide the importance of having this in the revised manuscript.

Second revision

Author response to reviewers' comments

Reviewer #1
1-1. I respectfully disagree with the Authors' suggestion that using "mapped reads" is more understandable to readers than unique molecular identifiers, "UMIs". The term UMI is commonly used when referring to the number of unique molecules detected per cell, both in the literature and the Cellranger pipeline documentation. The Authors also report in the methods section that "Cells were kept for downstream analysis if they had more than 500 genes expressed, less than 20,000 UMIs", rather than referring to a filtering cutoff based on "mapped reads". The Authors should be consistent between the methods section and QC plots in Figure S1.Response: We thank the Reviewer for pointing this out. After careful review, we realized that the plots were in fact reporting the UMIs, however the plot titles and figure legends needed to be edited, which we have now corrected in Suppl. Fig. 1B,D.

1-2. While I understand the Authors' response that the currently available doublet-detection tools are not perfect for finding true multiplets in all kinds of single cell datasets, I disagree that the number of cells affected is likely to be negligible. Given the number of recovered cells per 10X sample and expected multiplet rate (~7.6% for 10k cells recovered), it is a conservative estimate that > 5,000 detected "cells" are in fact multiplets. Even if the authors do not feel that current multiplet detection methods are satisfactory, they should acknowledge the true predicted multiplet rate for their samples in the text. Response: We thank the Reviewer for the helpful comment. We have updated Suppl Fig. 1A and text in the Methods that we estimated the multiplet

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rate per 10X Genomics' webpage: https://kb.10xgenomics.com/hc/en-us/articles/360001378811-What-is-the-maximum-number-of-cells-that-can-be-profiled-

1-3. To clarify, the authors do have a written legend for S1F-H, but there is no color legend for the cell cycle signature plots. The described method is insufficient to ascertain precisely how the scores were computed (scaled counts vs raw counts, averaging, etc). The Authors should add this to the methods section and additionally describe the human to chick ortholog conversion process, including the number of genes retained post-conversion for each score (G2/M, M/G1, S). Depending on how the signature scores were computed, using such a large number of genes to generate scores can result in biases from a few highly expressed genes; this could result in missing true proliferative signatures for these cells. One of the advantages of single cell experiments is the ability to capture heterogeneity in cell cycle phase in a population of cells. The result as displayed in Figure S1F-H suggests that all cells express somewhat equal levels of genes from all phases of the cell cycle; this seems biologically implausible. The authors should comment on using other proliferative signatures that have been applied to more similar systems (such as those from Kowalczyk, Tirosh et al, Genome Research, 2015) and are commonly used for measuring cell cycle phase in single cell RNA-seq data as an orthogonal metric for measuring proliferation in this dataset. Response: We appreciate the Reviewer's point to consider Kowalczyk, Tirosh et al, Genome Research, 2015. To clarify, we used ML Whitfield, Mol Biol Cell, 2002 for our analysis, which was the basis for Kowalczyk and colleagues work on the cell cycle signature. We recognize that Kowalczyk and colleagues did include genes that had a "cell cycle progress" GO annotation, but they subsequently filtered the Whitfield and 'cell cycle progress' gene lists to only include genes that were cycling in their (Kowalczyk) data set. We feel that a list of genes cycling in HSCs may not be informative to better understand what is cycling in the chick. To ensure our clusters were not the product of read depth or cell cycle variations (Kiselev et al., 2019; Whitfield et al.,2002), we performed several quality control analyses (Suppl. Fig. 1) and find continuity in both sequencing depth and cell cycle markers among our seven clusters. Feature plots of the mean normalized expression values for lists of genes known to be associated with different cell cycle phases are shown (Suppl. Fig. 1). The evenness of the expression of these sets of genes indicates a lack of cell cycle driven expression. We have included clarifying text to the Methods and Results.

Reviewer #2
2-1. Additional comparative analyses of scRNA-seq signatures of cardiac mesoderm and presumptive invasive NCCs within the cardiac mesoderm cluster have not been provided, so I am not convinced that any of these are NCCs. Response: We appreciate the Reviewer's request and have subclustered cluster 4 (cardiac mesoderm + invasive/trailblazer NC) into 4 annotated subpopulations (k means = 4) as shown in Suppl. Fig. 1J. We have also generated a list of markers for each subpopulation (Suppl Table 4) from cluster 4 and added text to the Results which supports and strengthens what is shown in Fig. 4B-C.

2-2. Insufficient data are provided to evaluate how the clustering in Figures 1 and 4 was generated and although the paper focuses on invasiveness and migration, rather than differentiation, the proposed transition from cluster 1 to 4 still makes no sense. This relates to comment #6 in the new set of Referee comments included with the Response to Reviewers. Response: We appreciate the Reviewer's comment. K-means clustering was performed for Fig 1 & 4 as stated in the Methods. We have removed the dashed lines and arrowheads from Fig 4G-I and edited the results section. We have also clarified in the text that Cluster 0 is largely undefined; with few (n=49) differentially expressed genes (see suppl table 1). As such, it occupies the center of the plot and comes between more distinctive clusters, such as clusters 1 & 4.

2-3. If the genes chosen for functional analysis are also expressed in cells surrounding the NCCs and knockdowns may be causing defects cell non-autonomously, as the authors acknowledge, this complicates interpretation of specific roles in NCC invasiveness. I agree with the Referee comment #1 doubting the specificity or statistical significance of the MO results. Response: We appreciate the Reviewer's comment. To clarify, we target knockdowns into the premigratory neural crest cells by microinjection into the lumen of the dorsal neural tube and electroporation. This delivers morpholinos or shRNA directly into premigratory neural crest cells or cells that remain in the neural tube and not the paraxial mesoderm or any microenvironment through which the neural crest cells will travel. This is a standard method that has been established and validated in several of our studies and those of others in the neural crest field.

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2-4. Without having resolved the issues with the scRNA-seq analyses of embryonic NCCs, interpreting similar invasive signatures in neuroblastoma cells in Figure 6 is problematic. Response: We appreciate the Reviewer's comment. To clarify the results presented in Fig. 6, the neural crest cell invasion signature of 964 enhanced genes was determined from migrating neural crest cells specifically isolated by microdissection – and validated in our previous publication (Morrison et al., 2017). Thus, we are confident this represents a valid invasion signature specific to the migrating neural crest cells. It was this invasion signature that was compared to 34 other published cell invasion signatures to arrive at a common set of 45 genes displayed in Fig. 6C and it was this list that was utilized for the comparison to genes enhanced with invasive human neuroblastoma cells presented in Fig. 6G.

Reviewer #3
3-1. This reviewer very strongly disagrees with the authors on their statistical approach. Using a simple multiple testing correction method called Bonferroni's, where the corrected p-value for significance is calculated by dividing the original p-value (in this case, 0.05) by the number of comparisons made (in this case, 5), only p-values less than 0.01 would be considered "significant," rendering two comparisons from 5H, both comparisons from 5I, one comparison from 5J, and both comparisons from 5K insignificant. In response to the authors' rebuttal to this reviewer's original comment, corrected p-values are also easily interpretable by a wide audience, especially in today's day and age of genomics-based research. Ultimately, this reviewer will leave it to the editor to decide how they would like to handle this disagreement. It is very important that correct statistical methods be used, and unfortunately, that is not the case in this particular instance. This reviewer thinks that the authors can either present their data as a qualitative difference or remove the comparisons that are insignificant from the main figure. Also, this reviewer couldn't find the number of embryos analyzed for the siRNA knockdown data. The plot itself only seems to have one raw data point, yet a widespread distribution is presented. Can the authors please clarify how many embryos were analyzed? Response: We thank the Reviewer for this suggestion and have edited Fig. 5 and its legend accordingly to state our use of Bonferroni's method. We analyzed n=12 embryos for each of the morpholiono (MO) and siRNA knockdown and control experiments.

3-2. Is the Sox10 expression in supplementary figure 2 using HCR? This reviewer was unable to find that information in the text. Response: The data in Supplemental Fig. 2A-D is using RNAscope. We have clarified this in the figure legend and Methods

3-3. Can the authors please comment on why they don't present cross-sections through the branchial arches to confirm the expression of their candidate genes? Response: We appreciate the Reviewer's question. We performed 3D confocal imaging on whole mount embryos such that we were able to visualize the entire dorsolateral migratory pathway of the neural crest cells and surrounding tissues in the head. This in vivo intact method allowed us to overcome 'ambiguities' in interpreting neural crest cell positions and gene expression in migratory streams captured in tissue sections since this often leads to inaccuracies from the inability to cut and capture clean sections that are not skewed.

3-4. If the original analysis was performed by the collaborator, they likely have R scripts for the downstream analysis, and therefore should not be time-intensive to re-align the data using a newer assembly. This reviewer appreciates that the authors have changed aspects of this figure by removing the "trajectories". However, if there is a potential to expand the number of genes identified in the data by using a newer assembly, it should be at least tried and presented as a supplementary figure. Once again, this reviewer will leave it to the editor to decide the importance of having this in the revised manuscript. Response: We appreciate the Reviewer's desire to identify additional genes using the most recent release of the Gallus genome. In this spirit, we will make the raw sequencing files publicly available for the community to analyze with newer genomes and software for individual research goals. The focus of this work, however, is the presence of a specific transcriptional signature that was defined using an earlier version of the genome.

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Third decision letter

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MS TITLE: Single-cell reconstruction with spatial context of migrating neural crest cells and their microenvironments during vertebrate head and neck formation

AUTHORS: Jason A Morrison, Rebecca McLennan, Jessica M Teddy, Allison R Scott, Jennifer C Kasemeier-Kulesa, Madelaine M Gogol, and Paul M Kulesa

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

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. Specifically, reviewer 2 remains concerned that clusters 1 and 3 do not correspond to NCCs. It would be helpful to provide some additional evidence that these are indeed NCCs or a discussion of the gene expression patterns of these clusters to help users navigate the resource.

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 1

Advance summary and potential significance to field

Please see summary in initial review submission.

Comments for the author

The authors have addressed the majority of my comments, and I have listed below a couple of minor changes that should be added prior to the publication of this study.
1-1. The correction is noted. However Supp. 1E is still titled “Mapped Reads” and should be corrected.
1-2. The corrections to the text and methods make this analysis more clear. It would still be helpful if the authors change the number of genes in the cell cycle analysis from 1134 to the retained ortholog number after converting from human to chick genes (if different; if not that should be noted as well).

Reviewer 2

Advance summary and potential significance to field

This is an scRNA-seq study of the cranial neural crest cells (NCCs) in chick embryos. The authors have separated distal and proximal migrating cranial NCCs and argue that a set of distal marker genes characterizes invasive cells (e.g. “trailblazer”) NCCs. They show that some of these genes are expressed in NCCs and that knocking down their expression disrupts NCC migration. They describe similar signatures in human neuroblastoma cells that correlate with invasiveness. scRNAseq studies of cranial NCCs or cancer cells that can be correlated with migratory behaviors address an important issue in embryonic morphogenesis as well as cancer metastasis.

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Comments for the author

The authors have addressed some of my comments (2-2–2-4), mainly by removing the controversial interpretations, but the major one remains.
The authors have provided the additional comparative analyses of scRNA-seq signatures of cardiac mesoderm and presumptive invasive NCCs within the cardiac mesoderm cluster that we requested. These additions help clarify what was previously impossible to decipher from the results presented. However, now looking at the list of markers I am concerned that the cells in subclusters 1 (neural crest cell, NCC) and 3 (trailblazer NCC) in Suppl. Fig. 1J of the original cluster 4 in Fig. 1 (cardiac mesoderm) are not NCCs. This is critical for assessing the importance for the community of the 'techniques and resources' reported in this paper. It really needs further in vivo validation using in situ hybridization particularly in light of previously published work.

For example, of the top 5 markers for subcluster 1 (NCC), only NGFR would be considered a relatively specific NCC marker, but it is also expressed elsewhere.
STRA6 is primarily mesodermal in chick [Reijntjes et al. Int J Dev Biol 54, 1267-1275 (2010)] and expressed in cardiac mesoderm (endocardium). JAM3 is a hematopoietic/vascular determinant in cardiac mesoderm with some later roles in NCC-derived melanophore survival and PNS myelination, but no evidence of expression during NCC migration. PRTG is also in cardiac mesoderm, and its branchial arch expression is restricted to the ectoderm [Vesque et al – Dev Dyn 235, 2836-2844 (2006)] not NCCs. COL1A2 is a later marker of skeletal and tendon derivatives of NCCs but not early migrating NCCs.

Showing in vivo expression of some of the top markers for subcluster 3 (trailblazers) other than KAZALD1 (which is fairly far down the list in Suppl Table 4) in leading NCCs would also make the key conclusion of the paper much more convincing that this is truly a distinct signature of a leading/invasive subpopulation of cells.

Reviewer 3

Advance summary and potential significance to field

Please see the previous review report.

Comments for the author

I am satisfied with all but one of the authors' comments. I encourage the authors to add a section on "Statistical Analysis" in the materials and methods section and describe their application of the Bonferroni method for multiple hypothesis correction. I was surprised to see that the exact p-values were removed from version 3 of the manuscript even though they were a part of the figure legend in version 2. Assuming that the actual p-values for Figure 5 did not change between versions 2 and 3 (p=0.006, 0.02, and 0.03), should the comparison between Control vs CST3 be reported as significant (the revised p-value cutoff should be 0.0167)?

Third revision

Author response to reviewers' comments

Response to Reviewers

Reviewer 1
1-1) The correction is noted. However Supp. 1E is still titled "Mapped Reads" and should be corrected.
> Response: Thank you. We have corrected this to read UMI's.

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1-2) The corrections to the text and methods make this analysis more clear. It would still be helpful if the authors change the number of genes in the cell cycle analysis from 1134 to the retained ortholog number after converting from human to chick genes (if different; if not that should be noted as well). >Response: We appreciate the Reviewer's comment and have added that from the 1134 cell cycle markers published by Whitfield et al., we used a list of 276 genes with matching gene symbols between human and chick (Galgal4; Ensembl release 84).

Reviewer 2
2-1) The authors have provided the additional comparative analyses of scRNA-seq signatures of cardiac mesoderm and presumptive invasive NCCs within the cardiac mesoderm cluster that we requested. These additions help clarify what was previously impossible to decipher from the results presented. However, now looking at the list of markers I am concerned that the cells in subclusters 1 (neural crest cell, NCC) and 3 (trailblazer NCC) in Suppl. Fig. 1J of the original cluster 4 in Fig. 1 (cardiac mesoderm) are not NCCs. This is critical for assessing the importance for the community of the 'techniques and resources' reported in this paper. It really needs further in vivo validation using in situ hybridization, particularly in light of previously published work. >Response: It was exciting to learn that some of the genes identified with the most invasive NCCs indeed overlap with mesoderm, suggesting common signals may guide the movements of the two cell populations. We believe that the current bioinformatics and multiplexed FISH bring this to light; further in-depth analysis of this is outside the scope of this paper. However, to provide additional evidence that subclusters 1 & 3 within cluster 4 are comprised of NC cells, we asked how many of our 314 NC markers (cluster 1 markers from Supplemental Table S1) were also markers of each of the 4, cluster 4 subclusters (Suppl Fig 1J; Supplemental Table S4). We find that none of the 64 or 147 markers of subclusters 0 or 2 (presumptive cardiac mesoderm) contain markers of NC cells. Conversely, 147 of the 390 markers (45%) of subcluster 1 (presumptive NC) also mark cluster 1 NC. Furthermore, 62 of the 197 (31%) markers of subcluster 3 (presumptive invasive NC) overlap with the markers of cluster 1 NC. We have plotted this information in two bar charts (Suppl Fig. 1K-L) and added to the text of the manuscript.

2-2) For example, of the top 5 markers for subcluster 1 (NCC), only NGFR would be considered a relatively specific NCC marker, but it is also expressed elsewhere. STRA6 is primarily mesodermal in chick [Reijntjes et al. Int J Dev Biol 54, 1267-1275 (2010)] and expressed in cardiac mesoderm (endocardium). JAM3 is a hematopoietic/vascular determinant in cardiac mesoderm with some later roles in NCC-derived melanophore survival and PNS myelination, but no evidence of expression during NCC migration. PRTG is also in cardiac mesoderm, and its branchial arch expression is restricted to the ectoderm [Vesque et al – Dev Dyn 235, 2836-2844 (2006)] not NCCs. COL1A2 is a later marker of skeletal and tendon derivatives of NCCs but not early migrating NCCs. Showing in vivo expression of some of the top markers for subcluster 3 (trailblazers) other than KAZALD1 (which is fairly far down the list in Suppl Table 4) in leading NCCs would also make the key conclusion of the paper much more convincing that this is truly a distinct signature of a leading/invasive subpopulation of cells. > Response: Please see the previous response for details.

Reviewer 3
3-1) I encourage the authors to add a section on "Statistical Analysis" in the materials and methods section and describe their application of the Bonferroni method for multiple hypothesis correction. I was surprised to see that the exact p-values were removed from version 3 of the manuscript even though they were a part of the figure legend in version 2. Assuming that the actual p-values for Figure 5 did not change between versions 2 and 3 (p=0.006, 0.02, and 0.03), should the comparison between Control vs CST3 be reported as significant (the revised p-value cutoff should be 0.0167)? > Response: We appreciate the Reviewer's point and have added details to the Methods section.

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Fourth decision letter

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ARTICLE TYPE: Techniques and Resources Article

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

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